Silke Rickert-Sperling · Robert G. Kelly
David J. Driscoll *Editors*

Congenital Heart Diseases: The Broken Heart

Clinical Features, Human Genetics and Molecular Pathways

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This book is dedicated to my mentors
Hanno D. Schmidt, Peter E. Lange, and Hans Lehrach.
Their training, support, and encouragement have
made this book possible.

Silke Rickert-Sperling

Foreword

As is indicated in its title, the book you are about to read is concerned with the congenitally malformed heart. Approximately eight neonates in every thousand born alive present with such a "broken heart". This number has changed little since Maude Abbott, when describing the first plate in her Atlas devoted to congenitally malformed hearts, commented that "An understanding of the elementary facts of human and comparative embryology is essential to an intelligent grasp of the ontogenetic problems of congenital cardiac disease". Paul Dudley White, when writing the foreword to her Atlas, commented that it had been left to Abbott to "make the subject one of such general and widespread interest that we no longer regard it with either disdain or awe as a mystery for the autopsy table alone to discover and to solve". It is perhaps surprising, therefore, to realise that it has taken nearly a century for us to achieve the necessary understanding of the "elementary facts" emphasised by Abbott. Indeed, it is not that long since, in company with my very good friend and collaborator Anton Becker, we suggested that interpretations based on embryology might prove to be a hindrance, rather than a help, in understanding the congenitally malformed heart. The contents of this book show how much has changed in the years that have passed since we made that comment, such that we now need to eat our words.

As is revealed by the multiple chapters of this book, the recent advances made in the fields of cardiac embryology and molecular genetics have been truly spectacular. It was these fields that were expertly summarised in the volumes edited by Rosenthal and Harvey. The details contained in the central part of this book, related to central molecular pathways, recapitulate and extend those reviews. Such extensive knowledge of the genetic and molecular background, however, is of limited value if these interpretations cannot properly be translated into the findings observed on a daily basis by those who diagnose and treat the individual cardiac lesions. The first part of this book, therefore, provides a necessarily brief overview of normal cardiac development, while the final chapters then incorporate the developmental and molecular findings into the clinical manifestations of the abnormal morphogenesis.

I know from my own experience how difficult it is to obtain such chapters from multiple authors, who nowadays are themselves under greater pressure to produce primarily in the peer-reviewed realm. The editors, therefore, are to be congratulated

on assembling such a panoply of authoritative texts. As might be expected, not all of the texts are of comparable length or content. The critical reader will note that several of the topics addressed remain contentious, and that opinions continue to vary between the chosen experts. This is no more than to be expected, since the topics remain very much moving targets. One hopes, therefore, that this is but the first edition of a work which itself, for the first time, seeks to provide in detail the scientific background to the specific lesions that continue to break the normal heart. As the pages of this book demonstrate, we still have much to do if we are fully to understand the mechanics of normal as opposed to abnormal cardiac development.

London, United Kingdom Robert H. Anderson
August 2015

Preface

Leonardo Da Vinci made the first drawing of partial anomalous pulmonary venous connection in the fifteenth century, and 300 years later Karl von Rokitansky described ventricular septal defects. Since then the history of clinical recognition, therapeutic opportunities, and understanding of the developmental and genetic origin of congenital heart diseases (CHDs) has evolved rapidly. The first wave of progress was dedicated to the improvement of clinical diagnosis and therapy based on anatomical, physiological, and surgical considerations. Thus, the mortality of patients with CHD declined below 1 in 100,000 cases and a new group of adult patients with corrected and palliated CHD was formed.

A second wave of progress focused on the developmental, genetic, and molecular aspects of CHDs. Here significant insights were gained by studying animal models along with human. A large collection of genes, signaling pathways, and other molecular or hemodynamic insults have been discovered, frequently considering the developmental perspective as a starting point.

After decades of basic research focusing on animal models, the human phenotype will be the central dogma in the following years. This shift is based on significant developments to overcome technological limitations now enabling studies addressing more and more complex biological questions and systems together with the recognition that improving human health is a central aim of life science research. This book brings together clinical, genetic and molecular knowledge starting from the perspective of the observed human phenotype during development and in the disease state. It aims to reach basic scientists as well as physicians and it might contribute to the current third wave of progression where basic science of cardiovascular development is translated into clinical diagnosis and therapy of CHDs.

To reach this goal, this book is structured in three main parts providing an introduction to the development of the heart and its vessels, an overview of molecular pathways affecting the development of multiple cardiovascular structures, and a textbook-like structure focused on the different types of congenital heart diseases with their clinical features, underlying genetic alterations and related animal models and pathways. We are grateful to all the contributors to this volume, who have provided state of the art accounts of their fields of expertise.

Berlin, Germany — Silke Rickert-Sperling
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Abbreviations

22q11DS	22q11 deletion syndrome
AAA	Aortic arch anomalies
ACTC1	Cardiac alpha-actin
ACVR	Activin A receptor
AD	Arterial duct
ADAM19	ADAM metallopeptidase domain 19
ADAR	Adenosine deaminase that acts on RNA
ADP	Adenosine diphosphate
AGS	Allagile syndrome
AICD	Automatic internal cardiac defibrillator
ALCAPA	Anomalous origin of the left coronary artery from the pulmonary artery
AKT	V-akt murine thymoma viral oncogene homolog
AngII	Angiotensin II
ANP	Atrial natriuretic peptide
ANK2	Ankyrin B
ANKRD1/CARP	Ankyrin repeat domain 1, cardiac muscle
Ao	Aorta
AP	Action potential
ARVC	Arrhythmogenic right ventricular cardiomyopathy
ASD	Atrial septal defect
ATFB	Atrial fibrillation
ATP	Adenosin triphosphate
AV	Atrioventricular
AVB	Atrioventricular bundle
AVC	Atrioventricular canal
AVN	Atrioventricular node
AVSD	Atrioventricular septal defect
BAF	Brg1-associated factor
BAV	Bicuspid aortic valve
BBS	Bardet-Biedl syndrome
BET	Bromodomain and extra terminal
BMP	Bone morphogenetic protein

BNP	Brain natriuretic peptide
BRAF	v-Raf murine sarcoma viral oncogene homolog B
BRG1	SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily a, member 4 (also known as brahma-related gene 1)
BRGDA	Brugada syndrome
BWIS	Baltimore Washington Infant Study
CAA	Coronary artery anomalies
CACN	Calcium channel, voltage-dependent, L type
CAD	Coronary atherosclerotic disease
CaMK	Calmodulin dependent kinase
cAMP	Cyclic adenosine monophosphate
CALM	Calmodulin
CASQ	Calsequestrin
CAT	Common arterial trunk
CBP	CREB-binding protein
CC	Cardiac crescent
CCDC	Coiled-coil domain containing
CCS	Cardiac conduction system
CCVA	Congenital coronary vascular anomalies
CF	Cephalic folds
CFC1	Cripto, FRL-1, Cryptic family 1 (CRYPTIC)
CGH	Comparative genomic hybridization
CHARGE	*C*oloboma of the eye, *H*eart defects, *A*tresia of the nasal choanae, *R*etarded growth and/or development, *G*enital and/or urinal abnormalities, and *E*ar anomalies
CHD	Congenital heart disease
CHD7	Chromodomain helicase DNA binding protein 7
CHF	Congestive heart failure
ChIP	Chromatin immunoprecipitation
CITED2	Cbp/P300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain 2
CNCCs	Cardiac neural crest cells
CNV	Copy number variation
CoA	Coarctation of the aorta
CPVT	Catecholaminergic polymorphic ventricular tachycardia
CRE	Cre recombinase
CRELD1	Cysteine-rich protein with EGF-like domains 1
CRISPR	Clustered regularly interspaced short palindromic repeats
CTD	Conotruncal defects
CTGF	Connective tissue growth factor
CTVM	Canine tricuspid valve malformation
CX	Connexin
DCM	Dilated cardiomyopathy
DGC	Dystrophin-glycoprotein complex

DGS	DiGeorge syndrome
DMP	Dorsal mesenchymal protrusion
DNAH	Dynein, axonemal, heavy chain
DNMT	DNA methyltransferases
DORV	Double outlet right ventricle
DPF3	D4 Zinc and double PHD fingers family 3 (also known as Baf45c)
DSC2	Desmocollin 2
DSG2	Desmoglein 2
DSP	Desmoplakin
Dvl2	Dishevelled segment polarity protein 2
E	Embryonic day
ECs	Endocardial cushions
ECG	Electrocardiogram
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
ELC	Essential myosin light chain
ELN	Elastin
EMT	Epithelial-to-mesenchymal transition
ENU	N-ethyl-N-nitrosourea
ET1	Endothelin 1
EPDC	Epicardially derived cells
ErbB	Erythroblastic leukemia viral oncogene homolog
ERK	Extracellular signal-regulated kinase
ERS	Early repolarization syndrome
ESC	Embryonic stem cells
EVC	Ellis-van-Creveld
FA	Folic acid
FACS	Fluorescence-activated cell sorting
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FHF	First heart field
FHL1	Four and a half LIM domains protein 1
FISH	Fluorescence in situ hybridization
FOX	Forkhead box
FOG2	Friend of GATA 2
GATA	GATA binding protein
GBX	Gastrulation brain homeobox
GDF1	Growth differentiation factor
GFP	Green fluorescent protein
GJA5	Gap junction protein, alpha 5, 40 kDa (connexin 40)
GPCR	G-protein coupled receptors
GRP	Gastrocoel roof plate
GWAS	Genome-wide association study

H3K4me3	Trimethylation of histone H3 at lysine 4
H3K4me2	Dimethylation of histone H3 at lysine 4
H3K4me1	Monomethylation of histone H3 at lysine 4
H3K24ac	Acetylation of histone H3 at lysine 24
H3K27ac	Acetylation of histone H3 at lysine 27
H3K27me3	Trimethylation of histone H3 at lysine 27
HAND	Heart and neural crest derivatives expressed
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HCM	Hypertrophic cardiomyopathy
HCN4	Hyperpolarization activated cyclic nucleotide-gated potassium channel 4
HE	Haematoxylin and eosin
HES1	Hes family BHLH transcription factor 1
HEY	Hes-related family bHLH transcription factor with YRPW motif
HLHS	Hypoplastic left heart syndrome
HOX	Homeobox genes
HT	Heart tube
IAA	Interrupted aortic arch
IC	Inner curvature
IGF1	Insulin-like growth factor 1
INO80	Inositol requiring 80
IP3	Inositol-1,4,5-triphosphate
IPCCC	International Pediatric and Congenital Cardiac Code
iPSCs	Induced pluripotent stem cells
IRX	Iroquois homeobox
ISL1	ISL LIM homeobox 1 (Islet 1)
IVF	Idiopathic ventricular fibrillation
IVS	Interventricular septum
JAG1	Jagged 1
JNK	c-Jun N-terminal kinase
JUP	Junctional plakoglobin
KLF	Kruppel-like factor
KCNE	Potassium channel, voltage gated subfamily E regulatory beta subunit
KCNJ	Potassium channel, inwardly rectifying subfamily J
KCNQ	Potassium channel, voltage gated KQT-like subfamily Q
LA	Left atrium
LBB	Left bundle branch
LCC	Left common carotid
LEFTY	Left-right determination factor
LEOPARD	*L*entigenes, *E*CG conduction abnormalities, *O*cular hypertelorism, *P*ulmonic stenosis, *A*bnormal genitalia, *R*etardation of growth and sensorineural *D*eafness

LIF	Leukemia inhibitory factor
LMNA	Lamin A/C
lncRNA	Long non-coding RNA
LPM	Lateral plate mesoderm
LLPM	Left lateral plate mesoderm
LQTS	Long QT syndrome
LRO	Left-right organizer
LSA	Left subclavian artery
LTCC	L-type calcium channel
LV	Left ventricle
LVNC	Left ventricular noncompaction
MAPCA	Major aortopulmonary collateral arteries
MBD	Methyl-CpG binding domain-based
MDM2	Murine double minute 2
MED13L	Mediator complex subunit 13-like
MEF2C	Myocyte enhancer factor 2C
MEK2	kinase 2
MESP1	Mesoderm posterior 1 homolog
MHC	Myosin heavy chain
MI	Myocardial infarction
MMP	Matrix metalloproteinases
MLC	Myosin light chain
MLL2	Mixed-lineage leukemia protein 2
miRNA	MicroRNA
MOs	Morpholino oligonucleotides
MPES	Mid-pharyngeal endothelial strand
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MS	Mass spectrometry
MSX	Msh homeobox
MuRF	Muscle RING finger protein
MyoD	Myogenic differentiation factor
MYBPC3	Cardiac myosin-binding protein C
MYH6	Cardiac alpha-myosin heavy chain
MYH7	Cardiac beta-myosin heavy chain
NADPH	Nicotinamide adenine dinucleotide phosphate
NCC	Neural crest cells
NCCM	Noncompaction cardiomyopathy
NCX	Sodium/Calcium exchanger
ncRNA	Non-coding RNA
NF1	Neurofibromatosis type 1
NFAT	Nuclear factor of activated T-cells
NGS	Next-generation sequencing
NKA	Sodium/Potassium ATPase
NKX2-5	NK2 homeobox 5

NKX2-6	NK2 homeobox 6
NMD	Nonsense mediated decay
NMR	Nuclear magnetic resonance spectroscopy
NODAL	Nodal growth differentiation factor
NOS3	Nitric oxide synthase 3
NPC	Nuclear pore complexes
NPHP	Nephronophthisis
NPPA	Natriuretic peptide A
NRG1	Neuregulin 1
NR2F2	Nuclear receptor subfamily2/group F
NVP	Nodal vesicular parcels
OFT	Outflow tract
OR	Odds ratios
p300	E1A binding protein p300
PA	Pulmonary atresia
PAAs	Pharyngeal arch arteries
PACHD	Pancreatic agenesis and congenital heart defects
PAPVC	Partial anomalous pulmonary venous connections
PAX3	Paired box 3
PCD	Primary ciliary dyskinesia
PCP	Planar cell polarity
PCR	Polymerase chain reaction
PDA	Patent ductus arteriosus
PDGF	Platelet-derived growth factor
PDK1	Phosphoinositide-dependent kinase1
PECAM1	Platelet endothelial cell adhesion molecule 1
PFO	Patent foramen ovale
PI3K	Phosphoinositide 3-kinase
PITX2	Paired-like homeodomain 2
PKA	Protein kinase A
PKC	Protein kinase C
PKD2	Polycystic kidney disease 2 (polycystin 2)
PKP2	Plakophilin 2
PLN	Phospholamban
PLXN	Plexin
PRDM16	PR domain containing 16
PTA	Persistent truncus arteriosus
PTPN11	Protein tyrosine phosphatase, non-receptor type 11
PTC	Premature termination codon
PVCS	Peripheral ventricular conduction system
QT	QT interval (electrocardiogram)
RA	Right atrium
RA	Retinoic acid
RAA	Right aortic arch
RAAS	Renin-angiotensin-aldosterone system

Ras/MAPK	Rat sarcoma viral oncogene homolog/mitogen-activated protein kinase
RBB	Right bundle branch
RBPs	RNA-binding proteins
RCC	Right common carotid
RCM	Restrictive cardiomyopathy
RLC	Regulatory myosin light chain
RNAi	RNA interference
RNP	Ribonucleoprotein
ROS	Reactive oxygen species
RSA	Right subclavian artery
rRNA	Ribosomal RNA
RR	Relative risk
RV	Right ventricle
RVOTO	Right ventricular outflow tract obstruction
RYR	Ryanodine receptor
S1	First heart sound
S2	Second heart sound
S3	Third heart sound
SAN	Sinoatrial node
SCN	Sodium channel, voltage gated
SCN5A	Sodium channel voltage gated type V alpha subunit
SCN10A	Sodium channel voltage gated type X alpha subunit
SDF1	Stromal cell-derived factor 1
SENP	SUMO/sentrin-specific proteases
SEMA3C	Semaphorin 3C
SERCA	Sarcoplasmic/endoplasmic reticulum calcium ATPase
SHF	Second heart field
SHH	Sonic hedgehog
SHOX2	Short stature homeobox 2
SNPs	Single nucleotide polymorphisms
snRNA	Small nuclear RNA
snoRNA	Small nucleolar RNA
SQTS	Short QT syndrome
SMAD4	SMAD family member 4
SMC	Smooth muscle cells
SRF	Serum response factor
STAT3	Signal transducer and activator of transcription 3
SUMO	Small ubiquitin-like modifier
SV	Sinus venosus
SVC	Superior vena cava
SVAS	Supravalvar aortic stenosis
SWI/SNF	Switching defective/sucrose nonfermenting complex
TA	Truncus arteriosus
TAC	Transverse aortic constriction

TALEN	Transcription activator-like effector nucleases
TAPVR	Total anomalous pulmonary venous return
TBX	T-box
TF	Transcription factor
Tg	Transgenic
TGA	Transposition of the great arteries
TGFβ	Transforming growth factor-β
TMEM43	Transmembrane protein 43
Tn	Troponin
TNFα	Tumor necrosis factor alpha
TOF	Tetralogy of Fallot
tRNA	Transfer RNA
TTN	Titin
UPS	Ubiquitin proteasome system
UTRs	Untranslated regions
VACTERL	*V*ertebral defects, *A*nal atresia, *C*ardiovascular anomalies, *T*racheo-*E*sophageal fistula, *R*adial and renal anomalies, and *L*imb defects
VANGL2	VANGL planar cell polarity protein 2
VCFS	Velocardiofacial syndrome
VEGF	Vascular endothelial growth factor
VP	Venous pole
VSD	Ventricular septal defect
VSMC	Vascular smooth muscle cells
VT	Ventricular tachycardia
VUS	Variants of unknown significance
WBS	Williams-Beuren syndrome
WPW	Wolff-Parkinson-White syndrome
WNT	Wingless-type MMTV integration site family
XB	Cross-bridges
Y2H	Yeast-two-hybrid
ZIC	Zic family member
ZFN	Zinc finger nucleases
ZFPM2	Zinc finger protein, FOG family member 2

Part I

Introduction

Cardiac Development and Animal Models of Congenital Heart Defects

1

Robert G. Kelly

Contents

Abstract

The major events of cardiac development, including early heart formation, chamber morphogenesis and septation, as well as conduction system and coronary artery development, are briefly reviewed together with a short introduction to the animal species commonly used to study heart development and model congenital heart defects.

1.1 Introduction

This chapter provides a brief overview of the major events of cardiac development as well as the animal models used to study the developing heart. We will focus on the sequence of events that occur during mouse heart development, the principal animal model used for genetic studies of heart development. Mouse heart development and the structure of the definitive mouse heart are highly conserved with humans, with evident

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differences in scale, both temporal and spatial [1]. The advantages of the mouse are discussed in the animal models section below (1.5) and have led to the generation of numerous mouse congenital heart disease (CHD) models [2]. The goal here is to provide an introduction to the chapters in Part I as well as to the pathways and models sections; the majority of the references in this introductory chapter are reviews, to which the reader seeking further information is directed. For a detailed description of human heart development, the reader is referred to embryology textbooks such as *Larsen's Human Embryology* or *Langman's Medical Embryology* [3, 4].

1.2 From the Cardiac Crescent to the Embryonic Heart Tube

The central week of mouse gestation, corresponding approximately to weeks 2–5 of human embryonic development, spans the onset of cardiac differentiation through to the completion of cardiac septation (Fig. 1.1). Cardiomyocytes first differentiate in the most anterior lateral region of splanchnic mesoderm underlying the neural folds [5]. Bilateral cardiac progenitor populations converge at the ventral midline to generate the cardiac crescent by embryonic day (E) 7.5 (Fig. 1.1a). These myocardial cells are specified by a combination of positive intercellular signals from surrounding embryonic tissues, including foregut endoderm and surface ectoderm, and negative signals from midline structures. These inputs result in the precise activation of cardiac transcription factors that combinatorially drive the cardiomyogenic program in the cardiac crescent [6].

These early differentiating myocytes form the linear heart tube, with a posterior venous pole and anterior arterial pole, surrounding an endocardial tube; the linear heart tube gives rise to the apex of the left ventricle in the definitive heart (Fig. 1.1b). Cardiac progenitor cells in pharyngeal mesoderm adjacent to the early heart tube subsequently add to the growing cardiac poles, giving rise to right ventricular and outflow tract myocardium at the arterial pole and atrial myocardium at the venous pole [7]. These progenitor cells, known as the *second heart field* (Chap. 3) and discovered in 2001, thus form the major part of the heart, including the entire outflow tract or conotruncal region, connected to the pharyngeal arch arteries. Progressive addition of second heart field cells is regulated by diverse signals from pharyngeal epithelia and neural crest-derived mesenchyme that are in turn integrated by transcription factors such as that encoded by the 22q11.2 deletion (or DiGeorge) syndrome gene *T-box 1* (*TBX1*) [8–10]. Defective addition of second heart field cells results in a spectrum of common congenital heart defects, ranging from conotruncal anomalies such as tetralogy of Fallot and double outlet right ventricle to venous poles defects including atrial and atrioventricular septal defects. As the heart tube elongates, it loops to the right, the first morphological manifestation of the embryonic left-right axis (Fig. 1.1c). The *left-right axis* (Chap. 7) is established in the node during gastrulation and results in the activation of lateralized signaling information that is transmitted to asymmetrically developing organs such as the heart [11]. Dissection of the regulation of second heart field deployment and the establishment and transmission of laterality information are considered to be key steps in understanding the mechanisms underlying common forms of CHD [12].

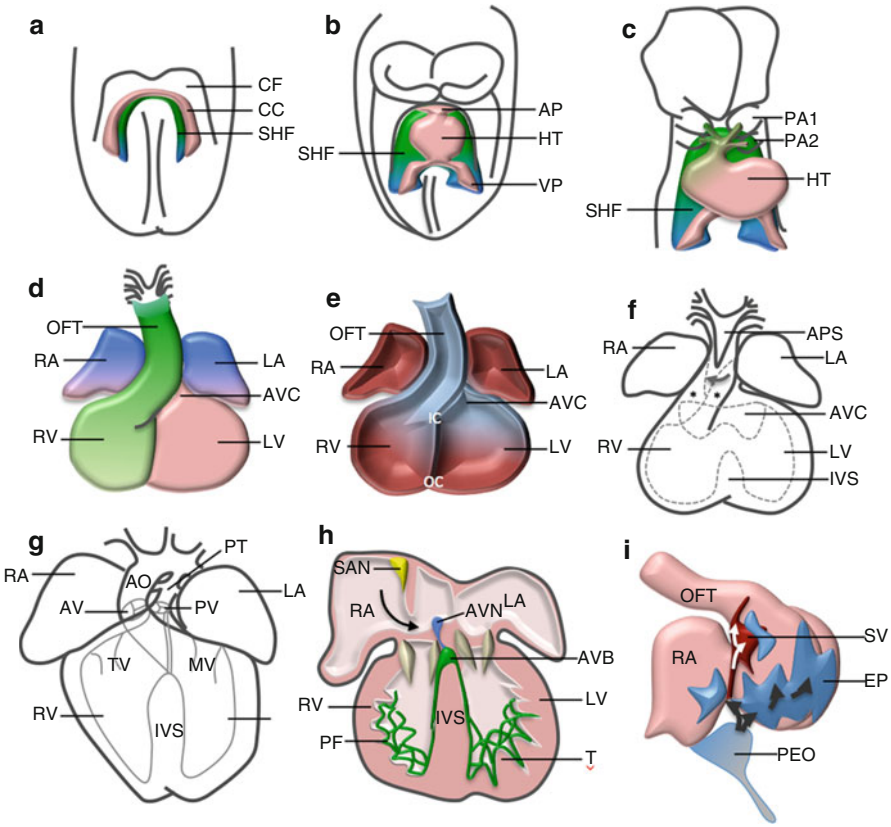

Fig. 1.1 Mouse heart development. Cartoon showing the major events in mouse heart development. (**a**–**d**) From the cardiac crescent (E7.5, **a**), through linear (E8, **b**) and looping (E8.5, **c**) stages to the midgestation embryonic heart tube (E10.5, **d**). The cardiac crescent and its linear heart tube and left ventricular derivatives are *pink* and the second heart field and derivatives *green* (arterial pole) and *blue* (venous pole). At midgestation, atrial and ventricular chamber myocardium (*red*) expands by proliferation at the outer curvature of the heart tube (**e**). Cardiac septation is underway at E11.5 (**f**); the interventricular and aorticopulmonary septa and outflow tract cushions (*asterisks*) are evident and the outflow tract rotates in a counterclockwise direction (*arrow*). Cardiac septation is complete by E14.5 (**g**), including the division of the outflow tract into the ascending aorta and pulmonary trunk. Cardiac cushions have remodeled to form atrioventricular (tricuspid and mitral) and outlet valves (aortic and pulmonary). At this stage, the differentiation of the cardiac conduction system is underway (**h**); the *arrow* shows the direction of propagation of the electrical signal in atrial myocardium. Epicardium spreads over the ventricular surface from the pro-epicardial organ from E10.5 (**i**, right lateral view), and sinus venosus-derived cells contribute to coronary vascular endothelium (Cartoons **a**–**i** adapted from [1]). Abbreviations: *CF* cephalic folds, *AO* aorta, *AP* arterial pole, *APS* aorticopulmonary septum, *AV* aortic valve, *AVB* atrioventricular bundle, *AVC* atrioventricular canal, *AVN* atrioventricular node, *CC* cardiac crescent, *EP* epicardium, *IC* inner curvature, *IVS* interventricular septum, *LA* left atrium, *LV* left ventricle, *MV* mitral valve, *HT* heart tube, *OC* outer curvature, *OFT* outflow tract, *PA1* first pharyngeal arch, *PA2* second pharyngeal arch, *PEO* pro-epicardial organ, *PF* Purkinje fiber network, *PT* pulmonary trunk, *PV* pulmonary valve, *RA* right atrium, *RV* right ventricle, *SAN* sinoatrial node, *SHF* second heart field, *SV* sinus venosus-derived endothelial cells, *T* trabeculae, *TV* tricuspid valve, *VP* venous pole

1.3 Ballooning Morphogenesis and Cardiac Septation

By midgestation (E10.5), looping and heart tube extension are complete (Fig. 1.1d). Subsequent growth of the heart occurs by proliferation of atrial and ventricular cardiomyocytes in the outer curvature of the heart tube, a process known as the ballooning model of chamber morphogenesis (Fig. 1.1e). Patterned proliferation in the embryonic heart is regulated by transcription factors, in particular members of the T-box family, that are expressed in overlapping patterns in the early heart and define the atrioventricular canal and outflow tract as regions of low proliferation where endocardial cushions develop [13]. Cushions contain mesenchymal cells that are derived from the endocardium by a process of epithelial to mesenchymal transformation in response to overlying myocardial signals. The atrioventricular canal and outflow tract regulate directional blood flow through the early heart by valve-like cushion function and reduced myocardial electrical coupling. At this stage, cardiac septation initiates, dividing the heart into separate left and right chambers. The primary atrial septum converges with the developing endocardial cushions in the atrioventricular region. The addition of second heart field progenitor cells at the *venous pole* (Chap. 5) of the heart is essential for the formation of the muscular base of the primary atrial septum and plays a critical role in atrioventricular septation [14]. Atrioventricular cushions also connect with the interventricular septum, developing at the interface between linear heart tube and second heart field-derived myocardium, i.e., future left (systemic) and right (pulmonary) ventricles. At the arterial pole, the influx of *cardiac neural crest cells* (Chap. 4) is essential for the division of the outflow tract into the ascending aorta and pulmonary trunk (outlets of the left and right ventricles) through the formation of the aorticopulmonary septum and outflow tract cushion fusion (Fig. 1.1f). Defects in neural crest cell development result in a spectrum of conotruncal defects including common arterial trunk [15].

The endocardial cushions are remodeled during septation to form the definitive tricuspid, mitral, aortic, and pulmonary valves that regulate blood to and from the ventricular chambers (Fig. 1.1g). The conotruncal region rotates in a counterclockwise direction under the influence of the embryonic laterality cascade, and the aorta becomes aligned with the left ventricle through a process termed wedging. Perturbation of cardiac septation results in failure to make the exclusive connection between the ascending aorta and pulmonary trunk that is essential to separate the systemic and pulmonary circulatory systems at birth. Defects in either heart tube elongation that generates the template for septation or in the septation process itself thus result in a range of CHD. Systemic and pulmonary circulatory systems become fully isolated at birth by the closure of the oval foramen and ductus arteriosus.

1.4 Development of the Conduction System and Coronary Circulation

Chamber development is accompanied by establishment of the definitive *cardiac conduction system* (Chap. 8) that regulates cardiac rhythm (Fig. 1.1h). Electrical activity is triggered by the pacemaker, or sinoatrial node, in the sinus venous region.

Conduction between the atria and ventricles is delayed by the atrioventricular node, ensuring sequential atrial and ventricular contraction. The electrical signal initiates apical ventricular contraction through the atrioventricular bundle, bundle branches, and Purkinje fiber network. Conductive cells are specialized myocytes and share common progenitor cells with nonconducting myocytes [16]. Defects in the establishment of the electrical wiring of the heart result in arrhythmias and conduction blocks. The Purkinje fiber system develops adjacent to the ventricular lumen in trabecular myocytes, transient projections of myocardium that form by signal exchange between the myocardial wall and endocardium. Trabeculae are present in the mouse heart from midgestation to fetal stages when a process of compaction generates the definitive ventricular wall. Failure of this step results in noncompaction cardiomyopathy.

From E10.5, a third cell layer, the *epicardium* (Chap. 6), covers the external surface of the heart (Fig. 1.1i). The epicardium originates in the pro-epicardial organ and forms an epithelium from which cells undergo a mesenchymal transition and invade the myocardium, giving rise to smooth muscle cells associated with coronary arteries and cardiac fibroblasts. Signal exchange between the epicardium, epicardially derived fibroblasts, and myocardium plays an important role in regulating myocardial proliferation and differentiation. Coronary vasculature, essential for the delivery of oxygen and nutrients to the myocardium, develops during fetal stages [17]. The origin of coronary endothelial cells is controversial; however, sinus venosus endothelium and endocardial trapping during compaction are thought to be two major sources. Coronary smooth muscle cells originate from the epicardium as well as neural crest-derived cells. Coronary arteries connect with the base of the ascending aorta to form right and left coronary ostia. Abnormal proximal coronary artery patterning, often associated with conotruncal CHD, is a cause of sudden cardiac death.

1.5 Animal Models of Heart Development

The above summary of heart development focuses on the mouse, where cardiogenesis is highly conserved with the human embryo. The mouse provides the advantages of a highly tractable organism for genetic studies [18, 19]. Gene targeting, either using homologous recombination in embryonic stem cells or the more recent transcription activator-like effector nucleases (TALEN) or clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 strategies, allows detailed investigation of loss of function or potential disease causing alleles [20, 21]. Conditional and inducible gene inactivation using, for example, Cre recombinase, permits precise temporal and spatial evaluation of gene function. In addition to these reverse genetic approaches, forward genetic screens following mutagenesis with, for example, N-ethyl-N-nitrosourea (ENU), allow the identification of novel developmental regulators. Powerful state of the art genotyping and phenotyping technologies for characterizing mouse mutants with congenital heart defects are detailed in Chap. 18. In addition, transgenic mice can be used to study the function of gene regulatory sequences, modify gene dosage, or trace genetic lineages to distinct cardiac structures [22]. While mouse embryos can be cultured in a rolling bottle system for short periods of time, in utero embryonic development makes the mouse a poor model for

experimental embryology. In contrast, the avian system is a model of choice for manipulation of the developing embryo such as, for example, ablation studies, electroporation or chick to quail transplantation experiments [23]. The avian heart is also four chambered, although great artery development diverges from the mammalian configuration. Although transgenic chick lines are now available and gene and regulatory sequence function can be evaluated by electroporation, the chick is currently not a good model for genetic studies.

Other vertebrate models that will be referred to in the following chapters include the frog and fish [24]. *Xenopus laevis*, the clawed frog, has a three-chambered heart, ventricular septation being incomplete; xenopus is however, like the chick, a good model for experimental embryology. Zebrafish has two muscular cardiac chambers and is a highly tractable model for questions concerning the formation and looping of the heart tube, atrial and ventricular patterning, or myocardial differentiation, but not for studies of cardiac septation, the defects of which account for the majority of common human CHD [25]. The zebrafish is also an extremely powerful system for forward genetic screens and has become a model of choice for the study of cardiac regeneration that can occur in adult zebrafish, yet is restricted to the days after birth in mammals [26, 27]. Finally, two invertebrate models have provided very important insights into the genetic regulation of heart development. The protochordate *Ciona intestinalis* has recently been shown to have two cardiac precursor cell populations, similar to the mammalian heart, regulated by conserved transcription factors and signaling pathways. Evolutionarily more distant, dissection of cardiac development in the fruitfly *Drosophila melanogaster* has revealed that fly and mammalian heart development are regulated by a set of homologous genes [28, 29]. Furthermore, the convergence of cardiac progenitor cells in the dorsal midline of the Drosophila embryo is similar to that of cardiac progenitor cells during the formation of the vertebrate linear heart tube. The discovery of the fly *Nkx2-5* homologue *Tinman*, a gene essential for the formation of the fly heart, was a founding event in the molecular study of heart development [30, 31]. Subsequent analysis of *NKX2-5* (NK2 homeobox 5) led to the first mutations identified in human CHD patients, and NKX2-5 remains the most commonly mutated gene in patients with a diverse spectrum of heart defects, illustrating the importance of animal models of heart development for the study of CHD [32, 33].

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Normal Cardiac Anatomy and Clinical Evaluation

2

David J. Driscoll

Contents

2.1 Introduction Normal Heart Anatomy

The heart is a midline structure which is located in the middle, superior, and posterior regions of the mediastinum. Although it is a midline structure the apex of the heart, normally it is positioned to the left of the midline (Fig. 2.1).

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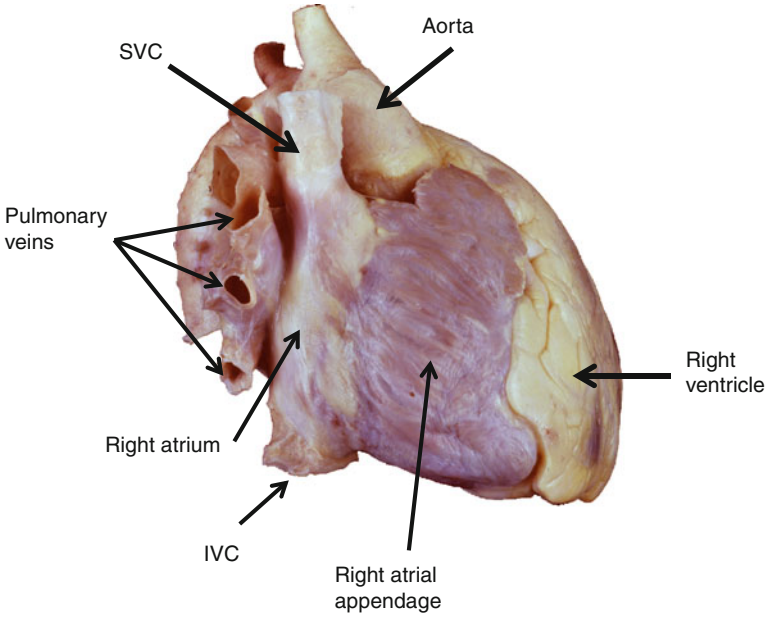

Fig. 2.1 Right anterior oblique external view of the human heart (Photographs the courtesy of Dr William Edwards)

2.1.1 Venous Return to the Heart

The heart receives venous return both from the body and lungs, and from the heart itself. Systemic venous return from the body is through the superior vena cava and inferior vena cavae. Venous return from the lungs is via pulmonary veins, and venous return from the heart is via the coronary sinus as well as sinusoidal veins in the right ventricle.

Normally, the superior vena cava is a right-sided structure as is the inferior vena cava. Usually, there are four pulmonary veins, two from the left and two from the right; however, it is not abnormal to have a total of three or five pulmonary veins. The superior and inferior vena cavae empty into the right atrium. The pulmonary veins empty into the left atrium. The venous return from the heart muscle itself enters the right atrium through a coronary sinus, the os of which is in the right atrium and to the right ventricle via the coronary veins and right ventricular sinusoids.

2.1.2 Atria

There is a right atrium and a left atrium. These structures are not named just for their relative position in the chest but rather for their morphological features. A morphological right atrium normally is on the right side of the body, and a morphological left atrium normally is on the left side of the body. However, there are a number of

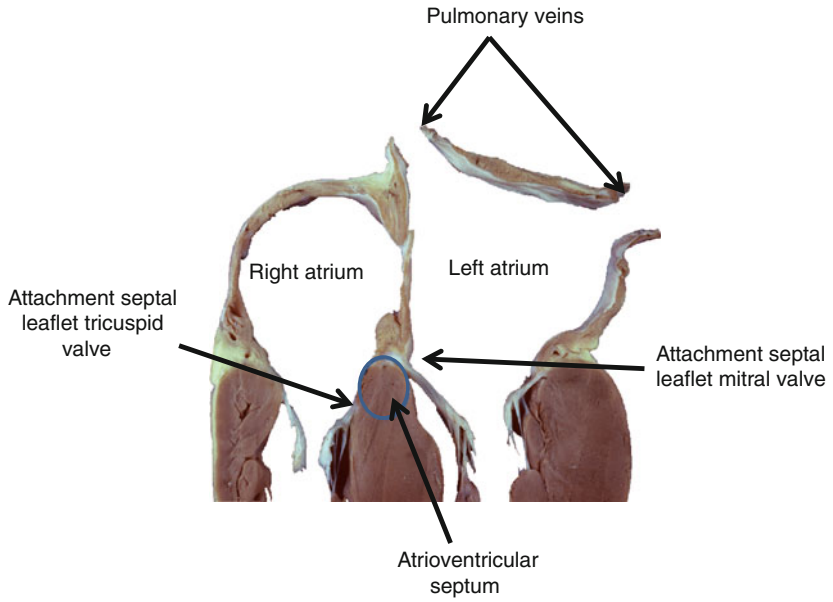

Fig. 2.2 Four-chamber view of a human heart. Note that the septal leaflet of the mitral valve attaches more cephalad than the septal leaflet of the tricuspid valve (Photographs the courtesy of Dr William Edwards)

cardiac anomalies in which the morphologically right atrium may be on the left side of the body and the morphologically left atrium may be to the right side of the body. A right atrium is characterized by the limbus of the fossil ovalis, a large pyramidal-shaped atrial appendage, the presence of the crista terminalis and pectate muscles, and the fact that it receives the vena cava and the coronary sinus. Alternatively, a left atrium is characterized by the presence of a small fingerlike atrial appendage, the absence of a crista terminalis, and the absence of pectinate muscles.

2.1.3 Atrioventricular Valves

There are two atrioventricular valves. The tricuspid valve allows blood to travel from the right atrium to the right ventricle, and the mitral valve allows blood to move from the left atrium to the left ventricle. The tricuspid valve, as its name implies, is a three-leaflet structure. The mitral valve, as its name implies, is a bileaflet structure. The annuli of both of these valves attach to the ventricular septum, but the mitral valve attaches in a more cephalad position than the tricuspid valve (Figs. 2.2 and 2.3). Hence, there is a septum which on one side is in the left ventricle and on the other side is in the right atrium. This is termed the "atrioventricular septum." Another feature of a tricuspid valve is that it has a papillary muscle attached to the ventricular septum, but the mitral valve does not. Mitral valves do not have papillary muscles on the ventricular septum.

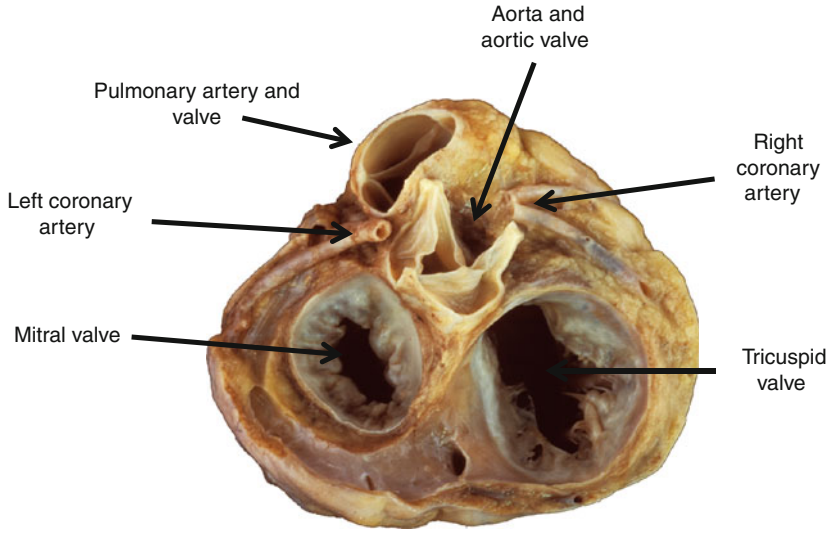

Fig. 2.3 Pathologic specimen demonstrating the relationships of the four valves of the heart to each other and the location of the proximal coronary arteries (Photographs the courtesy of Dr William Edwards)

2.1.4 Ventricles

There is a right ventricle and a left ventricle. These ventricles are not named simply because the right ventricle is on the right and the left ventricle is on the left. In fact the right ventricle is anterior and to the right of the left ventricle. They are morphologically dissimilar. A right ventricle is a thinner-walled structure than the left ventricle. A right ventricle has an inflow portion, body, and an outflow portion. The distribution of muscle in the right ventricle is arranged such that the right ventricle is a more compliant chamber than the left ventricle. It is designed to accommodate changes in volume. The left ventricle is a thicker-walled left compliant chamber than the right ventricle. The orientation of its muscle fibers is such that it is designed to pump higher pressure than the right ventricle is designed to pump.

2.1.5 Semilunar Valves

There are two semilunar valves: the pulmonary valve and the aortic valve (Fig. 2.3). Both of these valves have three cusps. The pulmonary valve has muscle beneath it (a "conus") such that the annulus of the pulmonary valve and the annulus of the tricuspid valve are not contiguous (Fig. 2.3). On the other hand, the aortic valve annulus is contiguous with the annulus of the mitral valve. This is an important distinction when dealing with a number of congenital anomalies of the heart. The

aortic valve is oriented to the right and posterior to the pulmonary valve. The coronary arteries arise from the aorta in the region of the cusps of the aortic valve. There is dilation in the area of the cusps of the aortic valve, and these areas of dilation refer to sinuses of Valsalva. Similarly, there are dilations by the cusps of the pulmonary valve. These are called the sinuses of the pulmonary valve and should not be confused with the term sinuses of Valsalva.

2.1.6 Great Arteries

There are two great arteries, the pulmonary artery and the aorta. The pulmonary artery arises from the right ventricle and divides into the right and left pulmonary arteries. The aorta arises from the left ventricle. The initial branches of the aorta are the right and left coronary arteries. The next branch is the innominate artery that gives rise to the right carotid artery and right subclavian artery. The next branch of the aortic arch is the left carotid artery and the next branch is the left subclavian artery.

The ductus arteriosus connects the aorta and the pulmonary artery. This structure is patent in the fetus but normally should close within several days of birth. The ductus arteriosus remains patent (PDA) in 1 of 2500–5000 live births, but, in infants born prematurely, the incidence is 8 of 1000 live births. It represents 9–12 % of congenital heart defects. Persistent patency of the ductus results in an excess blood volume transiting the pulmonary circulation, the left atrium, and the left ventricle. This can produce signs and symptoms of congestive heart failure. If the PDA is large and is allowed to persist for several years, it can cause pulmonary vascular obstructive disease. Thus, a clinically significant patent ductus arteriosus should be closed with a transvenous device or surgery.

2.1.7 Coronary Arteries

There are two coronary arteries, the right and the left (Fig. 2.3; see also Chap. 43). The left coronary artery rises from the left sinus of Valsalva, and the right coronary artery arises from the right sinus of Valsalva. There are two major branches of the left coronary artery, the circumflex coronary artery and the left anterior descending coronary artery. There are numerous additional branches of the left coronary artery. The right coronary gives rise to the posterior descending coronary artery in about 80 % of the cases. The right coronary artery is also characterized by a cone branch.

2.1.8 Electrical System

The electrical system of the heart consists of a sinus node and atrioventricular node and a His-Purkinje system. The electrical activity of the heart begins in the sinus node (Fig. 2.4). The sinus node is located at the junction of the SVC and

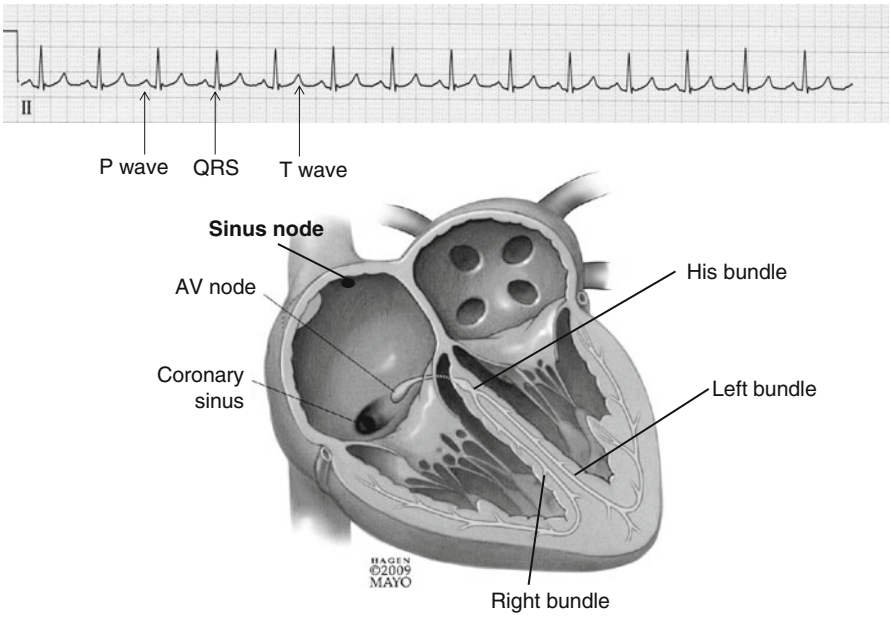

Fig. 2.4 Diagram of the cardiac conduction system

the right atria. The electrical activity travels through both atria. Normally, there is only one route of the electrical activity to travel from the atria to the ventricle and that is through the AV node and bundle of His. Conduction through the AV node is relatively slow and this accounts for the PR segment of the ECG. The AV node is located in the triangle of Koch which is boarded by the tricuspid valve annulus, the tendon of Todaro, and the coronary sinus. After the electrical activity travels through the AV node and His bundle, the electrical activity is distributed throughout the ventricles through the right and left bundle branches and the Purkinje system.

2.2 Techniques, History, and Physical Examination

2.2.1 The History

The historical points of interest, of course, will vary considerably depending upon the age of the patient and the presenting signs, symptoms, and complaints. For an infant, one wants to know if there is a family history of congenital heart disease or premature death and if the baby was exposed to any teratogenic agents and how well the baby is feeding.

For older patients, one needs to ascertain the presence of cardiac symptoms such as dyspnea, shortness of breath, palpitations, syncope, etc.

2.2.2 Physical Examination

Physical examination has four components: inspection, palpation, auscultation, and percussion.

The following can be ascertained by inspection: respiratory rate, chest retractions, pallor, clubbing, cyanosis and differential cyanosis, sweating, quality of fat stores, dysmorphic features, edema, and abnormal superficial vascular patterns

The following can be ascertained by palpation: pulse volume, precordial impulses, thrill, temperature, liver and spleen size, peripheral edema, and ascites.

Percussion is useful to determine the presence of pleural effusion, ascites, and size and position of the liver and spleen.

Cardiac auscultation involves more than just describing the presence of murmurs. It is used to describe the heart sounds, clicks, and rubs in addition to murmurs.

One needs to note if the intensity (i.e., loudness) of the first (S1) and second (S2) heart sound is normal, reduced, or increased. The intensity S2 will be increased if the chest wall is very thin, if there is pulmonary hypertension, or if the aorta is relatively anterior in the chest such as occurs with transposition of the great arteries, tetralogy of Fallot, and pulmonary atresia. S2 normally splits with inspiration and becomes single or nearly single with expiration. If S2 is widely split and never becomes single, one must suspect the presence of an ASD or right bundle branch block.

The presence of third heart sound (S3) is normal in children. It must be distinguished, however, from a mitral murmur or tricuspid diastolic flow murmur which are abnormal.

Heart murmurs result from turbulent blood flow within the heart. They are described by intensity (loudness), frequency or pitch, timing within the cardiac cycle, location on the chest wall, and effects of position of the patient on the quality of the murmur. For systolic murmurs, standard convention is to describe the intensity as from 1–6/6. A grade 4/6 murmur is accompanied by a palpable thrill which is a vibratory sensation felt by the examiner with the examiner's hand applied to the patient's chest. A 5/6 murmur can be heard with the stethoscope removed several centimeters from the chest, and a grade 6/6 murmur can be heard without the use of a stethoscope. For diastolic murmurs, the intensity is described as a range of 1–4/4.

The description of the change of the intensity of the murmur throughout the duration of the murmur is important. A "crescendo-decrescendo" murmur is typical of the murmur produced by aortic or pulmonary stenosis. A "decrescendo" murmur is typical of aortic or pulmonary valve insufficiency. The term "holosystolic" means that the murmur occurs throughout systole.

The frequency or pitch refers to the quality of the sound. Frequency and pitch are described as low, mid, or high. For example, the murmur of a VSD is a low-frequency murmur and that of mitral insufficiency is a high-frequency murmur. The murmurs of aortic and pulmonary stenosis are mid frequency.

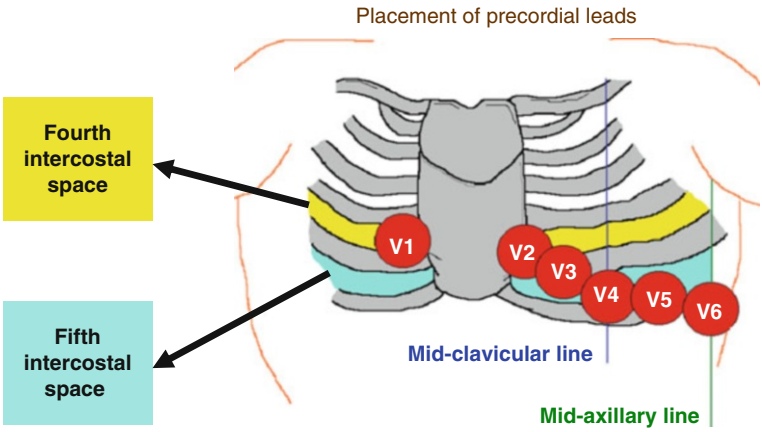

Fig. 2.5 Diagram of the placement of the precordial chest leads for recording an electrocardiogram

2.2.3 The Electrocardiogram

The electrocardiogram (ECG) is the measurement of the electrical activity of the heart. It allows the examiner to determine if the rhythm of the heart is normal. The voltage of the electrical signals is related to the thickness of the heart muscle so it allows the clinician to determine if the heart muscle is hypertrophied. To perform an ECG, leads are placed on all four extremities and six leads are placed on the chest (Fig. 2.5).

2.2.4 Chest X-Ray

The chest x-ray is useful to determine the presence of cardiac enlargement and whether or not there is increased or decreased pulmonary blood flow.

2.2.5 Echocardiography

Echocardiography (cardiac ultrasound) allows one to visualize the heart and to determine the anatomy and accurately define the presence of congenital cardiac anomalies (Figs. 2.6 and 2.7). In addition, using Doppler technology, one can map the flow of blood through the heard and calculate pressure gradients within the heart.

2.2.6 Cardiac Catheterization

Prior to the introduction of echocardiography, cardiac catheterization was necessary to determine the presence or absence and the severity of cardiac defects. It involves advancing a catheter from the femoral vein into the various chambers of the heart

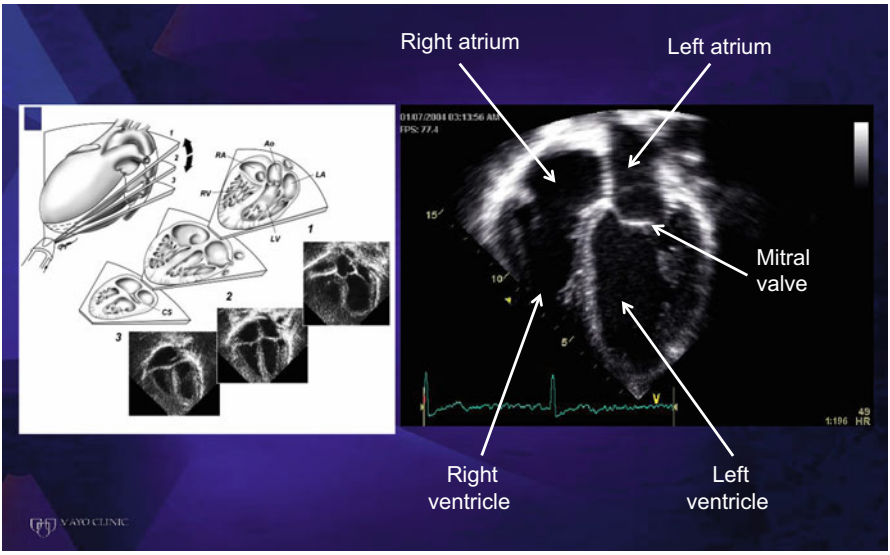

Fig. 2.6 Four-chamber echocardiographic view of the normal heart

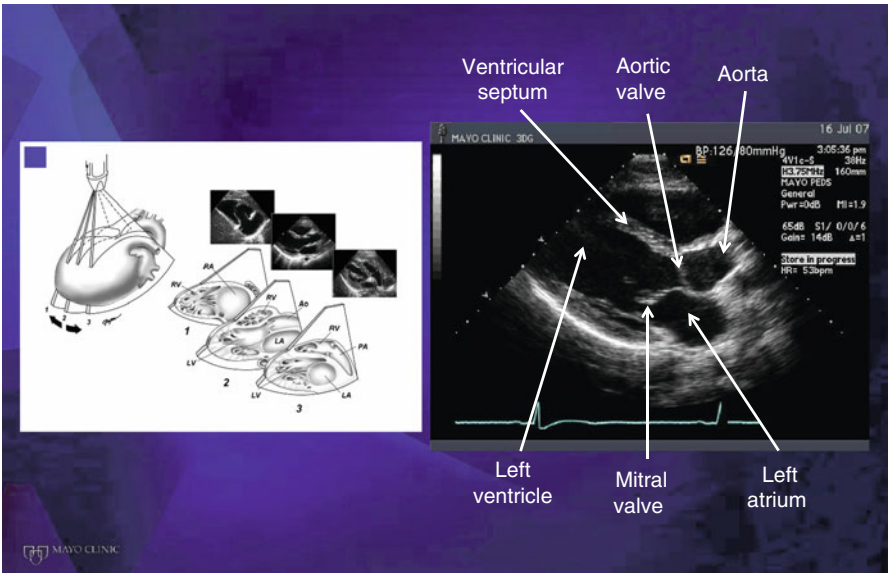

Fig. 2.7 Long-axis echocardiographic view of the normal heart

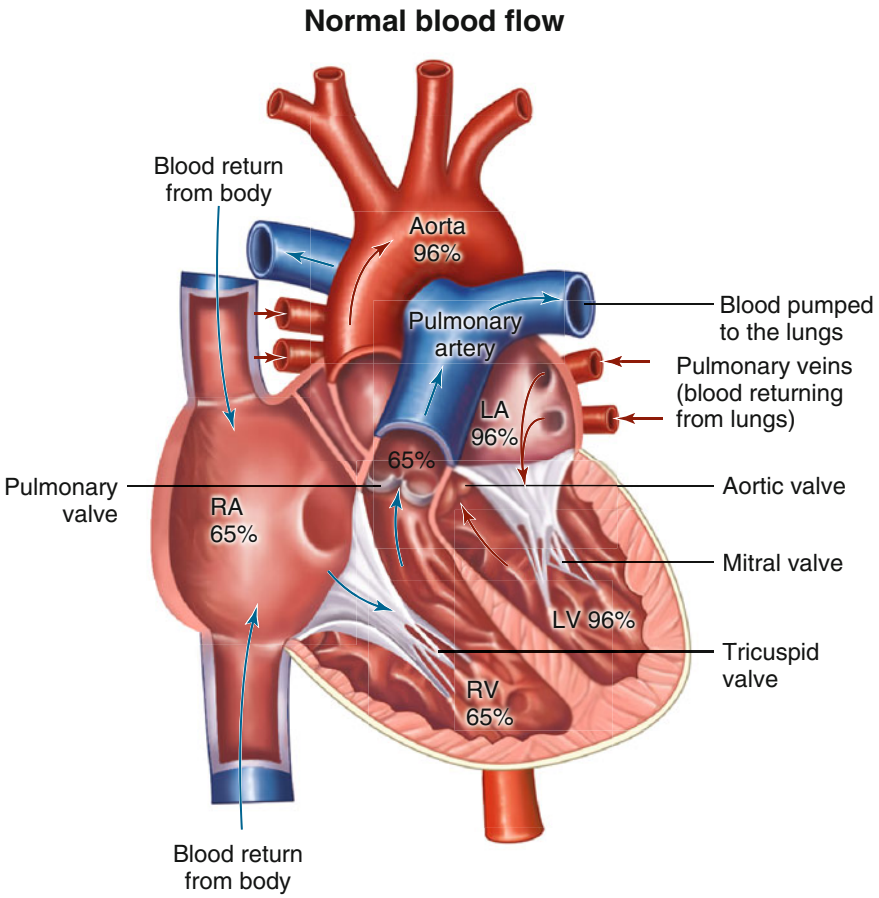

Fig. 2.8 Heart diagram demonstrating blood oxygen saturations in a normal heart. Note that the blood oxygen saturation is 65 % as it returns to the heart and remains at that level until it returns to the heart from the lung. Since, in the lungs, it became oxygenated, the blood returning to the left atrium from the lungs is 96 % and remains at that level as it passes through the left ventricle and is pumped into the aorta and subsequently delivered to the body. Abbreviations: *RA* right atrium, *RV* right ventricle, *LA* left atrium, *LV* left ventricle, *pulm v.* pulmonary valve, *ao v.* aortic valve, *tric v* tricuspid valve, *mitral v* mitral valve

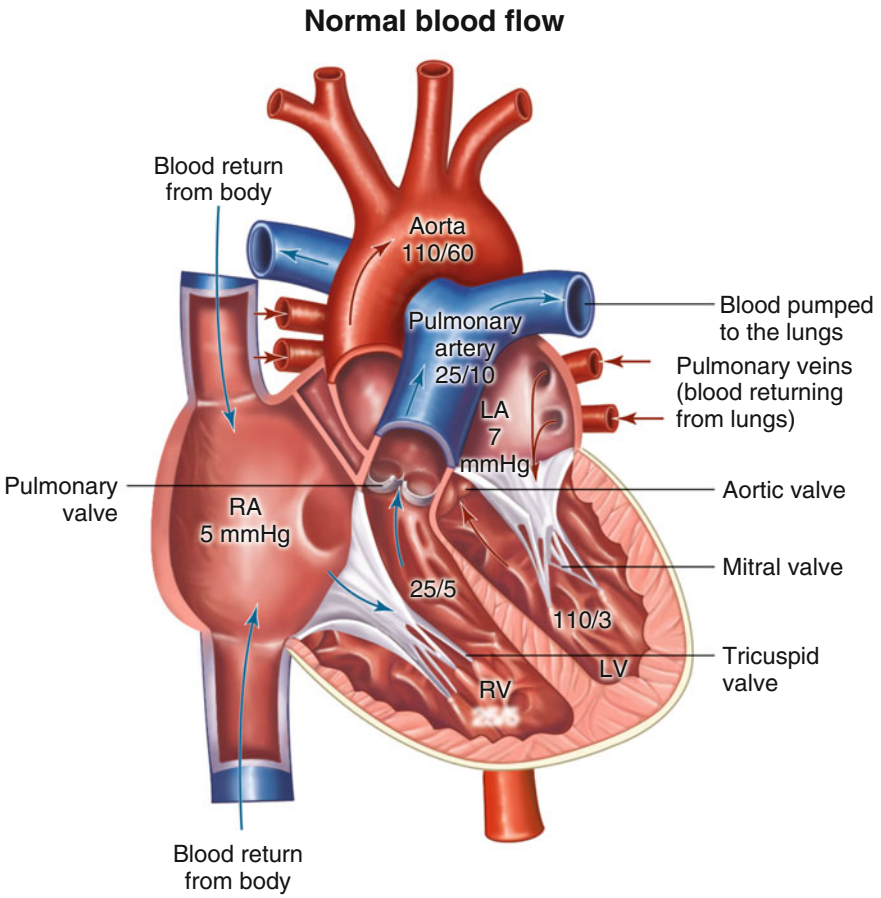

Fig. 2.9 Heart diagram with intracardiac pressures in a normal heart. Abbreviations: *RA* right atrium, *RV* right ventricle, *LA* left atrium, *LV* left ventricle, *pulm v.* pulmonary valve, *ao v.* aortic valve, *tric v.* tricuspid valve, *mitral v.* mitral valve

and measuring the blood oxygen saturations and pressures in the various parts of the heart and blood vessels (Figs. 2.8 and 2.9). In addition, contrast (dye) can be injected into the heart to produce angiographic pictures of the heart and blood vessels. With the advent of echocardiography, cardiac catheterization is used much less frequently to outline the extent of cardiac anomalies.

However, over the past 25 years, a number of "interventional" cardiac catheterization has evolved, and a number of congenital heart defects can be treated in the cardiac catheterization laboratory sparing the patient on open chest operation. Stenotic valves can be dilated; areas of vascular narrowing such as coarctation of the aorta can be dilated or held open with insertion of a stent. Certain atrial septal defects can be closed. Fistulas can be closed. In certain cases, tissue valves can be inserted.

Part II

Development of the Heart and Its Vessels

First and Second Heart Field

3

Margaret Buckingham

Contents

Abstract

The heart forms from the first and second heart fields which contribute to distinct regions of the myocardium. This is supported by clonal analyses which identify corresponding first and second cardiac cell lineages in the heart. Progenitor cells of the second heart field and its subdomains are controlled by a gene regulatory network and by signalling pathways which determine their behaviour. Multipotent cells in this field also can contribute cardiac endothelial and smooth muscle cells. Furthermore skeletal muscles of the head and neck are related clonally to myocardial cells that form the arterial and venous poles of the heart. These lineage relationships, together with the genes that regulate the heart fields, have major implications for congenital heart disease.

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3.1 Introduction and Historical Perspective

The heart is the first organ to form and plays a vital role in pumping nutrients and removing waste as soon as the embryo reaches a size where passive diffusion no longer is sufficient to ensure cell survival. Early heart defects are a frequent source of mortality, estimated to account for as many as 30 % of embryos/foetuses lost before birth in humans [1]. As many as 0.8 % of newborn children in developed countries have some form of congenital heart malformation. These figures reflect the complex process of cardiogenesis which involves the integration of different cell populations with precise spatial and temporal control as the heart undergoes morphogenesis [2] (Fig. 3.1). In the mouse embryo, which provides a mammalian model suitable for genetic manipulation, the early heart tube forms and begins beating at about embryonic day (E) 8 which is equivalent to 3 weeks of human gestation. At this stage the tube is linear, with an anterior outflow tract and posterior inflow region, connecting to the circulatory system. As it grows, the cardiac tube rapidly bends leftwards and then loops to the right so that the mature outflow and inflow tracts, the arterial and venous poles, are aligned anteriorly. The cardiac chambers grow out from the looped tube in a process described as ballooning [3], with formation of septa and valves to control blood flow within the heart. By this stage, neural crest cells of neurectodermal origin migrate into the cardiac tube which is composed of myocardium surrounded by an inner layer of endocardium and an outer epicardium, all of mesodermal origin.

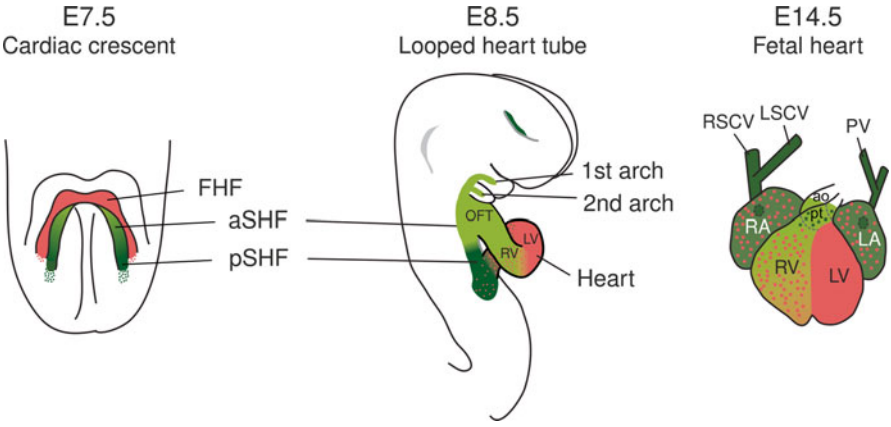

Fig. 3.1 Formation of the heart from the first and second heart fields. Schematic representation of cardiogenesis showing the contribution of the first heart field (*FHF – red*), located in the cardiac crescent, to the myocardium of the left ventricle and other parts of the heart and the anterior second heart field (*aSHF – brighter green*) and posterior second heart field (*pSHF – dark green*) to the myocardium of the arterial and venous poles of the heart, as well as the right ventricle and atria. *Abbreviations*: *LA* left atrium, *RA* right atrium, *LV* left ventricle, *RV* right ventricle, *OFT* outflow tract, *ao* aorta, *pt* pulmonary trunk, *PV* pulmonary vein, *RSV* right superior caval vein, *LSV* left superior caval vein, *E* embryonic day of mouse development

Cardiomyocytes proliferate within the heart tube, and it was thought previously that this accounted for heart growth, with different regions of the early tube prefiguring the future atria and ventricles, in a segmental model of cardiogenesis [4]. However classic experiments in the chick embryo had already pointed to the addition of cells to the growing tube, notably to the inflow region [2]. Investigators subsequently showed that cells are added to the outflow region of the avian heart from adjacent mesoderm [5, 6]. At the same time, in 2001, addition of cells from pharyngeal mesoderm, marked by expression of a transgene integrated upstream of the fibroblast growth factor (*Fgf*) 10 locus, was demonstrated to contribute to the outflow tract and right ventricular myocardium in the mouse embryo [7]. The transcription factor Islet1 also marks these cells, and its expression pattern, together with the *Islet1* mutant phenotype, indicated a contribution to the venous as well as the arterial pole of the heart [8]. This was confirmed later by explant experiments and tracking of cells labelled by dye injection in cultured embryos [9], which also showed that the early heart tube has a mainly left ventricular identity [10]. These findings regarding cardiac progenitor cells that form the developing heart tube were complemented by a different experimental approach examining myocardial cell lineages in the mouse embryo. Retrospective clonal analysis demonstrated that myocardium derives from two cell lineages. The first lineage is the source of left ventricular myocardium, whereas the second lineage is the source of the myocardium of the outflow tract and most of the right ventricle and also contributes to the atria which are derived from both lineages [11]. Thus, cardiac progenitor cells of the first heart field (FHF) form the early cardiac tube, whereas cells of the second heart field (SHF) subsequently enter the developing tube and constitute the second lineage contribution to the heart [2] (Fig. 3.2).

The distinction between these two cell populations is underlined by the early segregation of the first and second lineages, previously estimated to take place at the onset of gastrulation or earlier [11]. This conclusion was drawn from a genetic approach to clonal analysis that depends on a random recombination event in a progenitor cell that converts an inactive *laacZ* reporter to an active *lacZ* sequence. In this case the reporter was targeted to an allele of the cardiac actin gene resulting in b-galactosidase expression in clonally related daughter cells in the myocardium. The size of the clone gives an estimation of the time of recombination. However this is only approximative. Recently [12], another genetic approach to clonal analysis made it possible to have a more precise estimation of the timing of clonal segregation and also to follow all mesodermal cell types in the heart. This approach is based on a conditional *confetti* multicolour reporter and brief induction of a Cre recombinase that activates a single coloured reporter in a progenitor cell and its descendants. In this case the *Cre* is controlled by the mesoderm posterior 1 homolog (*Mesp1*) gene which is expressed in the primitive streak in gastrulating mesodermal cells that will contribute to all compartments of the heart [13]. These experiments confirmed that two myocardial cell lineages form the heart, as previously described, and demonstrated that the first lineage can be distinguished by E6.25–E6.75, at the onset of gastrulation, whereas the second lineage that gives rise to SHF derivatives is marked when the Cre is induced at E6.75–E7.25. A similar genetic approach based on a

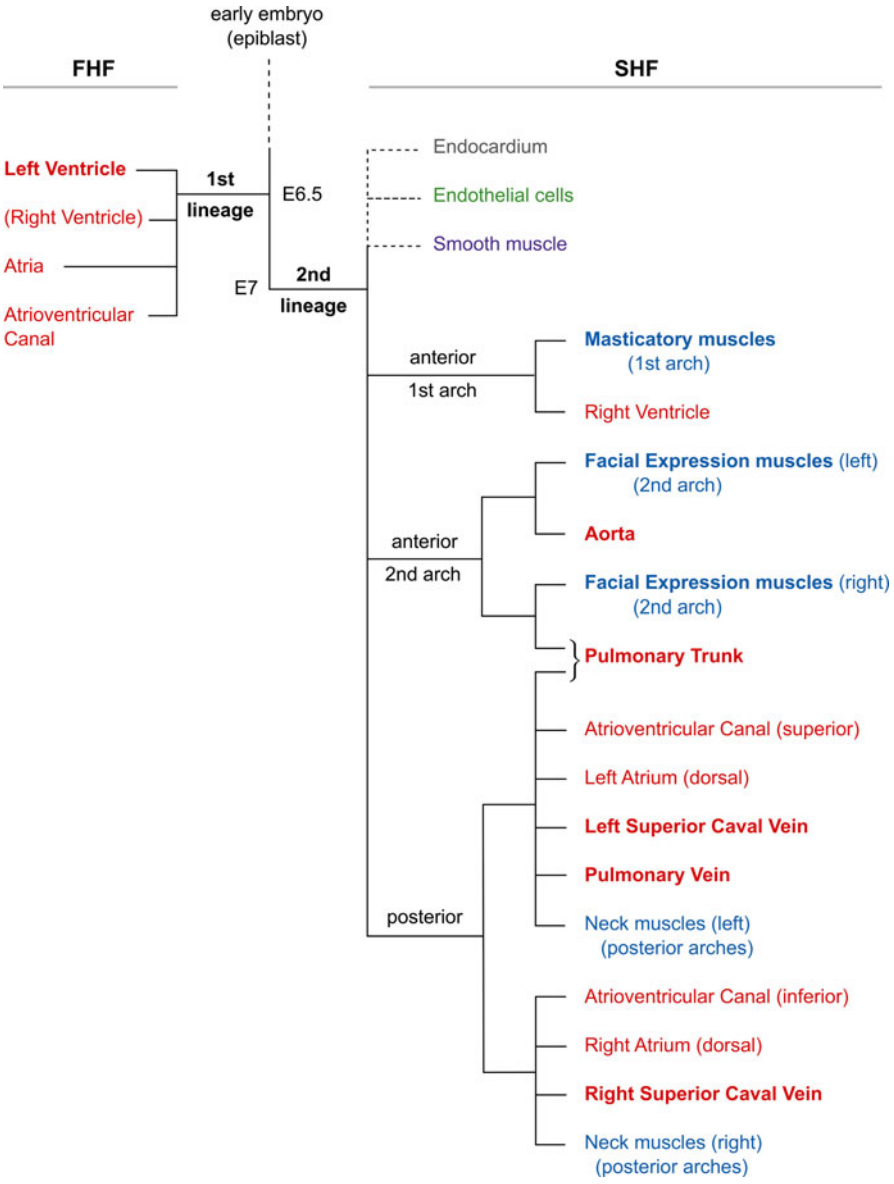

Fig. 3.2 A lineage tree for the heart. Schematic representation of the contribution of first and second cell lineages to the mouse heart. Myocardial derivatives are in *red* and skeletal muscle in *blue*, with bold type indicating that this is the major source, for example, the 1st lineage/first heart field for the left ventricle or the 2nd lineage/second heart field for head muscles, whereas not all neck muscles derive from this source. Other 2nd lineage (SHF) derivatives are indicted with *dotted lines* since their precise relation to each other or to the tree for myocardium and skeletal muscle is not yet clearly defined – endocardium in *brown*, endothelial cells in *green* and smooth muscle in *purple*. The approximate timing of lineage segregation from a common progenitor present in the epiblast is indicated. Abbreviations: *FHF* first heart field, *SHF* second heart field. *E* embryonic day of mouse development

mosaic analysis with double markers (MADM) also showed distinct segregation of two lineages [14]. The common progenitor that gives rise to the two lineages is therefore present prior to activation of *Mesp1* early in gastrulation, before day 12 of human embryonic development.

3.2 The First Heart Field

The first differentiated cells expressing cardiac muscle markers are detected prior to formation of the heart tube, in a region under the head folds that spans the midline of the embryo, known as the cardiac crescent. The early heart tube forms as a result of fusion of the crescent at the midline. This process reflects morphogenetic movements of the underlying endoderm as the embryo develops but also depends on genes expressed in cardiac mesoderm which control cell migration and fusion of the crescent. Not much is known about the underlying cell biology in mammals; however a number of mutations have been identified that perturb the process. Failure to migrate leads to cardiac bifida where two hearts begin to develop on either side of the embryo. Mutation of the gene encoding the transcription factor GATA binding protein 4 (*Gata4*) provides a classic example of this phenotype [2]. The early heart tube expresses transgenic markers for left ventricular myocardium, and dye labelling of these cells followed by embryo culture showed that they will mainly contribute to the left ventricle [10]. Markers of cells in the crescent that distinguish the first from the second heart field have been elusive. For example, the transcription factor T-box (Tbx) 5, implicated in Holt-Oram syndrome [15], is present in FHF cells, but it also marks subdomains of the SHF [16] and is expressed dynamically in the forming heart tube before showing left ventricular localisation. The hyperpolarization-activated cyclic nucleotide-gated potassium channel 4 (*Hcn4*) gene which is expressed later in the conduction system is transitorily activated specifically in the cardiac crescent and thus provides an interesting early marker [17]. However it is probably expressed in myocardial cells at this stage rather than in the FHF progenitors. Induction of a *Mesp1-GFP* (green fluorescent protein) transgene at the early (E6.5) and later (E.7.5) time points, when progenitors for the first and then the second heart fields can be distinguished [12], makes it possible to separate these cell populations by flow cytometry and to perform transcriptome analysis. This approach has already identified a number of differentially expressed genes and should lead to novel markers. Such experiments also open the way to functional analysis, not only for FHF cells present in the crescent but also at earlier stages as they progress through the primitive streak, exit it and then migrate anteriorly to take up their position under the head folds.

3.3 The Second Heart Field

The cardiac progenitor cells of the SHF (Fig. 3.1) are located medially to the cardiac crescent, extending posteriorly. As the crescent fuses to form the early heart tube, these cells come to lie behind the tube and also anteriorly, adjacent to the outflow

tract, as well as posteriorly, adjacent to the sinus venosus and venous pole as it develops. Initially the tube is not completely closed, and there is continuity with the underlying dorsal mesocardium which is attached to the pericardial wall, with some differentiating cells entering the heart dorsally from the SHF [11]. Subsequently the dorsal mesocardium breaks down, as the tube closes. The cardiac tube becomes suspended in the pericardial cavity, with cells of the SHF continuing to feed into the poles of the heart to form myocardium. This process continues over several days in the mouse embryo, with myocardium at the base of the pulmonary and caval veins still being formed as late as E12.5 (40 days of human development). The SHF provides a pool of cardiac progenitor cells which are maintained as a proliferating, non-differentiated population. Transcription factors and signalling pathways that mark these cells [18] play a critical role in regulating this behaviour. Mutations in these genes lead to major arterial pole defects and also to venous pole defects which may be more subtle, reflecting the fact that the outflow tract is derived exclusively from the second myocardial cell lineage and thus is dependent entirely on the SHF contribution. In newborn children with a congenital heart defect, 30 % have a malformation of the arterial pole of the heart. Fortunately this can usually be corrected surgically and the child survives. However, a small proportion of babies die after surgery, and re-examination of the records indicates that in a number of cases there was also a venous pole defect that had not been detected [19]. It is therefore very important to check for subtle venous pole defects which can be corrected at the same time as surgery is performed to correct a defect at the arterial pole.

3.3.1 Regulation of Proliferation and Differentiation

Cells in the SHF are highly proliferative so that the pool of cardiac progenitor cells is maintained as the heart grows. FGF, canonical WNT and Hedgehog signalling promote proliferation [18]. Differentiation of SHF cells is triggered as they enter the poles of the heart. BMP signalling plays an important role in this process, antagonising FGF signalling [20, 21] and promoting the deployment and differentiation of cells that will contribute to the arterial pole. Notch and non-canonical WNT signalling are implicated also in cardiac differentiation [18]. At the venous pole of the heart, canonical WNT, Hedgehog and BMP signalling orchestrate the critical balance between proliferation and differentiation, where Hedgehog signalling promotes the migration of cells to the heart tube as well as their proliferation [22, 23]. These pathways are activated by signals from surrounding tissues as well as by cell autonomous signalling within the SHF [24]. Thus, the midline structures (neural tube and notochord) are sources of canonical WNT and Hedgehog signalling, promoting SHF proliferation. Hedgehog signalling also comes from the endoderm, while FGF signals come from surrounding endoderm and ectoderm as well as from the SHF itself. Neural crest cells, which migrate through the SHF into the arterial pole, mediate BMP inhibition of FGF signalling, such that in the absence of neural crest, excessive SHF cell proliferation results in reduced cardiomyocyte differentiation and defective growth of the heart. BMP signals are also produced by cells in the

SHF, promoting neural crest survival, and by cardiomyocytes, promoting recruitment to the poles of the heart.

An extensive gene regulatory network operates in the SHF to control cardiac progenitor cell behaviour [18]. Key transcription factors modulate signalling pathways, illustrated by Tbx1 interference with the BMP signalling cascade to delay differentiation [24, 25] or Tbx1 activation of enhancers that control *Fgf8* and *Fgf10* expression in the SHF [24–26]. Islet1 also activates the *Fgf10* enhancer [26] and has a SHF enhancer which is itself controlled by forkhead box (Fox) c2 [18].

Some genes expressed in the SHF are specific to the progenitor cell population in this cardiac context. Foxc2, for example, marks SHF progenitors, and Islet1 has long been considered to be the key marker of this field. However, more sensitive reporters now indicate that *Islet1* is also expressed at a low level in FHF progenitors, although the *Islet1* mutant phenotype suggests that it is mainly functional in the SHF [18].

Genes that are involved in myocardial differentiation also are expressed in the SHF. These include genes coding for the transcription factors, NK2 homeobox 5 (Nkx2-5), Gata4, Mef2c, serum response factor (SRF), Tbx5 and the chromatin remodelling protein SWI-/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily D, member 3 (Smarcd3; also called 60 kDa BRG-1/Brm associated factor subunit C1; Baf60c) which marks a subset of early Mesp1-positive cardiac progenitors [18]. These genes are expressed in differentiating cells of the cardiac crescent and in regions of the heart, where combinations of them activate sarcomeric genes. Ectopic expression of Gata4, Tbx5 and Smarcd3 in non-cardiogenic regions of the mouse embryo has been shown to activate cardiac muscle formation [27], indicating the role of these factors in cardiac progenitor cell determination as well as in cardiac differentiation. Their targets in the SHF are not well understood, although transcriptome analysis of *Nkx2–5*-expressing cells gives some insights [28]. Notably, Nkx2–5 functions in a negative feedback loop with Bmp2/Smad1 signalling that controls cardiac progenitor cell behaviour, which is probably of importance in the aetiology of Nkx2-5-related human congenital heart defects. Different combinations of factors are one reason why targets may differ between cardiac progenitors and myocardium. Another consideration is the level of expression which may affect function. The potential importance of modulating the level of these factors is indicated by Tbx1 downregulation of SRF and Tbx5 in the SHF [16, 25]. In the *Tbx1* mutant, abnormal cardiac differentiation of SHF progenitors in the pericardial wall probably reflects this effect as well as deregulation of the balance between BMP and FGF signalling [24]. The SHF enhancer of the *Fgf10* gene provides an example of how levels and combinations of transcription factors can be functionally important [26]. In this enhancer both Islet1 and Nkx2-5 bind to the same sites. In the SHF, Islet1 levels are high, and it competes successfully for binding, leading to activation of *Fgf10*, which also depends on Tbx1. In the heart, on the other hand, Nkx2-5 levels are very high and it displaces Islet1. This leads to downregulation of *Fgf10* in keeping with the repressive function of Nkx2-5 observed for SHF genes, including *Islet1*, in the heart [28]. Since Nkx2-5 also functions as a transcriptional activator in the myocardium this dual role again depends on combinatorial effects of other factors.

Most transcription factors and components of signalling pathways that play a role in cardiogenesis are not cardiac specific. This is in contrast to skeletal muscle where the myogenic regulatory factors of the myogenic differentiation (MyoD) family are only expressed during myogenesis [29]. Analysis of mutant phenotypes is informative for the heart only when the gene does not play a vital role during early embryogenesis. Islet1 or Mesp1, for example, is mainly important for formation of the heart at early stages of development, whereas Fgf8 is required for gastrulation and conditional mutation of *Fgf8* directed to cardiac progenitors is essential in order to understand its role in the SHF. *Mesp1-Cre* lines [13] have proved to be very valuable in targeting all cardiac progenitors, whereas an enhancer of the *Mef2c* gene targets cells in the SHF [30]. It follows that in the human population, many mutations in cardiac regulatory genes may be early embryonic lethal or result in multiple effects as seen in a number of syndromes where the heart is affected. This is the case, for example, of mutations in *TBX1* which give rise to DiGeorge syndrome [31]. This includes major defects at the arterial pole of the heart, such as common arterial trunk or persistent truncus arteriosus as well as craniofacial abnormalities, due to the function of Tbx1 in the SHF as well as in endoderm and ectoderm in the pharyngeal region which is important for cardiac and cranial neural crest. Unless there is some cardiac-specific aspect of function, mutations in enhancers that direct gene transcription to the cardiac progenitors of the first or second heart fields are the best candidates for defects that are restricted to the formation of the heart. Many such enhancers, that specifically direct transcription in the SHF, have now been identified for genes such as *Fgf8*, *Fgf10*, *Islet1*, *Nkx2-5* or *Mef2c* [18]. They are regulated by combinations of SHF transcription factors, as for *Fgf10* [26].

3.3.2 Patterning the SHF

The SHF is patterned on the anterior/posterior axis. Although gene expression has not yet been compared at a single-cell level, regional heterogeneity is apparent. The anterior part of the SHF is marked by genes that are not expressed throughout the field [18]. This is the case for *Fgf8* or *Fgf10* and also for the SHF *Mef2c* enhancer which is not active in the posterior SHF. This anterior/posterior distinction correlates with the contribution of cells expressing these genes to the arterial or venous pole, respectively. This is illustrated in the anterior SHF also, where *Tbx1* is mainly expressed in cells that will contribute to myocardium at the base of the pulmonary trunk [24]; in contrast to *Fgf8*/*10* or the *Mef2c* enhancer which mark progenitors of the right ventricle as well as outflow tract myocardium, both of the pulmonary trunk and the aorta. Differences in the origin of myocardium at the base of these arteries are reflected by differences in gene expression [16]. Pulmonary trunk myocardium, for example, is marked by semaphorin 3c (*Sema3c*) gene expression. In *Tbx1* mutants pulmonary trunk myocardium is reduced [24], which is probably the early abnormality that underlies the development of tetralogy of Fallot and other cardiac defects in DiGeorge syndrome patients.

The posterior SHF is marked also by distinct gene expression profiles. Homeobox (*Hox*) genes, differentially expressed along the axis of the embryo, are classically involved in anterior/ posterior patterning. Anterior members of the *Hox* gene clusters such as *Hoxb1* are expressed in the posterior SHF, under the control of retinoic acid signalling. When this signalling pathway is perturbed, *Hox* gene expression is affected, and the posterior boundary of the SHF is modified [35]. *Tbx5* is expressed in cardiac progenitor cells in the posterior SHF. In addition to atrial myocardium, the dorsal mesenchymal protrusion [33] also derives from the posterior SHF, from cells expressing *Tbx5* and *Islet1*, and its development depends on Hedgehog and BMP signalling [22]. This structure is important in the context of congenital heart malformations since it provides the muscular base of the atrial septum, and anomalies in its formation from the posterior SHF lead to atrial and atrioventricular septal defects. *Tbx18* is expressed in a particular lateral domain of the posterior SHF which is distinguished also by lack of *Nkx2-5* and *Islet1* expression [34]. This domain, which has been proposed to constitute a third heart field, contributes caval vein myocardium, which is marked by continuing *Tbx18* expression. Retrospective clonal analysis distinguishes sub-lineages of the second myocardial cell lineage (equated with the SHF) which contribute to myocardium at the arterial or venous poles of the heart. However this clonal analysis does not distinguish a particular sub-lineage contributing to caval vein myocardium only, as might be expected if this arises from a third heart field [35].

The distinction between anterior and posterior domains of the SHF is made on the basis of gene expression; however, cells may move between these domains. This was first indicated by genetic tracing of cells that had expressed *Hoxb1* in the posterior SHF [32], leading to labelling of myocardial derivatives at the venous pole. Unexpectedly Tbx1-dependent myocardium at the base of the pulmonary trunk was labelled also [16]. In keeping with this result, dye labelling of small groups of cells in the posterior domain of the SHF showed, after embryo culture, that some labelled cells were located in the outflow tract and had therefore moved anteriorly. In so doing these cells activated markers of the anterior SHF, as indicated by *Fgf10* reporter gene expression [36]. Most cells do not appear to move extensively within the SHF and clonal analysis [2] suggests coherent, rather than dispersed, cell growth as the heart forms. However lineage studies for the venous pole of the heart showed unexpected clonality between myocardium at the left side of the venous pole (pulmonary vein, left caval vein and left atrium) and pulmonary trunk myocardium at the arterial pole [35]. This is consistent with anterior movement of a subset of progenitor cells located in the left side of the posterior SHF from which left venous pole myocardium derives. Unexpectedly, Cre genetic tracing experiments show that the classic anterior SHF markers, *Tbx1* [37] and the *Mef2c-AHF* enhancer [16], are transiently expressed in progenitor cells giving rise to the atrial septum and dorsal mesenchymal protrusion. Indeed, recent experiments have shown that loss of function of *Tbx1* impacts not only the anterior SHF but also the posterior SHF, resulting in venous as well as arterial pole defects [16]. The development of the dorsal mesenchymal protrusion is affected, leading to ostium primum and atrioventricular septal defects. Some anterior SHF cells may move posteriorly to contribute to parts of

the venous pole; however, there is no evidence to date for this. Alternatively, diminished proliferation and premature differentiation of cells in the SHF in the absence of Tbx1 may perturb the demarcation between anterior and posterior regions of the SHF. In addition Tbx1 may play a role in the segregation of cells to anterior and posterior domains from a common early progenitor population.

In addition to patterning on the anterior/posterior axis, the SHF also shows left/right patterning. This is driven by the Nodal signalling pathway which leads to expression of the transcription factor paired-like homeodomain 2 (Pitx2) in cells on the left side of the SHF. Pitx2 is highly expressed in the posterior SHF where it promotes a left myocardial cell fate by repressing the acquisition of right ventricular identity [9]. It also tends to repress proliferation so that the left SHF and sinus venosus are less proliferative than the right. At later stages it is implicated also, in conjunction with Nkx2-5, in initiating the formation of pulmonary vein myocardium at the left venous pole [38]. Pitx2 in the left anterior SHF also plays a role in the formation of the arterial pole of the heart. The rotation of the outflow tract, which is essential for the correct juxtaposition of the pulmonary trunk and aorta, depends on *Pitx2*, and, prior to this, genetic interactions with *Tbx1* have been demonstrated in the anterior SHF. Foxh1 is another transcription factor that plays a role in the development of the anterior SHF [39], and mutations in *FOXH1* have been linked to arterial pole defects in humans [40]. This reflects a modification of left/right signalling since Foxh1 functions in the Nodal pathway. Upstream of Nodal, Hedgehog signalling is important for the establishment of left/right asymmetry in the embryo and *Shh* mutants have arterial pole defects resembling tetralogy of Fallot [41] probably due to perturbation of left/right identity in the SHF. These mutants have bilateral expression of Pitx2 and left atrial isomerism [42].

3.4 Non-myocardial Derivatives of the First and Second Lineages

3.4.1 Endocardium, Epicardium and Vascular Endothelial and Smooth Muscle Cells

As the heart tube forms, other cell types of mesodermal origin, in addition to myocardial cells, participate in its development. The endocardium, marked by *Nfatc1* expression, constitutes the inner layer of the cardiac tube and chambers as they develop [18]. It provides an important source of signals that promote ventricular trabeculation and also is important for valve formation, initiated by delamination of endocardial cells from the cardiac cushions. The source of the endocardium has been controversial. It derives from cells that had expressed *Islet1* and *Nkx2-5* or activated the *Mef2c* enhancer, suggesting a SHF origin. Early segregating fetal liver kinase 1 (Flk1)-positive endocardial progenitor cells also have been documented [43]

The epicardium forms the outer layer of the cardiac tube [18]. Epicardial cells delaminate and enter the underlying myocardium where they form the smooth

muscle of the coronary blood vessels, as well as constituting the interstitial cells of the heart. The epicardium derives from the pro-epicardial organ, a transitory mesenchymal structure located near the venous pole of the heart. When cultured, epicardial cells can form myocardium; however whether this is the case *in vivo* is controversial. Cells in the pro-epicardial organ express *Islet1* and *Nkx2-5*, and the latter is required for its formation. However, the relation between the SHF and the epicardium remains unclear.

At the arterial pole of the heart, the arterial tree derives from pharyngeal arch arteries. Pharyngeal arches (1, 2, 3, 4 and 6) are transitory extensions of the pharyngeal region that bulge out as pouches on either side of the embryo from E9. Their mesodermal core can be regarded as an extension of the SHF where cells express anterior SHF genes [18]. Dye labelling of cells in arch 2, in cultured mouse embryos, has shown that they contribute outflow tract myocardium. The posterior arches give rise to endothelial cells of the arch arteries, and as expected for SHF derivatives, targeted mutations in genes like *Fgf8*/*10* [44] or positive regulatory domain I-binding factor 1 (*Prdm1*) [45] lead to malformations of the arterial tree, as well as affecting aorta and pulmonary trunk development.

Isolation of cells, marked by Islet1, for example, from the embryo shows that they give rise to myocardial, smooth muscle and endothelial derivatives, and this has also been reported at the single-cell level [24, 46]. Embryonic stem (ES) cell lines, when directed towards a cardiac fate, give rise to progenitors expressing *Islet1* and *Nkx2-5* [46]. When cloned, these cells prove to be multipotent again forming vascular and myocardial derivatives. However, this does not establish that cardiac progenitors *in vivo* give rise to more than one cell type. Recent clonal analysis using an inducible *Mesp1-Cre* and the conditional multicolour *confetti* reporter [12] now clarifies this issue during cardiogenesis. Cells of the first myocardial cell lineage, that constitute the first heart field, were found to give rise only to myocardial cells. In contrast, cells of the second lineage that populate the second heart field were found to be multipotent with clones contributing to the endocardium and to endothelial or smooth muscle of blood vessels, as well as to myocardium. Most clones in the epicardium did not show labelling with myocardium, pointing to the existence of a unipotent population of epicardial progenitors.

3.4.2 Skeletal Muscles of the Head and Neck

Skeletal muscles of the trunk and limbs derive from somites, segments of paraxial mesoderm located on either side of the neural tube along the anterior/posterior axis of the embryo. Their formation depends on a gene regulatory network in which the transcription factor paired box 3 (Pax3) acts upstream of the myogenic regulatory factors of the MyoD family that are required for cell entry into the skeletal muscle programme [29]. Head muscles have a non-somitic origin and although the myogenic regulatory factors also control myogenic determination and differentiation, Pax3 is not implicated in the upstream gene regulatory network [29]. Extraocular

muscles form from anterior pre-chordal mesoderm, but most head muscles derive from mesoderm of the first two pharyngeal (also referred to as branchial) arches, such that masticatory or facial expression muscles derive from the first or second arches, respectively. The progenitors of these muscles have expressed genes, such as *Islet1*, that characterise the anterior SHF. Mutation of *Tbx1*, for example, leads to abnormalities in jaw muscles as well as to malformation of the arterial pole of the heart [47]. Within the mesodermal core of the arches, cells that have activated myogenic regulatory factors are located in the more distal region compared to cardiac progenitors which continue to express SHF markers in the proximal pharyngeal mesoderm [48]. These observations point to cardiopharyngeal mesoderm as a source of cardiac and skeletal muscle, with overlapping expression of SHF genes in the progenitor cells that give rise to the heart and head muscles. Retrospective clonal analysis shows that both types of muscle derive from a common progenitor. The cardiac actin gene, which was used to drive transcription of the *lacZ* reporter, is expressed in developing skeletal muscles as well as throughout the myocardium. First arc-derived head muscles are clonally related to right ventricular myocardium, whereas second arc-derived head muscles share a common clonal origin with the arterial pole of the heart, masticatory muscles on the left side of the head with myocardium at the base of the pulmonary trunk and those on the right side with myocardium at the base of the aorta [49]. The clonal relationship between head muscles and the heart was also borne out by the observation of co-labelling of cells in the *Mesp1-Cre*/*confetti* system for genetically tracking clones. The results of a clonal analysis of myocardium at the venous pole of the heart indicated unexpected clonality between components of the left venous pole and pulmonary trunk myocardium. This sub-lineage does not include head muscles, leading to the conclusion that two progenitor cell populations contribute to the myocardium at the base of the pulmonary trunk. Since defects in the formation of the pulmonary trunk are frequent in congenital heart disease, this dual origin is important for diagnosis and prognosis.

The neck constitutes a transition zone where some muscles are somite-derived, and others, like the trapezius, sternocleidomastoid or larynx muscles, are marked by genetic tracing with regulatory elements of genes also expressed in the SHF [50]. Clonal analysis of these muscles shows that they share common progenitors with venous pole myocardium, derived from the SHF [51]. Again there is right/left lineage segregation such that muscles on the right side of the neck are clonally related to myocardium at the right venous pole and left neck muscles to the left venous pole. This sub-lineage includes pulmonary trunk myocardium at the arterial pole of the heart. SHF-derived neck muscles show no clonal relationship with head muscles. These lineage relationships further underline the dual origin of pulmonary trunk myocardium (Fig. 3.2).

A discussion of the evolution of the heart and heart fields is outside the scope of this chapter. It is notable that other vertebrates have a SHF. In birds and fish, this is an important source of cells for the arterial pole of the heart [24]. A basic gene

regulatory network, with similarities to that in the mammalian SHF, that involves genes like *Tbx1*, functions in cardiopharyngeal mesoderm in pre-vertebrates such as the ascidian *Ciona*, the sea squirt [52]. Progenitor cells in this mesoderm also give rise to muscle and heart-like structures, pointing to the ancient origins of the relation between cranial muscles and the myocardium [53].

Conclusion

The clonal relationships between progenitor cells that form the heart are important for understanding the potential links between different cardiac malformations. This is illustrated by the SHF contribution to both the arterial and the venous poles of the heart [19]. In the context of the SHF, perturbation of the two different sub-lineages that contribute to pulmonary trunk myocardium, one which also gives rise to head muscles and the other to the venous pole of the heart and neck muscles, will have different clinical manifestations. Effects on the myocardium derived from the second (SHF), as compared to the first (FHF) lineage, may also be associated with endocardial or cardiovascular defects. These considerations are important for diagnosis and prognosis. Defects that reflect cell lineage relationships also relate to gene expression. *Tbx1* mutations, for example, impact pulmonary trunk myocardium and also affect head muscles. Genes that regulate the posterior SHF and affect the venous pole may also affect the formation of pulmonary trunk myocardium and in this case be associated with neck muscle defects, processes also perturbed in *Tbx1* mutant embryos. Perturbation of mesodermal FGF signalling [44] provides an example of how interference with cells of the anterior SHF, where this signalling pathway plays a key role in proliferation, affects both myocardium at the base of the great vessels and the formation of the arterial tree by affecting endothelial cells derived from the posterior arches.

Congenital heart defects due to mutations in genes coding for regulatory factors or signalling pathways that control cardiogenesis are likely to reflect perturbations in the later SHF contribution to the poles of the heart, since earlier effects are more likely to result in embryonic lethality. However, perturbations in the FHF contribution to the atria or the right ventricle, for example, may be compensated by SHF-derived myocardium leading to less drastic malformations. Furthermore, depending on the mutation and on genetic compensatory mechanisms, the cellular source, whether FHF or SHF, may be compromised only partially. Continuing work on the mouse genetic model, as well as on organisms as apparently far-removed as *Ciona*, should continue to provide insight into the origins of congenital heart defects, their diagnosis and prognosis, with the possibility of correction by post- or prenatal surgery and in the future by gene or cell therapy, even perhaps in utero.

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Neural Crest

4

Bijoy Thattaliyath and Mary Hutson

Contents

Abstract

Cardiac neural crest cells (CNCCs) are a subpopulation of cranial neural crest cells that was first described 30 years ago to be required for cardiac outflow septation. Since then the role of the CNCCs in heart development and congenital heart disease has been explored extensively. This chapter will discuss the contributions of the CNCCs to cardiovascular development, the signaling pathways involved in neural crest development, and some of the human congenital heart diseases attributed to aberrant CNCC development.

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4.1 Introduction

The CNCCs were first defined by Kirby et al. 30 years ago [1]. The critical "cardiac" region of neural crest cells, extending from the axial level of the mid-otic placode to the third somite, was documented extensively using quail chick chimeras and ablation studies. This region was defined as "cardiac neural crest" [2] because the cells were found to be critical for several aspects of normal cardiovascular development. Significantly, ablation of this region resulted in persistent truncus arteriosus (failure of outflow tract septation), mispatterning of the great vessels, and hypoplasia or aplasia of the pharyngeal glands. This constellation of defects is observed in patients with congenital heart defects often associated with microdeletions of chromosome 22q11.2 (DiGeorge syndrome and velocardiofacial syndrome, VCFS) as well as CHARGE, Noonan, and LEOPARD syndromes. These initial observations were followed by multiple studies in the mouse using transgenic methods that allowed the neural crest cells to be marked genetically by neural crest-specific promoters driving *lacZ* or *GFP* expression, as well as by Cre recombinase (Cre-lox) technology to lineage trace neural crest cells in mouse embryos. The most commonly used promoters to drive Cre recombinase in neural crest cells are wingless-type MMTV integration site family, member 1 (*Wnt1*)-*Cre*, *paired box 3* (*Pax3*)-*Cre*, acidic ribosomal phosphoprotein P0 (*P0*)-*Cre*, and plexin-A2 (*Plxna2*)-*Cre* [3–5]. These genetic tools also allowed for tissue-specific ablation of many genes within the neural crest cells to address the functional importance of various transcription factors and signaling pathways that impact neural crest and cardiovascular development.

We begin with a description of CNCCs in normal development, including their function in remodeling the pharyngeal arch arteries, outflow tract septation, valvulogenesis, and development of the cardiac conduction system. We also discuss more recent data revealing the interaction of CNCCs with the second heart field, which is important for normal outflow tract alignment and septation. Finally, we discuss some of human syndromes associated with anomalous CNCC development.

4.2 Overview of CNCC Derivatives

The neural crest cells are a population of pluripotent cells that originate and delaminate from the dorsal neural tube, migrate widely throughout the body, and ultimately differentiate into diverse cells types, including melanocytes, cartilage, bone, connective tissue, smooth muscle, and nerves. Neural crest cells are divided broadly into two large axial regions, the cranial and trunk regions. CNCCs are a subpopulation of the cranial crest and represent a transitional region between the cranial and trunk neural crest. While both the cranial and trunk neural crest cells give rise to melanocytes and the peripheral nervous system, only cranial neural crest cells give rise to ectomesenchymal cells. Ectomesenchymal cells take part in the development of a wide variety of structures in the head, neck, and heart. The preotic cranial neural crest contributes significantly to development of skeletal structures of the face. The ectomesenchyme derived from the postotic or CNCC region migrates into the caudal pharyngeal arches 3, 4, and 6 and participates in cardiovascular development

as well as in thymus, thyroid, and parathyroid gland development (Fig. 4.1) [6]. Some of these CNCCs will condense to form the sheath of smooth muscle (or tunica media) around the pharyngeal arch arteries. Importantly, CNCCs also are critical for repatterning of the bilaterally symmetrical pharyngeal arch arteries to form the asymmetric great arteries of the head and neck (Fig. 4.1) [7]. A subpopulation of the

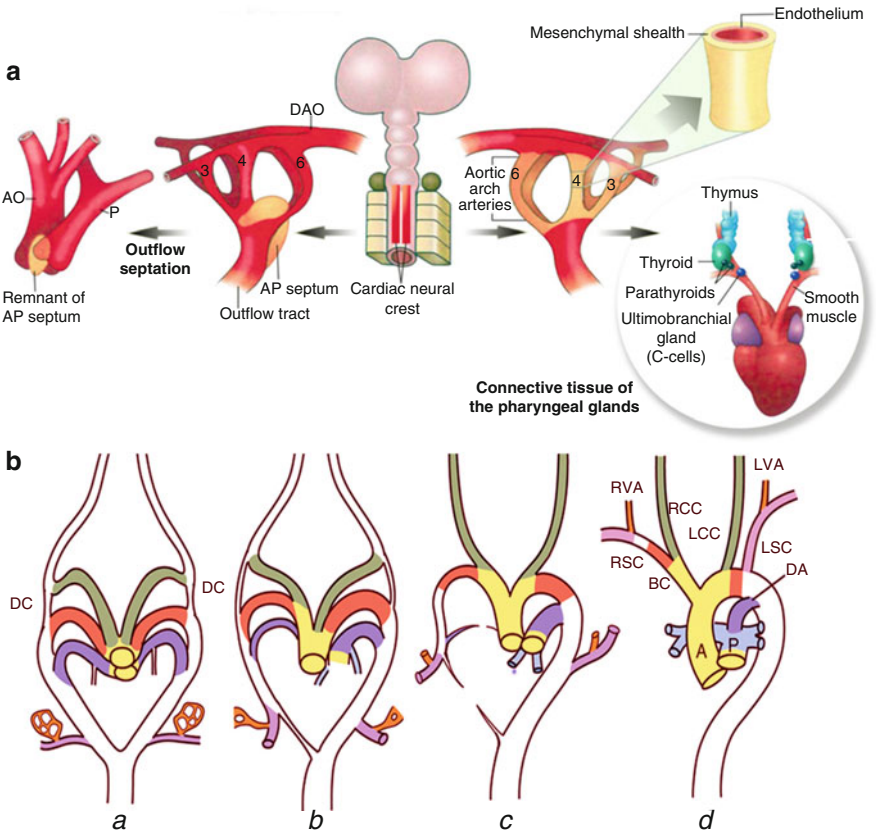

Fig. 4.1 (**a**) Derivatives of the cardiac neural crest cells (CNCC). CNCCs originate in the dorsal neural tube and migrate to pharyngeal arches 3, 4, and 6 and into outflow tract to form the aorticopulmonary septum (*AP*). The AP septum divides the single outflow tract into the aorta (*AO*) and pulmonary trunk (*P*). CNCCs form a mesenchymal sheath around the aortic arch arteries and the connective tissue of the pharyngeal glands. (**b**) Schematic of the development of the paired aortic arch arteries and the subsequent remodeling to form the mature pattern of the great vessels (*a–d*). (*a* and *b*) Diagram of the bilaterally symmetrical aortic arch arteries that carry the early cardiac output to the dorsal aorta which then distributes blood to the mouse or human embryo. (*c* and *d*) The early symmetry is lost when these vessels are remodeled to be the adult great arteries of the thorax. The 3rd aortic arch arteries (*green*) form the right and left common carotid arteries (*RCC* and *LCC*). The left 4th aortic arch artery (*red*) forms part of the transverse arch of the aorta. The right 4th forms the base of the right subclavian artery (*RSC*). The left 6th aortic arch artery (*purple*) forms the ductus arteriosus (*DA*). The 7th dorsal intersegmental arteries (*pink*) form most of the RSC and left subclavian (*LSC*) arteries. Abbreviations: *BC* brachiocephalic artery, *LVA* and *RVA* left and right vertebral arteries, *A* aorta, *P* pulmonary trunk, *DOA* dorsal aorta, *DC* ductus caroticus (Adapted from [50, 51])

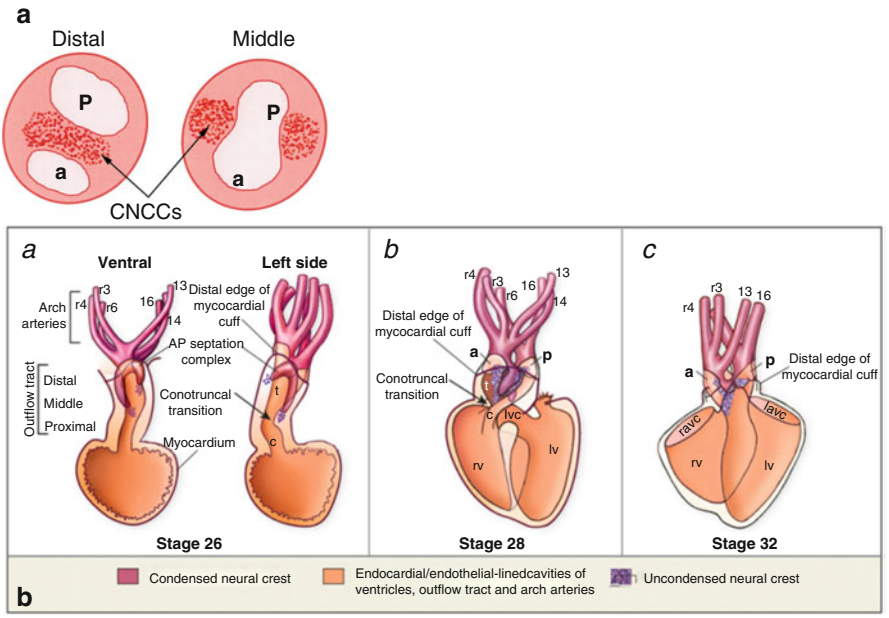

Fig. 4.2 (**a**) Cardiac neural crest cells (*CNCCs*) in the outflow cushions. Cross-section through the distal and middle outflow tract shows position of the condensed CNCC-derived mesenchyme within the cushions to form the future aorta (*a*) and pulmonary trunk (*p*). (**b**) Stages of outflow septation in the chick. (*a*) U-shaped septation complex straddles the aortic sac between the 4th and 6th aortic arch arteries. (*b*) The septation complex grows at the expense of the prongs. (*c*) Septation of the conus (Adapted from Hutson and Kirby [50])

CNCCs in the caudal pharyngeal arches will migrate into the cardiac outflow tract to form condensed mesenchyme at the junction of the presumptive aortic and pulmonary myocardium of the outflow tract (Figs. 4.1 and 4.2) [8, 9]. This condensed mesenchyme, termed the aorticopulmonary septation complex, will divide the most distal outflow into the base of the aorta and pulmonary trunk and the middle outflow into the aortic and pulmonary semilunar valve regions. CNCCs also migrate further into the most proximal outflow tract where they may be involved in closure of the membranous ventricular outflow septum. Recently, a population of preotic neural crest cells has been found to contribute to the smooth muscle of the coronary arteries in mouse and chick [10] and are, therefore, arguably cardiac crest as well. Finally, some of the CNCCs destined for the outflow tract will form cardiac ganglia as well as surround and insulate the bundle of His [11, 12].

4.3 Signaling Pathways Regulating Early CNCC Migration

CNCCs require a wide variety of environmental signals in order to be specified, migrate, proliferate, differentiate, and survive as they leave the neural tube and migrate into the pharynx and the outflow tact. The complex molecular cascade

required to establish the migratory and multipotent nature of neural crest cells is not fully understood. Neural crest cells form at the border of the non-neural ectoderm and the neural plate. Secreted extracellular signaling molecules such as *Wnts*, bone morphogenetic proteins (*BMPs*), and fibroblast growth factors (*Fgfs*) separate the non-neural ectoderm from the neural plate during neural induction [13]. For example, downregulation of *Bmp* signaling in the neural plate accompanied by an increase in *Bmp* signaling in the surface ectoderm results in the activation of the transcription factor *Slug* in the cells at the border between the two tissues. These cells become specified as neural crest. Induction is heralded by the expression of *Pax3* and Zic family member 1 (*Zic1*) transcription factors in the dorsal neural tube followed by activation of neural crest specification genes such as snail family zinc finger (*Snail*), *Slug*, transcription factor AP-2 (*AP-2*), SRY (sex determining region Y)-box 9 (*Sox9*), and forkhead box (*Fox*) D in the neural crest cells [13].

Once induced, neural crest cells require another set of overlapping signaling pathways to leave the dorsal neural tube in a process called epithelial-to-mesenchymal transition (EMT). During EMT the cells lose their cell-cell contacts, reorganize their cytoskeleton, and acquire a motile phenotype to leave the dorsal neural tube. One of the downstream effectors of BMP signaling is Smad-interacting protein 1 (*Sip1*). *Sip1*, an E-box binding zinc finger transcriptional repressor expressed by premigratory and migrating cranial neural crest cells, is known to specifically repress E-cadherin in epithelial cells and thus repress cell-cell adhesion [14]. Downregulation of cadherins is important for EMT and migration from the neural tube. Thus, one of the major roles of BMP in the CNCCs is to activate *Sip1*, which suppresses expression of E-cadherin, allowing the cells to detach from their neighbors.

Release from cell-cell contacts with adjacent cells allows the CNCCs to interact in three dimensions with extracellular matrix components. Migratory neural crest cells are now "mesenchymal" in that they express the intermediate filament protein vimentin and are flattened cells with filopodia and lamellipodia that facilitate movement. CNCCs express a complex collection of integrins, which are receptors that mediate attachment between cells and/or the extracellular matrix. Integrins are important for cell signaling and can influence cell shape and mobility and regulate the cell cycle. In addition to a role in induction, *Wnt* signaling also plays a role in CNCC migration. *Wnt1* is expressed in early migrating neural crest cells and is turned off as the cells migrate away from the neural tube. Mutations in the mouse *DISHEVELLED2* gene, another member of the *Wnt* signaling pathway, result in some of the same cardiac defects seen after cardiac neural crest ablation, including persistent truncus arteriosus [15].

Once the neural crest cells leave the neural tube, they travel along distinct migratory pathways to specific targets. In conjunction with *FGF* signaling, members of the *ephrin* family of ligands and receptors, semaphorins and connexin 43, define specific routes for the CNCC migration. *Ephrin* signaling is likely more important for directing migration into the pharyngeal arches rather than guidance away from the neural tube as seen with the semaphorins. Semaphorins, a group of secreted ligands, and their receptors are also important players in the targeted migration of

the CNCCs to the outflow tract of the heart. The semaphorin receptors plexin-A2, plexin-D1, and neuropilin-1 (*Nrp1*) are expressed by the CNCCs. *Sema6A* and *6B* ligands have been shown to repel neural crest cells, while *Sema3C* attracts them. *Sema3C* is expressed in the outflow tract, whereas *Sema6A* and *6B* are expressed in the dorsal neural tube and lateral pharyngeal mesenchyme [16]. This suggests that CNCCs are driven from the neural tube by *Sema6A* and *6B* and attracted to their target, the outflow tract, by *Sema3C*. Downregulation of each receptor in neural crest cells by RNAi knockdown results in the failure of the cells to migrate into the cardiac outflow tract [16].

4.4 CNCCs and Pharyngeal Arch Artery Remodeling

As described above, the neural crest cells migrate into pharyngeal arches 3, 4, and 6 to participate in the development of the pharyngeal arch arteries. Specifically the CNCCs migrate between the pharyngeal ectoderm and endoderm, proliferate, and envelope the endothelial strands of the nascent aortic arch arteries. The pharyngeal arch arteries initially form as a bilaterally symmetrical series of arteries that connect the aortic sac to the paired dorsal aortas (Fig. 4.1). Remodeling of the pharyngeal arteries requires programmed asymmetrical expansion, regression, persistence, and change of relative position of the different vascular segments (Fig. 4.1). Pharyngeal arch arteries 3–6 are remodeled into the asymmetric great arteries, including the common carotid, definitive aortic arch, and the ductus arteriosus. The right and left common carotid arteries are derived from the right and left 3rd arch artery. The arch of the aorta is derived from the left 4th arch artery. The left 6th arch artery gives rise to the ductus arteriosus. The CNCCs form the smooth muscle tunica media of these arteries [17]. Interestingly, CNCC ablations studies show that CNCCs are not required for aortic arch artery formation but are required for their repatterning [7]. Defects in 4th arch artery remodeling include interrupted aortic arch (IAA), double aortic arch, and aberrant (retroesophageal) subclavian artery. Patent ductus arteriosus and proximal pulmonary artery hypoplasia arise from defects in the 6th arch artery.

Several signaling pathways have been implicated in aortic arch artery patterning. Mutations in the endothelin 1 (*ET1*) pathway in mouse models result in patterning defects of the great arteries and outflow despite normal migration of the neural crest cells [18–22]. Haploinsufficiency for the transcriptional regulator T-box (*Tbx*) 1, by contrast, affects the initial growth and patterning of the arch arteries, the 4th arch artery in particular. Vessel growth failure, poor smooth muscle differentiation as well as abnormal CNCC migration have all been proposed as factors contributing to the defects [23]. The transcription factor encoding gene *Tbx1* is not expressed by the CNCCs but rather by the pharyngeal endoderm, ectoderm, and second heart field (see below). Loss of *Tbx1* indirectly affects the neural crest cells by impacting tissue-specific gene expression and altering the environment through which the neural

crest cells migrate [21, 24, 25]. This in turn influences signaling between the crest cells and surrounding tissues, ultimately leading to altered pharyngeal arch patterning.

4.5 CNCCs and Septation of the Cardiac Outflow Tract and Valvulogenesis

After CNCCs migrate into the caudal pharynx, a subpopulation of cells continues to migrate into the cardiac outflow cushions (Fig. 4.2). The cushions are cardiac jelly-filled ridges that spiral within the outflow tract. The outflow cushions are largely populated by two distinct groups of mesenchymal cells, depending on the proximal-distal location within the outflow tract. In the proximal or conal outflow tract, the cushions are populated by endocardial cells that undergo an EMT induced by the conal myocardium. These mesenchymal cells will form the bulk of the semilunar valve leaflets. CNCCs move into the distal or truncal cushions and form two condensed columns of cells that are joined by a shelf of condensed CNCCs at the aortic sac (Figs. 4.1 and 4.2). The horseshoe-shaped aorticopulmonary septation complex is positioned between the origins of the 4th and 6th pairs of arch arteries where it will divide the pulmonary and systemic circulation [27]. Septation proceeds as the aorticopulmonary septation complex elongates into the distal outflow tract at the expense of the prongs [8, 28]. This splits the common arterial trunk into the aorta and pulmonary trunk (Fig. 4.2). Once the aortic sac and truncal portion of the outflow is divided, the proximal outflow septum in the conus closes zipper-like from distal to proximal toward the ventricles. This is accomplished by the invasion of myocardial cells into the cushions in a process called myocardialization [29]. After division of the outflow tract, cushion mesenchyme is remodeled to form the aortic and pulmonary semilunar valves. Each valve has three cusps or leaflets. The valve leaflets receive a major contribution from the endocardial-derived mesenchymal cells. Neural crest cells are observed at the tip of the semilunar valve leaflets and have been shown play a role in remodeling and maturation of the valves [4, 30, 31]. However, it is unclear to what extent CNCCs play a role in initial valve formation.

4.6 Role of Interaction of CNCCs and the Secondary Heart Field

An unexpected finding from the CNCC ablation experiments in chick was that crest cells are required not only for outflow septation but also for normal heart looping. Abnormal looping is one of the earliest defects seen after cardiac neural crest ablation and can be observed days before the crest cells reach the outflow. Defective looping is caused by a failure of addition of outflow myocardium to the heart tube

from the secondary heart field, a subpopulation of the second heart field [32]. During looping stages, the secondary heart field is located in the ventral caudal pharynx, just behind the outflow attachment to the pharynx. The secondary heart field-derived cells add to the outflow and lengthen heart tube to ultimately form the myocardium and smooth muscle at the level of the semilunar valves. Lengthening of the outflow tract is required for the proper alignment of the aorta and pulmonary trunk over the left and right ventricles. An abnormally shortened heart loop results in arterial pole malalignment defects, such as double outlet right ventricle (DORV) and overriding aorta [32]. Thus the looping defects observed after CNCC ablation suggest that CNCCs [26] are required for normal deployment of the second heart field.

As discussed above, outflow alignment and outflow septation involve distinct cell populations, the second heart field derivatives and CNCCs. The ablation studies suggest that both these cell populations must act together to form a divided outflow tract. Indeed, after CNCC ablation, proliferation in the secondary heart field is increased due to elevated levels of FGF8 indicating that the CNCCs influence FGF signaling in the pharynx [33, 34]. Conversely, inhibition of Notch signaling within the second heart field results in abnormal migration of CNCCs into the outflow tract, suggesting that a Notch-dependent signal from the SHF can modulate CNCC behavior [35]. Thus, coordinated morphogenesis of the two cell populations is essential to form the myocardium and the septation complex to divide the outflow tract.

4.7 CNCCs in Cardiac Innervation and Conduction

CNCCs form the entire parasympathetic innervation of the heart [1]. While the components of the cardiac conduction system are largely innervated by neural crest-derived cardiac ganglia, the essential components of cardiac conduction system are myocardial in origin [36, 37]. Lineage analysis showed that some cells of His-Purkinje fibers lack mesoderm posterior 1 homolog (*Mesp-1*) implying that some of these cells may be of neural crest in origin, but this may just reflect variation in Cre expression [38]. Retroviral studies in chick and Cre-lineage analysis in mouse show a contribution of neural crest cells to the inflow region near the cardiac conduction system [39, 40], but a neural crest contribution to the inflow has not been demonstrated in quail chick chimeras [8]. Another lineage analysis using a *Wnt1-Cre* mouse suggested that the neural crest cells contribute to the central conduction system [41].

Whether or not CNCCs contribute directly to the His-Purkinje conduction system, they are required for normal development and maturation of the cardiac conduction system. A large number of fibroblasts derived from the epicardium, endocardium, and neural crest, are found within the mature conduction system and that insulate the ventricular pathways. Following ablation of CNCCs in chick embryos, there is a delayed maturation of conduction system function stemming from a failure of the conduction bundles to condense [12]. This finding is supported by mouse studies in which deletion of Sp4 transcription factor

(*Sp4*/*Hf1b*) in neural crest cells resulted in atrial and atrioventricular conduction dysfunction due to deficiencies in the neurotrophin tyrosine kinase receptor 3 (*Ntrk3*/*trkC*) [42].

4.8 Failure of Outflow Septation: Persistent Truncus Arteriosus

Persistent truncus arteriosus (PTA) is found in 1 % of patients with congenital heart disease. PTA results from a failure of outflow septation stemming from a failure of the CNCCs in the formation/function of the aorticopulmonary septation complex. This defect is characterized by a single vessel and common valve that originates from the base of the heart supplying the systemic, coronary, and pulmonary circulation. A ventricular septal defect (VSD) and an abnormal truncal valve usually accompany PTA. Further, a substantial proportion of patients with PTA have IAA, which almost always occurs between the left common carotid and left subclavian arteries.

4.9 Examples of Human Syndromes with Defective CNCC Development

4.9.1 22q11.2 Deletion Syndromes

Two overlapping syndromes, DiGeorge syndrome (DGS) and velocardiofacial syndrome (VCFS), are caused by hemizygous deletions of chromosome 22q11.2 [43]. The cardiovascular defects in these syndromes include IAA, PTA, tetralogy of Fallot, or DORV, implicating defective neural crest development. DGS is also often associated with a hypoplastic thymus and varying levels of immunodeficiency. In VCFS varying degrees of craniofacial defects, VSD, aberrant origin of right subclavian artery, hypothyroidism, psychosis, cleft lip, and cleft palate are usually seen. Besides deletions of 22q11.2, these syndromes can also be caused by other deletions or translocations involving chromosome 22q11 [44]. Occasionally no deletions are found. Gene penetrance is variable, and phenotypic expression of the same deletion may have a variable clinical picture. Interestingly, *Tbx1* is one of the genes included in the deleted region. *Tbx1* deficiency in mouse models recapitulates the cardiovascular phenotypes of DGS. As mentioned above, while *Tbx1* is not expressed by CNCCs, it is expressed in pharyngeal epithelia and the second heart field and impacts tissue-specific gene expression altering the environment through which the neural crest cells migrate and differentiate.

4.9.2 CHARGE Syndrome

CHARGE syndrome is characterized by the following defects: *c*oloboma of the eye, *h*eart defects, *a*tresia of the nasal choanae, *r*etarded growth and/or development,

*g*enital and/or urinal abnormalities, and *e*ar anomalies. The collective anomalies associated with *CHARGE* syndrome overlap with DGS-/VCFS-associated defects. The thyroid and parathyroid glands often are absent and accompany aortic arch artery and outflow defects, suggesting neural crest development is affected in this syndrome. Over 90 % of individuals affected with CHARGE syndrome have heterozygous mutations in *CHD7*, a member of the chromodomain helicase DNA-binding gene family [45]. Animal studies show that *CHD7* is required for activation of critical neural crest genes, including *Sox9*, Slug, and twist family bHLH transcription factor (*Twist*), and interacts genetically with Tbx1 [46, 47].

4.9.3 LEOPARD and Noonan Syndromes

Noonan and Leopard syndromes have considerable phenotypic similarities. Noonan syndrome manifests as growth retardation, facial dysmorphism, skeletal abnormalities, hypertrophic cardiomyopathy, and congenital heart defects including pulmonary stenosis, ventricular septal defect, and aortic coarctation. *LEOPARD* syndrome is characterized by *l*entigenes, *E*CG conduction abnormalities, *o*cular hypertelorism, *p*ulmonic stenosis, *a*bnormal genitalia, *r*etardation of growth, and sensorineural *d*eafness. Both these syndromes are caused by defects in the *PTPN11* gene that encodes for the protein tyrosine phosphatase, non-receptor type 11 (SHP2 phosphatase). Approximately half of Noonan syndrome patients have an activating mutation in the PTPN11 gene causing increased SHP2 phosphatase activity and signaling through the Ras/mitogen-activated protein kinase (Ras/MAPK) pathway. Conversely, the vast majority of individuals with *LEOPARD* syndrome carry mutations in *PTPN11* [48, 49] that produce catalytically inactive SHP2 with dominant-negative effects. In animal models, abnormal Shp2 activity results in defective neural crest migration and differentiation.

Conclusion

Over the last 30 years since the discovery of the CNCC, we have come to better understand its role in cardiovascular development. Like any other organ system or pathway, the functions of neural crest cells cannot be completely understood in isolation – rather the role of CNCCs in the heart reflects dynamic interactions with other cells and tissues. The varying severity of cardiac defects with single-gene mutations or identical chromosomal microdeletions reflects the importance of other factors that form the pharyngeal microenvironment with the neural crest cells. The future directions of this field will include deciphering the crosstalk between CNCCs and other cells in the pharyngeal region and heart.

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Inflow Tract Development

5

Andy Wessels

Contents

Abstract

The development of the venous pole of the heart is undoubtedly one of the most complex remodeling events during the formation of the 4-chambered heart. It involves the creation of two separate atrial chambers, the development of the atrial/atrioventricular septal complex, the processes that lead to the incorporation of the pulmonary venous return to the left atrium, and the incorporation of the caval veins and coronary sinus into the right side of the heart. In many of these processes, the dorsal mesenchymal protrusion, a derivative of the second heart field, plays a crucial role.

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5.1 Introduction

During the initial phase of heart development, the first heart field (FHF; see Chap. 3) provides the cells that form the primary heart tube. In subsequent stages, this tubular structure undergoes remodeling and looping, and during this process the heart grows and extends at both ends by addition of cells from the second heart field (SHF). The addition of SHF cells is especially prominent at the arterial pole of the heart (see Chap. 3). Using mouse models that allow the tracing of SHF-derived cells in the developing heart, it has been demonstrated that the entire outflow tract (OFT), right ventricle, and a significant part of the interventricular septum (IVS) are derived from the SHF [1]. The contribution of the SHF to the venous pole, while clinically very important, is less well documented and will be discussed in more detail below.

5.2 Structures Found at the Venous Pole

Before discussing the events involved in venous pole development, we will first discuss the normal features of this complex anatomical area of the heart. In this chapter we consider the venous pole to be that region of the heart containing cardiovascular structures that receive the blood returning from the rest of the body. These structures include the left atrium, receiving oxygenated blood that returns from the lungs via the pulmonary veins, and the right atrium that collects deoxygenated blood returning from the rest of the body via the superior and inferior caval vein and the coronary sinus returning deoxygenated blood from the coronary circulation. In a properly formed 4-chambered heart, oxygenated and deoxygenated blood are kept physically separated at atrial level by the atrial septal complex which is comprised of various components.

5.3 Development of the Venous Pole

In this chapter some of the most relevant events in the development of the venous pole of the heart are reviewed. Because of the complexity of the matter, it is only possible to touch the surface. It also is important to note that there is no universal agreement among cardiovascular developmental biologists regarding the respective mechanisms involved in all the individual steps involved in atrial development or heart development in general. Some of the statements provided in this chapter thus reflect our own insights into this topic and others may have different views and/or different interpretations.

5.3.1 The Dorsal Mesocardium

After its formation, the tubular heart consists of an outer myocardial wall and an inner lining of endocardial cells. These two cell layers are separated by an acellular extracellular-rich space, generally referred to as the cardiac jelly. In the earliest stages of heart development, the primary heart tube hangs suspended over its entire length from the rest of the embryo by a structure called the dorsal mesocardium. As

the heart starts to expand and loop, the dorsal mesocardium disintegrates in the anterior and midsection of the tube, the only remaining part of the dorsal mesocardium being found at the venous pole at the junction of the sinus venosus and atria. At the venous pole, the dorsal mesocardium is a conspicuous structure and a crucial anatomical structure in a number of remodeling events that determine the final architecture of the four-chambered heart.

5.3.2 The Atrioventricular Cushions

As the heart tube remodels, the atrial and ventricular chambers expand by a process sometimes referred to as ballooning [2]. During this process the chambers gradually lose most of the cardiac jelly that was seen between the myocardium and endocardium in earlier stages. However, in the subendocardial spaces at the AV junction and the outflow tract (OFT), an accumulation of cardiac jelly is observed resulting in the formation of prominent swellings, generally referred to as "cushions." While the extracellular matrix-rich cushions in the early stage of their development do not contain any cells, an endocardial epithelial-to-mesenchymal transformation (endo-EMT) generates a cohort of endocardially derived mesenchymal cells that gradually migrate into and populate the cushions. In this process, numerous molecular mechanisms appear to be involved, including the bone morphogenetic protein 2 (BMP2) and transforming growth factor-β 2 (TGFb2) signaling pathway [3–5]. The first AV cushions to develop are the two major AV cushions: the inferior and the superior AV cushion. The inferior AV cushion develops adjacent to the dorsal AV myocardium, while the superior AV cushion is attached to the ventral AV myocardium. As the major AV cushions grow in size and eventually fuse, they divide the common AV canal into the left and right AV orifice. As this process is taking place, two smaller AV cushions start to develop at the lateral walls of the AV canal. These AV cushions are known as the lateral AV cushions [6, 7]. Each of the four AV cushions plays a specific and important role in valvuloseptal development and has a specific fate. However, in particular, the major cushions play a central role in atrial and atrioventricular septation, as they, together with the dorsal mesenchymal protrusion (DMP) and the mesenchymal ridge ("cap") on the leading edge of the primary atrial septum (aka septum primum), form the AV mesenchymal complex which is a crucial structure in atrial and atrioventricular septation [8].

5.3.3 The Primitive Pulmonary Vein

After formation of the looped heart, a strand of endothelial cells can be found within the persisting dorsal mesocardium at the venous pole. This midline structure, which is contiguous with the endocardial lining of the heart, is known as the midpharyngeal strand [9]. The invagination of the endocardium/endothelium into the dorsal mesocardium creates an indentation, known as the "pulmonary pit" that easily can be seen in histological specimens (Fig. 5.1a, b) and by scanning electron microscopy [10, 12]. The midpharyngeal strand will eventually form a lumen and become

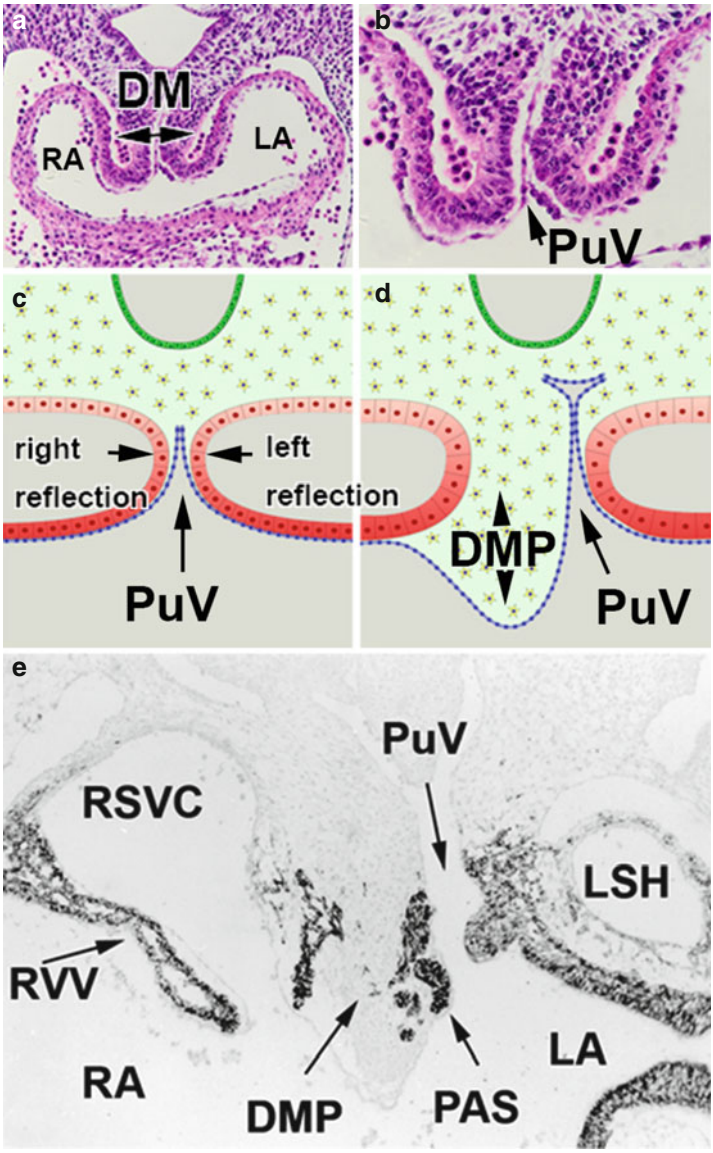

Fig. 5.1 The dorsal mesocardium and development of the dorsal mesenchymal protrusion. Panels **a** and **b** show the dorsal mesocardium at the venous pole of an E9.5 mouse heart. The pulmonary vein is situated in the midline of this structure. Panels **c** and **d** are cartoons depicting the venous pole of the mouse heart at E9.5 (**c**) and E11 (**d**), illustrating how the dorsal mesenchymal protrusion develops between the right dorsal mesocardial reflection and the developing pulmonary vein. Panel **e** shows a section of a human embryonic heart during the 7th week of development, immunostained for atrial myosin heavy chain [10], demonstrating the wedged position of the DMP and the displacement of the mouth of the central pulmonary vein into the left atrium. Abbreviations: *DM* dorsal mesocardium, *DMP* dorsal mesenchymal protrusion, *LA* left atrium, *LSH* left sinus horn, *PAS* primary atrial septum, *PuV* pulmonary vein, *RSVC* right superior vena cava, *RA* right atrium, *RVV* right venous valve (Adapted from: [11])

the primitive (or common) pulmonary vein. During this process, the primitive pulmonary vein also will coalesce with the endothelial network in the developing lung eventually resulting in the formation of the network of pulmonary veins that return oxygenated blood from the lung to the heart [13]. Whereas the primitive pulmonary vein is initially located in the midline of the venous pole [10], the development of the dorsal mesocardial protrusion (see below) to the right of the pulmonary vein [14] and a series of subsequent remodeling events eventually translocate the orifice of the pulmonary vein into the left atrium [15].

5.3.4 The Sinus Venosus

As described in the introduction, after the initial formation of the tubular heart, tissues subsequently are added at the arterial and venous pole during looping. During this process the sinus venosus is formed. Once formed, the sinus venosus consists of a left and right sinus horn and can be seen as a prominent structure in the developing human heart [15], receiving the venous return from the left and right anterior and posterior cardinal veins. As development progresses, the sinus venosus becomes largely incorporated into the back wall of the right atrium, resulting in the formation of the sinus venarum. This process also leads to the incorporation of the orifices of the superior and inferior caval veins, derivatives of the right-sided cardinal veins, and the orifice of the coronary sinus into the right atrium. In the human embryo, the connections of the left-sided cardinal vein and other-left sided venous structures associated with the left horn of the sinus venosus normally regress. This process is responsible for the formation of the coronary sinus. Thus, in the normal human heart, the coronary sinus is the derivative of the left horn of the sinus venosus. Failure of the left sinus horn to regress leads to persistence of the left superior caval vein. It is noteworthy that this regression of the left sinus horn does not occur in the mouse and that a left superior caval vein is part of the normal murine cardiovascular anatomy.

5.3.5 The Dorsal Mesenchymal Protrusion

The atrial septal complex functionally separates the left from the right atrium. In recent years it has become increasingly clear that atrial septation is intrinsically associated with the development of the tissues at the atrioventricular junction [8, 11]. Incomplete formation of elements of the atrioventricular septal complex results in atrial and atrioventricular septal defects [16–19]. The dorsal mesenchymal protrusion (DMP, also known as the vestibular spine) plays a critical role in atrial and atrioventricular septation. The mesenchymal structure that we now know as the DMP was first described in detail in studies of the developing human heart [15]. In these studies it was reported that the mesenchyme of the DMP was immunohistochemically distinct from the endocardially derived mesenchyme of the atrioventricular cushions, suggesting a different origin. With the development of transgenic mouse technology that enabled developmental biologists to perform cell fate studies, it

was determined that the mesenchyme of the DMP, indeed, is not an endocardially derived tissue [8, 20] but instead a derivative of the SHF [16–18]. During its development, the DMP extends ventrally into the common atrium using the dorsal mesocardium as its portal of entry (Fig. 5.1c, d). From the earliest stages at which it can be recognized, the DMP is continuous with two endocardially derived cell populations: the mesenchymal cap that extends along the leading edge of the septum primum and the inferior AV cushion. Whereas the DMP initially develops as a mesenchymal tissue, after completion of atrial septation, the SHF-derived mesenchyme of the DMP eventually undergoes a mesenchymal-to-myocardial differentiation, characterized by a decrease in the expression of islet 1 (Isl1) and an increase in NK2 homeobox 5 (Nkx2-5) in DMP-derived cells. This myocardialized DMP becomes the muscular inferior rim at the base of the oval fossa [18]. In the setting of *ostium primum* defect, a structural heart defect that allows shunting between the left and right atrium and a common abnormality found in all patients with atrioventricular septal defects (AVSDs), this DMP-derived structure is typically missing [14].

5.3.6 The Components of the Atrial Septum

Atrial septation comprises of a series of complicated events that can be summarized (and simplified) as follows. It all starts with a common atrium in which no atrial septal structure can be identified. The first event in the septation process is the development of the primary atrial septum (*septum primum*). The base of the primary atrial septum develops in close association with the left mesocardial reflection of the dorsal mesocardium and grows from the dorsal wall of the common atrium (Fig. 5.1e). As mentioned above, a mesenchymal "cap" is located on the leading edge of the primary atrial septum which is contiguous with the developing DMP. The left and right atria at this stage communicate with each other through the opening under the primary atrial septum, the primary interatrial foramen (*ostium primum*) (Fig. 5.2a). As the mesenchymal tissues of the major AV cushions, the mesenchymal "cap," and the DMP fuse and close the primary foramen [8], a secondary interatrial foramen (*ostium secundum*) forms in the body of the primary septum, allowing continuous shunting of blood from the right to left atrium, critical for the embryonic circulation (Fig. 5.2b). This step is followed by the development of the secondary atrial septum (*septum secundum*). As the secondary septum grows in, it descends into the atrial cavity to cover the secondary foramen (Fig. 5.2c). The mechanism by which the secondary septum develops appears different between man and mice. In the human heart, the secondary septum primarily develops by infolding of the atrial roof, whereas in the mouse active myocardial outgrowth forms the base of its development. After its formation, the secondary septum overlaps with the upper part of the primary atrial septum. As a result, the primary atrial septum basically becomes a temporary one-way valve allowing shunting from right to left but limiting/preventing left-to-right shunting. In the majority of the human population, the secondary and the primary septum eventually fuse, sealing off the potential of left-right

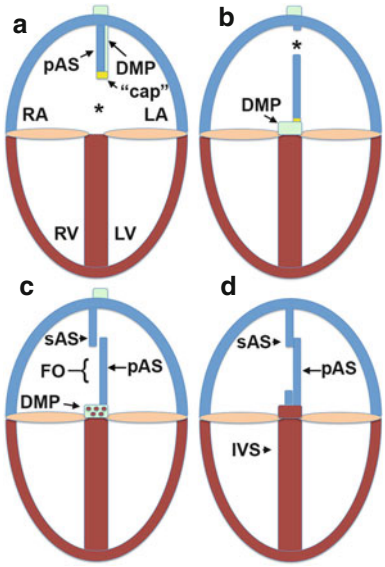

Fig. 5.2 Atrial septation. In this cartoon the series of events that lead to the formation of the atrial septal complex are presented in simplified diagrams. A detailed explanation of the individual steps is provided in the text of this chapter. It is important to note that atrial septation is a complex 3-dimensional event and that the panels in this figure do not truly do justice to this event (additional information can be found in [8]). The *asterisk* in **a** marks the primary foramen (*ostium primum*); the *asterisk* in **b** marks the secondary interatrial foramen (*ostium secundum*). Abbreviations: *DMP* dorsal mesenchymal protrusion, *FO* foramen ovale, *IVS* interventricular septum, *LA* left atrium, *LV* left ventricle, *PAS* primary atrial septum, *RA* right atrium, *RV* right ventricle, *sAS* secondary atrial septum

shunting at the atrial level (Fig. 5.2d). However, in a significant percentage (25 %) of human individuals, the two septa will not fuse, resulting in a condition known as patent foramen ovale (PFO).

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Epicardium and Coronary Arteries

6

José C. Martín-Robles and José M. Pérez-Pomares

Contents

Abstract

Congenital and acquired anomalies and diseases of coronary blood vessels are of paramount clinical relevance. Although important advances have been made in the diagnoses of these conditions, our knowledge of the molecular mechanisms that control the development of the coronary vascular bed and their involvement in

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coronary blood vessel pathophysiology remains incomplete. The aim of this chapter is to provide a succinct account of the key elements of coronary blood vessel development, especially in relationship to the epicardium and its epicardially derived cells. We will discuss the importance of the epicardial cell lineage in coronary blood vessel morphogenesis, from the contribution of epicardially-derived mesenchymal cells to these blood vessels to its role as an instructive signaling center, attempting to relate these concepts to the origin of coronary disease.

6.1 Introduction

Coronary artery disease is a prevalent condition in Western countries. It is intimately associated with risk factors including dietary habits, smoking, and sedentary life, all of which are difficult to manage even in the context of general policies to prevent cardiovascular disease. Quite obviously, disability and early death are the two most important consequences of cardiovascular disease. These chronic conditions significantly contribute to the estimated expense of 192,000 million €/year invested by the European Union to treat cardiovascular diseases. Together with adult coronary artery disease, widely regarded as an "acquired" ailment, congenital anomalies of coronary blood vessels (arteries and veins) have a significant incidence in newborns, with a prevalence ranging between 0.21 and 5.79 %, depending upon the definition of the anomaly and the diagnostic criteria [1].

Coronary anomalies strongly correlate with adult sudden death [2]. However, despite the enormous clinical relevance of coronary blood vessel disease, not much is known about the embryonic developmental mechanisms that control the formation of coronary arteries and veins. Moreover, the genetic networks and molecular signals involved in normal coronary blood vessel morphogenesis and coronary disease pathophysiology are poorly understood.

6.2 The Embryonic Epicardium and Forming Coronary Vessels Constitute a Developmental Unit

6.2.1 Tissue Interaction During Embryonic Development

A major challenge in research into the origins of cardiovascular disease is to increase the tissue-specific resolution of our genetic/molecular analyses. This is so because the molecular interaction between different tissues is instrumental to understand embryonic development, organ homeostasis, and the origin of disease. Accordingly, non-cell-autonomous mechanisms often are involved in the origin of diseases, the instructive tissues carrying the genetic defect and the adjacent, target tissues displaying the morphological and/or physiological anomalies. Several investigators have demonstrated how our knowledge of normal embryonic development is essential to guide us toward the causes of postnatal and adult cardiovascular disease [3, 4]. Thus, understanding coronary blood vessel development will prove critical to improve our diagnostic and clinical approaches to coronary diseases.

As suggested by a myriad of publications, the developing coronary vascular system and the epicardium, the outermost tissue layer of the heart, form a developmental unit [5–7]. The epicardium is required for coronary blood vessels to develop, since it materially contributes cells to the coronary vasculature and establishes the molecular microenvironment necessary for blood vessels to form, grow, and remodel. In order to complete these fundamental participations in coronary development, two essential cellular processes need to be initiated and progress: (1) formation of the epicardium from its extracardiac progenitors and (2) epicardial epithelial-to-mesenchymal transition (EMT).

6.2.2 Formation of the Epicardial Epithelium from Pro-epicardial Progenitor Cells

The origin of the epicardium remained a mystery for decades. We now know that the epicardial epithelium forms from the pro-epicardium, a proliferative mass of mesodermal cells protruding from the coelomic epithelium that covers the *septum transversum* area, i.e., the planar sheet of splanchnic mesoderm that separates the thoracic and abdominal cavities. In order to form the primitive monolayered epicardium, constituted of squamous epithelial cells, pro-epicardial cells, located caudally to the early embryonic heart, are transferred to the myocardial surface. Transference, adhesion, and spreading of pro-epicardial cells over cardiomyocytes are governed by poorly known mechanisms that differ between the animal models considered [8, 9]. In the case of mammals, integrins, especially α4β1 integrin, have been suggested to be critical to these processes [10, 11], but further, systematic studies are required to improve our current knowledge on these critical steps in coronary development.

6.2.3 Epicardial EMT

Once the epicardium has formed over the myocardium, it undergoes a process of EMT that transforms subsets of epicardial epithelial cells into a heterogeneous population of mesenchymal cells referred to as epicardially-derived cells (EPDCs). Although for the sake of simplicity it is stated that the EMT is initiated after epicardium formation, multiple lines of evidence suggest that events required for EMT to occur (such as loss of cell-cell adhesion and disruption of apicobasal polarity) can already be identified in the epithelial component of pro-epicardial cells [12, 13]. It is not the aim of this chapter to define the starting point for EMT in the epicardial cell lineage, but it seems important to consider that some of the molecular mechanisms governing epicardial EMT might have been activated before epicardial cells make contact with the heart muscle. Such mechanisms include the controversial involvement of the cadherin repressor Snail in the loss of cell junctions [14, 15], the activation of extracellular matrix degradation necessary to enable mesenchymal cell migration [16, 17], or even complex transcriptional changes following β-catenin translocation to the cellular nucleus [18]. Still, the possible role played by molecules involved in EMT regulation in specifying the developmental fate of EPDCs remains to be investigated.

6.2.4 Epicardium-Myocardium Molecular Crosstalk

Epicardial-myocardial juxtacrine/paracrine interactions are crucial to the development of these two tissues and have a clear impact on coronary vascular morphogenesis. First, the epicardium is known to produce a largely uncharacterized retinoic acid- and erythropoietin-dependent secretome which is necessary for the compact ventricular myocardium to grow. Different epicardially secreted growth factors like some fibroblast growth factors (FGF9, FGF16, and FGF20) or insulin-like growth factor 2 (IGF2) have been reported to form part of this secretome [19, 20]. The myocardium also secretes pro-vascular vascular endothelial growth factor (VEGF) under the control of HEDGEHOG [21], directly modulating coronary vascular development and patterning by defining a transmural growth factor gradient [22, 23] (see Chap. 11). The complexity of the molecular networks that control epicardial-myocardial interaction is emphasized by the severe epicardial and coronary defects found in mice mutant for the myocardially (but not epicardially) expressed genes friend of GATA 2 (FOG2) and VANGL planar cell polarity protein 2 (VANGL2) [24, 25], encoding a cofactor for Gata transcription factors and a transduction element of non-canonical (PCP) WNT signaling, respectively.

6.3 Diversity of Coronary Cell Lineages

6.3.1 Developmental Potential of Epicardial Cells

Most of the focus of recent epicardial/coronary research has been on the developmental potential of epicardial cells or, more precisely, on cells in the epicardial lineage, from pro-epicardial progenitors to EPDCs. Controversies remain as to whether the pro-epicardium/epicardium contains a multipotent progenitor population able to differentiate into multiple cell types or, on the contrary, cell fate commitment precedes the pro-epicardial stage. This point is indeed of interest if we consider current efforts to identify reliable sources for cardiovascular cells that can be used to repair or regenerate the injured heart and blood vessels.

The pro-epicardium has been shown to display quite different abilities *in vivo* and *in vitro*. Whereas cultured pro-epicardial cells can differentiate into endothelium, smooth muscle, fibroblasts, and myocardium, *in vivo* genetic tracing of (pro) epicardial cells in mouse embryos supports an extensive contribution of this lineage to coronary smooth muscle cells (CoSMC) and interstitial (valvular and ventricular) fibroblasts, and a proportionally lower but conspicuous number of coronary endothelial cells (CoE), the specific contribution reported for this latter cell type strongly depending on the Cre driver used to trace them (Fig. 6.1) [5]. Similar controversy concerns *in vivo* myocardial differentiation from epicardial cells, which is much debated on the grounds that some of the so-called epicardial genes used to drive Cre, like the transcription factor T-box (TBX) 18 [26], are normally expressed in the myocardium or are expressed in cardiac mesodermal progenitors prior to the pro-epicardial stage [5].

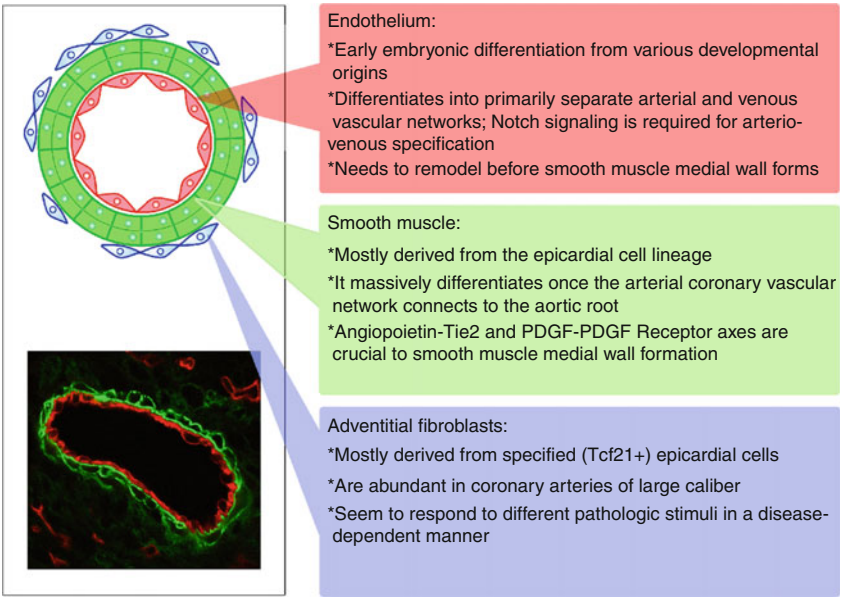

Fig. 6.1 Graphic summary of coronary blood vessel development in relation to epicardial-derived cell lineages. The *upper left* cartoon reproduces the characteristic concentric arrangement of coronary blood vessel cell types (*lower left pannel*; *red*, CoE; *green*, CoSMC)

6.3.2 Hierarchical Differentiation of Coronary Blood Vessel Cell Progenitors

An intense debate about the arterio-venous patterning of the coronary vascular system remains open. Coronary arteries and coronary veins are morphologically different and join the systemic circulation at opposed anatomical positions, since coronary veins drain to the coronary sinus, derived from the posterior (venous) pole of the embryonic heart, and coronary arteries are connected to the aortic root, derived from the anterior (arterial) pole of the developing heart. Not only are coronary arteries and veins anatomically distinct entities, but anomalies in these two vessel types are linked to different cardiac conditions, arteries to sudden death [27] and veins to arrhythmias [28].

A possible strategy to understand the mechanics of coronary artery versus vein development is to dissect the diversity of cell lineages contributing to coronary blood vessel morphogenesis. Recent data suggests that the coronary endothelium (CoE) develops from multiple embryonic cell sources, including different endocardial subpopulations (sinus venosus and ventricular endocardium), (pro)epicardial cells, and blood-borne cells. Indeed, it is difficult to exclude contributions from other tissues to CoE since vascular progenitors are known to be extremely plastic in their phenotype. In this context, it is logical to think that building the complex

arteriovenous coronary vascular tree, which develops independently from the aortic root (the point of eventual connection of the coronary circulation to the systemic one), may require a variety of cell progenitors. This is in accordance with the differentiation mechanics of the primitive coronary arterial vascular plexus, which forms isolated from the coronary venosus component, with a large contribution of ventricular endocardial cells as well as endothelial cells of different origins as mentioned above. However, some authors argue against a multilineage origin of CoE, favoring the reprogramming of arterial CoE cells from a single endocardial source, namely, the sinus venosus endocardium [29]. These authors used a clonal analysis approach to report that 30 % of CoE clones were shared with endocardial endothelium. Unfortunately, the lack of specific Cre lines for genetic tracing of defined cardiac chamber-specific endocardial populations prevents us from making definitive conclusions on this point.

Definitive patterning of coronary blood vessels requires the stable formation of the blood vessel medial wall (Fig. 6.1). Coronary smooth muscle cells (CoSMCs) are, for the major part, epicardial derivatives, although other alternative sources (e.g., neural crest) have been suggested [30]. CoSMC follow CoE differentiation being the maturation of the vessel smooth muscle wall, a late event that is triggered by the initiation of effective coronary circulation. This physiological delay in the differentiation of CoSMC is regulated by epicardial retinoids, working synergistically with VEGF to promote early vascular morphogenesis at the expenses of blood vessel muscularization [31]. It has been interpreted that late formation of the vascular wall permits CoE remodeling and proper patterning from an intricate, immature capillary network, to give rise to the characteristic “inverted crown”-shaped adult coronary vascular system. In this regard, anomalous muscularization of CoE during embryonic development could explain part of the known coronary congenital malformation phenotypes, especially those involving anomalous coronary artery course and termination [1].

A much neglected coronary cell type is that of coronary fibroblasts (Fig. 6.1). Differentiation of coronary adventitial fibroblasts (CoF) takes place once CoSMC have initiated their maturation to form the fibrous adventitia layer that appears in coronary arteries of large caliber. This adventitial layer includes a high number of epicardial-derived fibroblasts, a cell type characterized by the early expression of the transcription factor TCF21 [32], although it cannot be ruled out that heart fibroblasts of a non-epicardial origin, such as the endocardium, neural crest, or bone marrow (see [33]), can also locally contribute to the formation of the coronary adventitia.

Epicardial-derived CoF are ontogenetically related to ventricular interstitial fibroblasts [34], but their distinct location and different response to cardiovascular disease (e.g. perivascular fibrosis followed by diffuse interstitial fibrosis in pressure-overload models versus the massive, acute inflammation-induced fibrosis of the ischemic heart [35, 36]), suggests these two cardiac fibroblasts populations have diverged during their differentiation and maturation from common progenitors. Deciphering the signals that elicit specific fibrotic responses from different cardiac fibroblast populations is an important future challenge in the cardiovascular field, and some recent reports have undertaken the important task of identifying the origin of distinct populations of CFs as related to specific cardiac disease [37]. An important step will be

overcoming the limitations raised by the poor specificity of current so-called fibroblast markers such as vimentin, fibroblast-specific protein 1 (Fsp1), discoidin domain receptor 2 (Ddr-2), or cluster of differentiation 90(CD90/Thy1) [33].

Conclusion

We have shown that multilineage contribution to coronary blood vessel development during the normal patterning of the embryonic and prenatal coronary blood vessel system may be a substrate for the initiation of adverse responses to pathologic stimuli. Furthermore, we have highlighted the importance of cell and tissue interactions during coronary blood vessel formation and identified the characteristic transcriptional and signaling networks whose disruption could drive diverse forms of coronary disease, to finally conclude that extensive research in the field is required to increase our knowledge on coronary blood vessel disease.

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Establishment of Cardiac Laterality

7

George C. Gabriel and Cecilia W. Lo

Contents

Abstract

The formation of the heart with its complex arterial and venous connections in vertebrates is critically dependent on patterning of the left-right axis during early embryonic development. Abnormalities in left-right patterning can lead to a variety of congenital heart defects, life-threatening birth defects present in nine out of every one thousand infants born each year in the United States. Recent work has uncovered a highly conserved pathway responsible for left-right axis specification. This pathway involves ciliated cells in the embryonic node that induce asymmetric activation of a nodal signaling cascade ultimately driving left-sided expression of the Pitx2 transcription factor. We summarize recent insights gained from studies in animal models, and present a brief overview of the complex processes regulating the specification of cardiac asymmetry, and its contribution to congenital heart defects.

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7.1 Introduction

Studies using animal models rapidly have advanced our knowledge of the mechanisms driving cardiac morphogenesis, a process in which a left-right asymmetric, four-chambered heart is formed from an initially symmetric heart tube. It is now recognized that the developmental specification of cardiac asymmetry is a complex and dynamic process that begins very early in development with breaking of symmetry at the embryonic node. This comprises an organizing center that induces asymmetric gene expression that is propagated to the surrounding tissue to define distinct asymmetric patterning of visceral organ situs. Normal development results in visceral organ situs referred to as situs solitus where the heart, stomach, and spleen are on the left, with three lung lobes on the right and two on the left, and a stereotypical pattern of liver lobation and gut rotation. With defects in left-right patterning, the left-right body axis can become mirror symmetric, referred to as situs inversus totalis, or the situs can be affected differently across multiple organs in the thoracic and abdominal cavities, causing heterotaxy.

Heterotaxy patients are known to suffer high morbidity and mortality. This often is due to the co-occurrence of congenital heart defects. In fact, a recent epidemiological study found that of 517 cases of laterality defects, 82.2 % had some form of congenital heart disease [1]. More specifically, of the heterotaxy patients examined, 96.6 % presented with congenital heart disease [1]. These findings highlight the importance of left-right patterning in cardiac morphogenesis. In addition to congenital heart disease, heterotaxy patients also may have other visceral organ defects such as gut malrotation, biliary atresia (the bile duct is considered to be a right-sided structure), or asplenia/polysplenia (the spleen is a left-sided structure) [2]. High morbidity and mortality also can be seen with gut malrotation and biliary atresia, with biliary atresia often requiring liver transplant as the only effective palliation.

7.2 Cardiac Asymmetry and Oxygenation of Blood

The heart is in fact an asymmetric organ, sitting in the left side of the chest cavity with the apex pointing to the left, i.e., levocardia (Figs. 7.1 and 7.2). The pattern of blood flow through the four-chambered heart highlights the striking anatomical and functional asymmetries that have evolved to provide separate systemic vs. pulmonary circulation. Thus, deoxygenated blood flows from the body to the right side of the heart and then to the lung, and oxygenated blood flows from the lung back to the left side of the heart and systemically back to the rest of the body. This pattern of systemic and pulmonary blood flow connected as a circuit in series allows efficient oxygenation of blood from the lung. A disruption of this pattern of segregated blood flow from the right vs. left side of the heart can cause high morbidity and mortality. This can arise from failure in partitioning of the atrial and/or ventricular chambers, outflow tract malalignment, septation defects, or defects in cardiac valve development required for unidirectional blood flow.

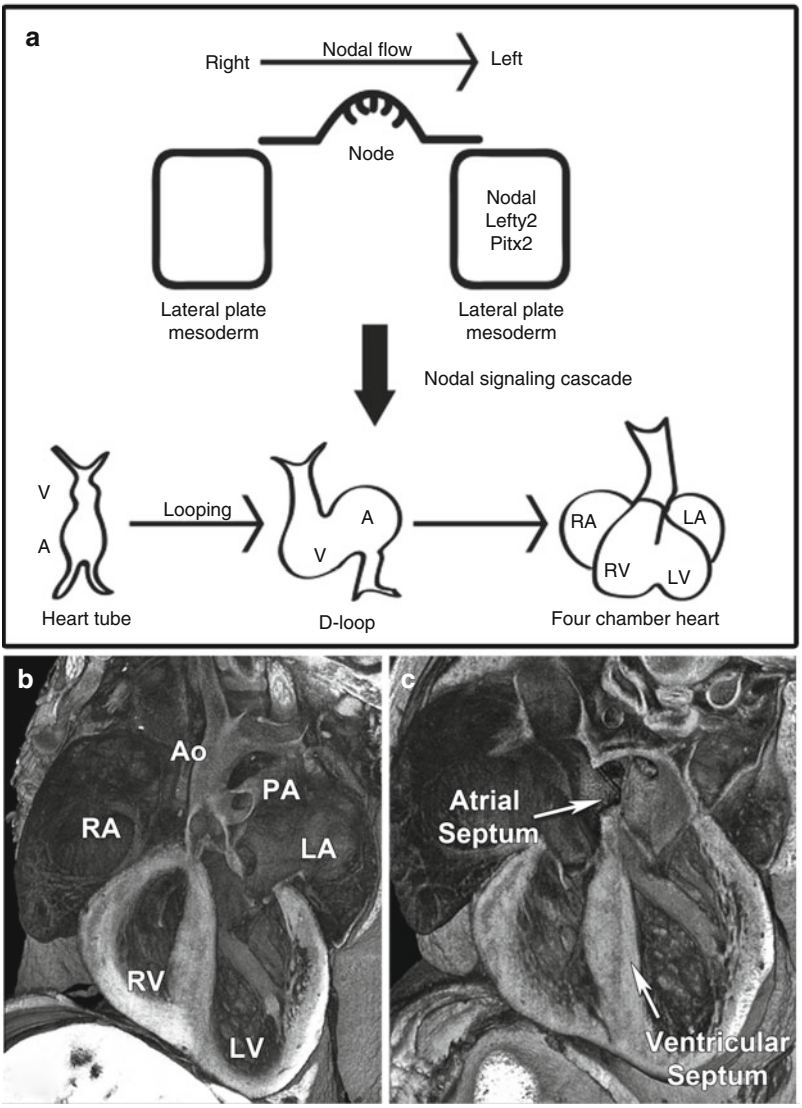

Fig. 7.1 Specification of cardiac asymmetry. (**a**) Cilia generated leftward flow at the embryonic node initiate an asymmetric signaling cascade in which *nodal, lefty2, and pitx2* are expressed exclusively in the left lateral plate mesoderm. This left-sided expression has important consequences for heart looping, a process in which the initially symmetric heart tube undergoes dextral looping to form the four-chambered heart positioned on the left side of the thoracic cavity. (**b**) Normal intracardiac anatomy in the newborn mouse heart visualized by histopathology examination using episcopic confocal microscopy reveals the arrangement of four cardiac chambers (*RV/LV*, *LA/RA*) with two great arteries (*Ao/PA*), with the aorta (*Ao*) seen arising from the left ventricle (*LV*) [45]. (**c**) Histopathology examination of a newborn heart at a different episcopic confocal microscopy imaging plane showed the normal intracardiac anatomy of the interatrial septum separating the right and left atria and the interventricular septum partitioning the left and right ventricles. Abbreviations: *V* ventricle, *A* atria, *LA* left atrium, *RA* right atrium, *LV* left ventricle, *RV* right ventricle, *Ao* aorta, *PA* pulmonary artery

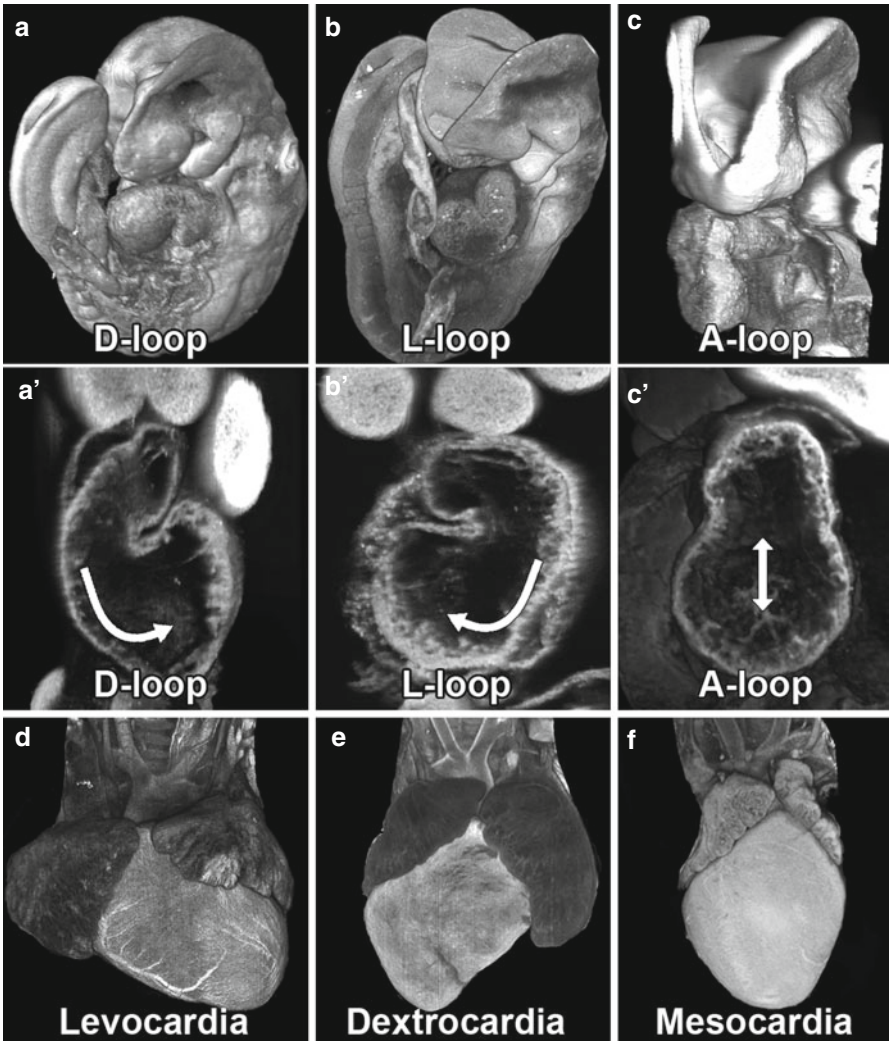

Fig. 7.2 Heart looping and heart situs. The linear heart tube undergoes dextral looping (**a**, **a**') yielding levocardia with heart apex pointing to the left and positioned on the left side of the chest cavity (**d**). Defects in left-right patterning can perturb heart looping, resulting in reversed looping (L-loop); **b**, **b**' with heart apex pointing to the right, or dextrocardia (**e**), or abnormal looping (A-loop, **c**, **c**'), with midline cardiac situs, or mesocardia (**f**). 2D images and 3D reconstructions were obtained by episcopic confocal microscopy. *Arrows* specify direction of heart rotation [45]

7.3 Complex Congenital Heart Disease and Heterotaxy

The finding of complex congenital heart disease in patients or mice with heterotaxy is well described. Mice are readily suited for modeling human congenital heart disease; as being air-breathing animals, they have the identical four-chambered

cardiac anatomy as humans [3]. Some examples of congenital heart defects found in mutant mouse models with heterotaxy are shown in Fig. 7.3. Compared to normal levocardia (Fig. 7.3a), mice with laterality defects can exhibit mirror-image dextrocardia, which is the complete reversal of normal heart structure (Fig. 7.3b). Aberrant left-right patterning also can lead to more complex phenotypes, such as ventricular dextroversion in which the heart exhibits dextrocardia, but the ventricular chamber placement and outflow tract alignment are not altered (Fig. 7.3c). Other examples of phenotypes associated with abnormal left-right patterning include transposition of the great arteries (TGA), a condition in which the connections between the ventricles and the outflow tracts are reversed (Fig. 7.3d), double-outlet right ventricle (DORV) in which both outflow tracts originate from the right ventricle (Fig. 7.3e), and atrioventricular septal defect (AVSD) with complete failure of the atrioventricular septum, resulting in a hole that allows communication between all four cardiac chambers.

7.4 Modular Assembly of Cardiac Asymmetry

While surgical palliation now allows many patients with congenital heart disease to survive their initial acute cardiovascular deficits, even those with complex structural heart defects, there remains high morbidity and mortality in these patients. To track and study the emergence of cardiac asymmetry and its disturbance, Van Praagh developed the "segmental" approach for the classification of cardiac asymmetry [4]. This approach utilizes a three-part lettering system to describe: (1) the atrial situs, (2) the direction of heart looping, and (3) the relationship of the great arteries [5]. In this manner, atrioventricular and inflow/outflow tract identities can be summarized and tabulated across a patient cohort. Thus normal cardiac situs would be described as (S,D,S) – solitus (S) or normal atria situs, normal dextral (D) looping of the heart, and solitus (S) or normal arrangement of the great arteries. What is striking with regard to patterning of cardiac asymmetry captured by the Van Praagh classification scheme is that left-right patterning of the different segments of the heart, i.e., the atria, ventricle, and outflow tract, can be specified in a modular fashion independent of each other. Thus it is possible to have a heart with two left or two right atria (referred to as left or right atrial isomerism), or a left atrium connected to a right ventricle, but on the body's right or left side. It also is possible to have the pulmonary artery connected to the left ventricle and the aorta to the right ventricle, i.e., transposition of the great arteries, and this can occur with the ventricles positioned in their normal left-right position (d-TGA) or in an inverted orientation (l-TGA). Such complex shuffling of cardiac situs can cause not only mixing of oxygenated with deoxygenated blood, but in some instances, blood flow is routed in two parallel circuits such that the systemic blood flow bypasses the lung altogether. These findings suggest that understanding how left-right asymmetry is established during cardiac morphogenesis is of great importance for elucidating the etiology of congenital heart disease.

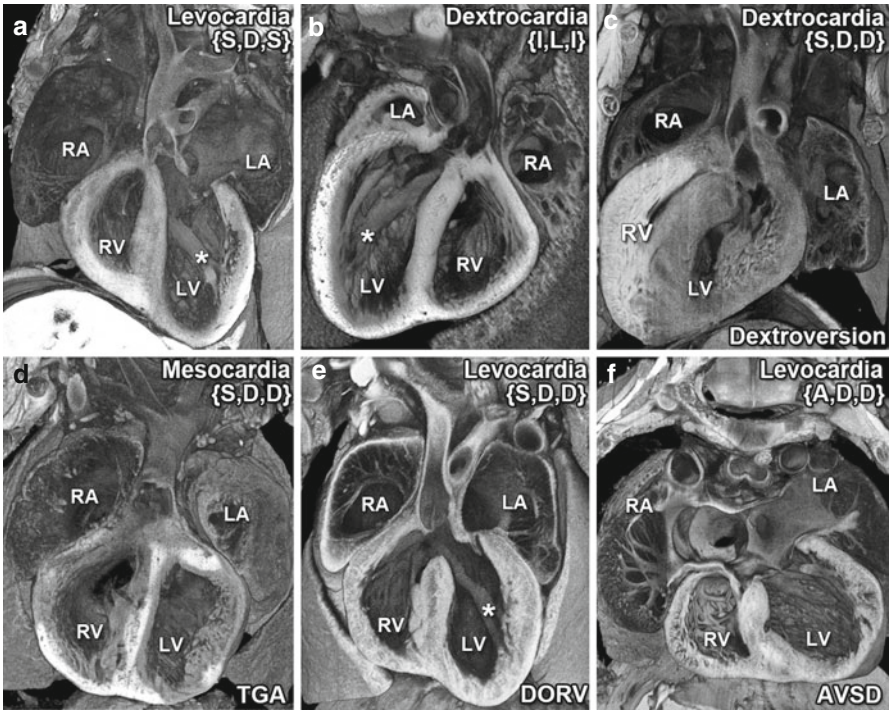

Fig. 7.3 Congenital heart defects associated with left-right patterning defects. Complex congenital heart defects are often found in conjunction with heterotaxy. Shown are five (**b–f**) examples of structural heart defects observed in mouse mutants with heterotaxy as compared to the normal cardiac anatomy of a wild-type mouse (**a**). In the wild-type heart (**a**), the heart apex points to the left, indicating levocardia, and the two atria and two ventricles are in their normal left-right position. Also seen are the papillary muscles attached to the free wall in the *LV* and the aorta emerging from the *LV*. The mutant heart shown in (**b**) exhibits mirror-image dextrocardia with the apex pointing to the right. Thus the four chambers and the intracardiac anatomy are entirely normal but are mirror symmetric in orientation. The mutant heart shown in (**c**) also exhibits dextrocardia but is said to have dextroversion, as the morphologic right and left ventricles/atria remain in the anatomic right and left, respectively, with correct outflow tract orientation. Other complex congenital heart defects commonly observed with heterotaxy are transposition of the great arteries (*TGA*), in which the connections between the ventricles and the outflow tracts are reversed (**d**), double-outlet right ventricle (*DORV*), in which both outflow tracts originate from the *RV*, or atrioventricular septal defect (*AVSD*), in which the atrial and ventricular septum fail to form, giving rise to abnormal common atrioventricular valves (**f**). Note that the heart exhibiting *TGA* also is characterized by abnormal midline positioning or mesocardia (**d**). For each heart, the Van Praagh segmental classification is indicated in brackets {} to denote the situs of the atria, direction of heart looping, and relations of the outflow tract (*S* solitus, *I* inverted, *D* dextral looping, *A* ambiguous). Normal cardiac situs is denoted by {S,D,S}, while mirror symmetric situs is indicated by {I,L,I}. The * denotes papillary muscle attached to the free wall, a characteristic of the left ventricle. 3D reconstructions were generated from serial 2D image stacks obtained by episcopic confocal microscopy [45]

7.5 Left-Right Patterning

Patterning of left-right asymmetry is a developmental process highly conserved in evolution. In the vertebrate embryo, the initial break in symmetry occurs via signaling mediated by a transient, ciliated organ, known as the embryonic node in mouse embryos. At the embryonic node, monociliated pit cells have rotating motile cilia and are surrounded by perinodal crown cells harboring nonmotile primary cilia [6, 7]. Motile cilia of the pit cells rotate clockwise, generating a leftward flow, resulting in propagation of a signal that establishes the left-right axis through asymmetric expression of nodal, a transforming growth factor (Tgf) ligand, on the left side, and its inhibitor DAN domain family member 5, BMP antagonist (Dand5) on the right side of the embryonic node [8]. Further studies investigating how the leftward nodal flow is perceived and propagated have led to three distinct models: (1) an accumulation of morphogens on the left side of the embryo, (2) an asymmetric distribution of node vesicular parcels containing hedgehog proteins and retinoic acid, or (3) detection of fluid flow by the nonmotile cilia of the crown cells leading to asymmetric calcium signaling via polycystic kidney disease (Pkd) 2 and the Pkd1-related locus Pkd1l1 [9–13]. Most intriguing is recent evidence from our laboratory resulting from analysis of a novel dynein, axonemal, heavy chain 5 (*Dnah5)* mutant, indicating that nodal flow may be necessary but not sufficient for establishing the left-right axis.

7.6 Heart Looping and Development of Cardiac Asymmetry

Cardiac morphogenesis begins with migration of cells comprising the primary heart field in the cardiac crescent to the axial midline, forming a hollow linear heart tube. This is followed by a distinct rightward or dextral (D) looping of the heart tube, the first evidence of cardiac asymmetry. Defects in the specification of left-right patterning can perturb heart looping, causing reversed looping (L-loop) or no looping (A-loop) which can result in dextrocardia (L-loop) or mesocardia (A-loop), respectively (Fig. 7.2) [14]. The process of heart looping also serves to initiate cardiac chamber formation such that the initially posteriorly positioned atrial segment is brought to a cranial position relative to the primitive ventricles, establishing the anterior or superior positioning of the atrial segment of the heart tube relative to the ventricle as seen in the mature heart (Fig. 7.1) [15]. While the primitive heart tube is formed by cells from the primary heart field, these cells will only give rise to the future left ventricle, while most of the remainder of the heart, the right ventricle, outflow tract, and atria, are derived from another cardiac progenitor cell population, the second heart field cells [16, 17]. These cells migrate into the looped heart tube via the dorsal mesenchyme protrusion and are added onto the arterial and venous pole of the heart over a period of several days during embryogenesis [18]. Second heart field cells play an important role in elaboration of the four-chambered heart and contribute cells that form the atrial septum [19].

Studies using zebrafish, a model organism which breaks symmetry via a ciliated organ functionally homologous to the mouse embryonic node, the Kupffer's vesicle (KV), have helped to elucidate the connection between early asymmetries in gene expression and heart looping [20]. While the zebrafish has only a two-chambered heart, it still undergoes cardiac looping and exhibits cardiac and visceral organ asymmetry. The transparency of the zebrafish embryo and its development externally has made it possible to study cardiac looping at the single-cell level. Such studies have indicated that activation of nodal signaling in the left lateral plate mesoderm (LPM) may direct heart looping by modulating directional migration of cardiac progenitor cells [21]. This is mediated through left-sided inhibition of bone morphogenetic protein (BMP) signaling by a nodal-regulated extracellular matrix protein, Has-2. As a result, a left-right difference in cell motility emerges in the zebrafish embryo, with the left side of the embryo exhibiting increased cell motility driving asymmetric looping of the heart tube [22]. Whether the same mechanism is conserved in the regulation of heart looping in the mouse embryo is yet to be determined.

7.7 Developmental Signaling in the Specification of Cardiac Asymmetry

The specification of cardiac asymmetry occurs during early embryogenesis, mediated by the same developmental processes that specify the entire left-right body axis. While the initial breaking of symmetry occurs at the embryonic node, signaling initiated at the node must be propagated to the LPM, resulting in left-sided LPM expression of Nodal, followed by left-sided expression of left-right determination factors Lefty1/Lefty2 (nodal inhibitors), and then left-sided expression of Pitx2, a paired-like homeodomain transcription factor [7]. This asymmetric gene expression cascade is essential for left-right organization of the entire embryo, with left-sided Pitx2 expression persisting in all of the visceral organ primordia throughout development. This is thought to regulate a gene expression program specifying left-sided differentiation (Fig. 7.1) [7, 23]. Since cells of the first and second heart field are found in both the left and right lateral plate mesoderm, it is likely that the early LPM asymmetric gene expression pattern may drive cardiac asymmetry and the coding of left-right patterning in the first and second heart field cells [24]. Consistent with this, Pitx2 expression persists exclusively on the left side of the developing heart, impacting patterning of the second heart field, which originates closer to the embryonic midline than the first heart field [25]. Expression of Pitx2 is also required later in development for formation of the aorta and pulmonary artery from the pharyngeal arches [26]. In addition, Pitx2 has been shown to regulate cell proliferation and cell migration, which may also impact heart looping and contribute to specification of cardiac left-right asymmetries (Fig. 7.1) [27].

7.8 Cilia, Left-Right Patterning, and Congenital Heart Defects

The important role of cilia in left-right patterning and in establishing cardiac asymmetry has implicated disruption of cilia as a potential key contributor to congenital heart disease. Mutations in Zic family members Zic2 and Zic3, transcription factors known to regulate cilia formation in the embryonic node, can cause cardiac situs anomalies and congenital heart defects in conjunction with heterotaxy [28, 29]. Consistent with the important role of the cilium in cardiac morphogenesis and the establishment of cardiac asymmetry, we recovered many ciliome genes from a large-scale mouse mutagenesis screen for mutations causing congenital heart disease [30]. This included both genes required for motile and primary cilium function. Some examples include *Mks1, Cep290, Dnah5, Dnai1, Dnah11, Drc1, Dyx1c1, Ccdc151,* and *Armc4* [31–37]. Many of these genes are known to cause primary ciliary dyskinesia (PCD), a sinopulmonary disease arising from mucociliary clearance defects due to dyskinetic or immotile cilia in the respiratory tract (see Chap. 38). This reflects the shared requirement for motile cilia in airway clearance and left-right patterning. Hence, some PCD patients can exhibit situs inversus totalis (Kartagener syndrome), or heterotaxy, with the heterotaxy/PCD patients usually also exhibiting congenital heart defects [38] (see Chap. 38). As expected, we observed congenital heart defects in conjunction with laterality defects, but interestingly we also found several mutations affecting ciliogenesis or cilium formation that caused isolated congenital heart disease without laterality defects, such as WD repeat containing planar cell polarity effector (*Wdpcp)* and Joubert syndrome 17 *(Jbts17)* [39]. These findings suggest that the cilium plays a fundamental role in cardiac morphogenesis beyond its role in patterning cardiac asymmetry. This may perhaps reflect the essential role of the cilium in modulating Tgf, Wnt, and Shh signaling, all cell-signaling pathways known to play important roles in cardiovascular development [40]. Disruption of these cell-signaling pathways has been shown to cause congenital heart defects in mutant mouse models [41–44].

Conclusion

Patterning the left-right asymmetries of the heart is a complex and dynamic process dependent on early embryonic events that break symmetry and establish the left-right body axis. Disruption of this process can result in congenital heart disease comprised of a wide spectrum of defects involving atrial, ventricular, or outflow tract anomalies. Of significance is the fact that defects in left-right asymmetry in the heart have been observed to present in a modular fashion, such that each segment of the heart (atrium, ventricle, outflow tract) structurally can be either left or right sided. Thus like LEGO constructions, complex congenital heart disease associated with heterotaxy can be comprised of distinct right- and/or left-sided atria/ventricle/outflow combinations. Thus it perhaps is not surprising that structural heart defects associated with heterotaxy are associated with high morbidity and mortality.

Although further work is needed to elucidate how the earliest events in left-right axis specification are transmitted to the cardiac precursor cells, and how the different segments of the heart are patterned, recent studies in animal models have suggested that cilia, both motile and nonmotile, play an important role in these processes. A better understanding of the mechanisms underlying the specification of cardiac asymmetry will help elucidate the developmental etiology of complex congenital heart disease. This will have important implications for the diagnosis and treatment of patients with congenital heart disease.

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Cardiac Conduction System

8

Rajiv Mohan and Vincent M. Christoffels

Contents

Abstract

The atria and the ventricles of the heart contract rhythmically and sequentially to achieve efficient blood flow. This contraction pattern is orchestrated by the cardiac conduction system, comprising specialized cardiomyocytes that initiate and propagate the cardiac electrical impulse. Genetic defects cause dysfunction of the cardiac conduction system leading to arrhythmias, emphasizing the need to understand the molecular and cellular mechanisms involved in its development and function. In the adult heart, the electrical impulse is generated in the sinoatrial node and traverses slowly through the atrioventricular node and rapidly through the atrioventricular bundle, the left and right bundle branches, and the peripheral ventricular conduction system. All components have a unique function, shape, and molecular composition but share particular properties acquired during embryogenesis. During embryonic development, the components are gradually formed from embryonic cardiomyocytes involving conserved molecular

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regulatory networks. In this chapter, the developmental origin, known signaling pathways, transcription factors, ion channels, and gap junctions involved in the development and functioning of the cardiac conduction system will be addressed.

8.1 Introduction

The heart is a muscle-driven pump that generates the blood flow necessary to sustain multicellular life. In order to function efficiently, a specialized group of cardiomyocytes generates and orchestrates the electrical impulse that initiates the contraction of the atria and the ventricles. These specialized cardiomyocytes comprise the cardiac conduction system (CCS). The CCS can be subdivided into pacemaker-like components that generate and slow down the impulse, the sinoatrial node (SAN), and atrioventricular node (AVN) and junction (AVJ), respectively, and components that rapidly propagate and distribute the impulse in the ventricles, the atrioventricular bundle (AVB), left and right bundle branches (L/RBBs), and the peripheral ventricular conduction system (PVCS). The AVB and PVCS are also known as the Bundle of His and the Purkinje fiber network, respectively (Fig. 8.1). The SAN is located at the border of the superior caval vein and the right atrium and is the dominant pacemaker of the heart. The electrical impulse is generated in the SAN and propagated to the atria. Before the atria contract, the electrical impulse travels rapidly through the atrial working myocardium and reaches the AVN, the only myocardial connection between the atria and ventricles were conduction is slow. This allows enough time for contraction of the atria and filling of the ventricles before the ventricles themselves are activated. The electrical impulse subsequently is conducted through the fast-conducting AVB and spreads with high velocity into the BBs and PVCS to activate the ventricular muscle mass, resulting in ventricular contraction and expulsion of blood into the aorta and pulmonary artery.

Although the SAN is the dominant pacemaker, the other components of the CCS also have intrinsic pacemaker activity. Pacemaker activity or automaticity is the capacity to generate an action potential autonomously. The SAN is dominant because its firing rate is the highest compared to the other components, which are stimulated before they will initiate an action potential autonomously. Because of this so-called overdrive suppression, these components are latent pacemakers that can take over pacing in case of dysfunction of the SAN or AV block [1].

8.2 Development of the Cardiac Conduction System

Early in development, during folding of the embryo, two cardiac mesodermal regions fuse and form a heart tube. At this stage, the heart tube contracts slowly in a peristaltic pattern from the inflow tract towards the cranially located outflow tract. All cardiomyocytes are able to depolarize spontaneously; however, already at this stage, dominant pacemaker activity resides at the inflow tract of the heart. The phenotype of the primary myocardium of the embryonic heart resembles that of the

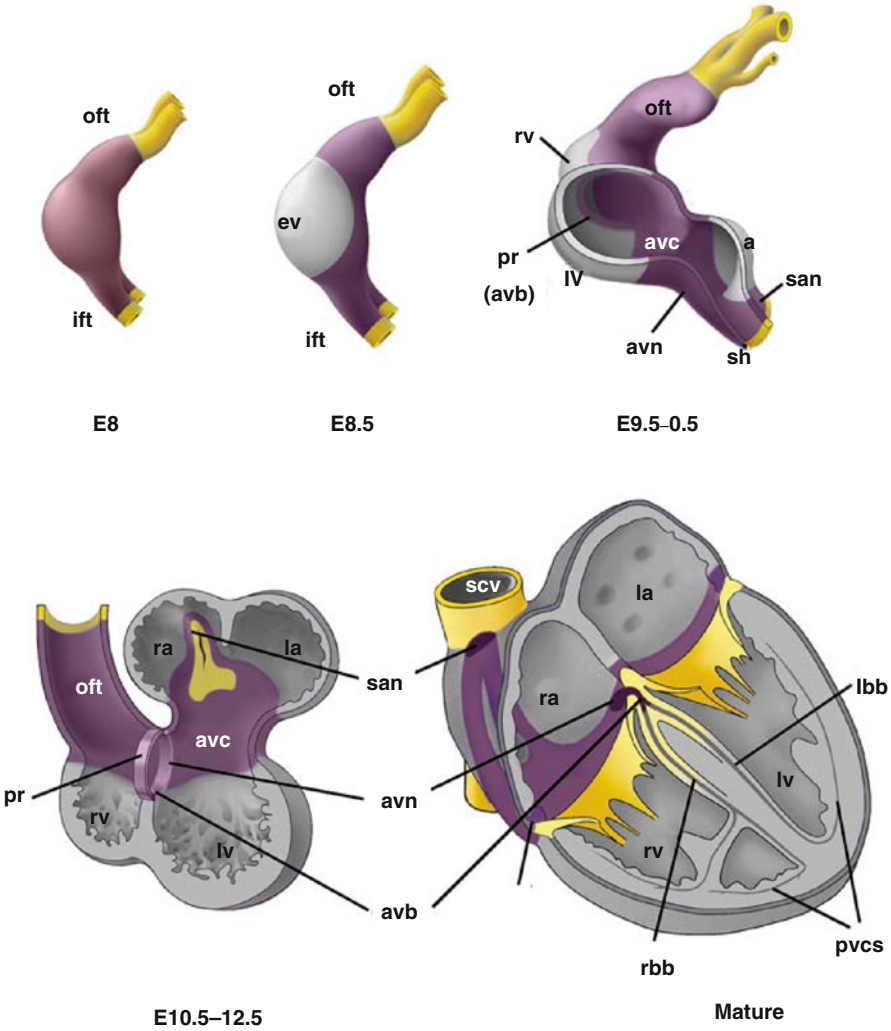

Fig. 8.1 Schematic overview of heart development in higher vertebrates. The early heart tube has a primitive phenotype (*light purple*). Chamber myocardium (*gray*) expands rapidly, whereas primary myocardium (*purple*) of the sinus horns (*sh*), atrioventricular canal (*avc*), primary ring (*pr*), and outflow tract (*oft*) retain a more primitive phenotype. Abbreviations: *a* atrium, *avn* atrioventricular node, *avb* atrioventricular bundle, *lv* left ventricle, *rv* right ventricle, *la* left atrium, *ra* right atrium, *san* sinoatrial node, *avj* atrioventricular junction, *lbb* left bundle branch, *rbb* right bundle branch, *pvcs* peripheral ventricular conduction system, *scv* superior caval vein, *ift* inflow tract, *ev* embryonic ventricle

nodal tissues in displaying automaticity a poorly developed contraction apparatus and slow conduction of the electrical impulse [2–4].

Lineage analyses have revealed that the initial heart tube only contributes to the LV and the AVC [5–7]. All other structures within the heart are formed by a continuous process of addition of cells at the inflow and outflow tract and dorsal mesocardium until this structure is ruptured [5, 8]. During the process of looping, a

working myocardial gene program is activated at specific sites, resulting in an increase in the proliferation rate and the expression of genes associated with the early chamber myocardium, including natriuretic peptide A (*Nppa)* and connexin 40 *(Cx40)*. These specific regions will give rise to the future atria and ventricles by rapid expansion (Fig. 8.1). Conduction in these structures is fast [9, 10]. However, they are separated by flanking myocardial cells that retain the primitive slow-conducting properties, the sinus venosus (SV), atrioventricular canal (AVC) inner curvatures, and outflow tract (OFT) [2–4]. The dominant pacemaker activity still resides in the SV, which implies that the newly added cells at the inflow tract immediately acquire this property [11]. The AVC now functions to slow the impulse before it reaches the ventricles, thus ensuring the atria and ventricles to contract in a subsequent pattern. The ECG derived from this configuration of alternating slow- and fast-conducting components resembles that of the adult heart, implying that conduction system function has been established before its morphologically distinguishable parts have been formed [12].

The SAN forms within the SV myocardium; the embryonic AVC myocardium forms the definitive AV node, AV junction (rings), and a large part of the base of the LV. The primary myocardium of the OFT differentiates to ventricular myocardium and forms the RV (and smaller LV) outflow tract. At the border between the LV and RV, an interventricular ring of primary myocardium is present (Fig. 8.1). Here, the interventricular septum will be formed containing the future AVB at its crest. The BBs arise from subendocardial cardiomyocytes in the septum, whereas the PVCS will form from the embryonic trabeculae. The origin and known signaling pathways, transcription factors, ion channels, and gap junctions involved in the development and functioning of the components of the CCS will be addressed in the following paragraphs and are summarized in Fig. 8.2.

8.3 Development of the Sinus Venosus and the Sinoatrial Node

The SAN is located at the superior venous entrance of the right atrium. It comprises a few thousand cardiomyocytes forming a “comma-shaped” elongated structure with a head and a tail [13, 14]. The periphery contains connective tissue which protects the SAN from the hyperpolarized right atrium and which dictates the electrical connections with the atrium [15]. SAN cardiomyocytes are glycogen rich and have poorly developed myofibrils and low intercellular conductance properties, which are the result of the expression of the connexin proteins Cx45, Cx30.2, and Cx30, subunits for low conductance gap junctions [16, 17]. Cx40 and Cx43 subunits for high conductance gap junctions are expressed in the atria but not in the SAN [18–21]. Furthermore, the essential cardiac voltage gated sodium channel Nav1.5 (encoded by *SCN5A*) is expressed at low levels in the SAN, whereas the hyperpolarization activated cyclic nucleotide-gated potassium channel 4 (Hcn4), a key player in the pacemaker potential, is expressed in the mature SAN and absent from the atria [1, 20, 22, 23].

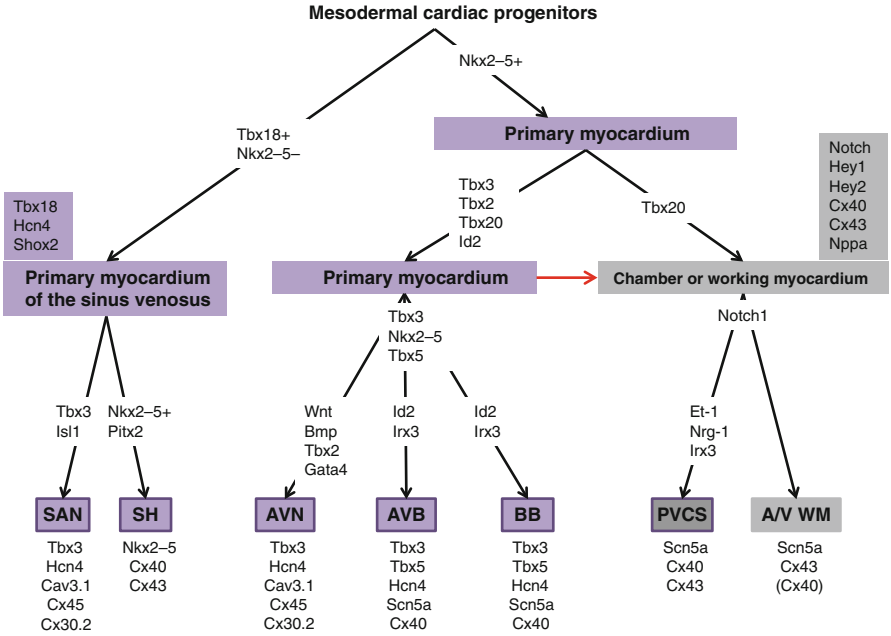

Fig. 8.2 Model for cardiac progenitor and cardiomyocyte differentiation into the myocardial components of the heart and the proteins involved in the regulation and/or correct functioning. The *red arrow* indicates continuous differentiation of primary cardiomyocytes into chamber myocardium. Nkx2-5, Gata4, and Tbx5 have a broad role in many processes of heart development. They are only included in the scheme when a specific role in CCS formation has been experimentally determined

The SV myocardium upstream of the right atrium comprises the SAN, the venous side of the venous valves, and both the right and left sinus horns. The latter will form the sinus coronaries in human. The myocardium of the SV is formed from mouse E9-9.5 onwards from T-box transcription factor (Tbx) 18 and Hcn4-expressing and NK2 homeobox 5 transcription factor (Nkx2-5)-negative precursors. The SV myocardium initially maintains this profile, does not express atrial working myocardial genes such as *Cx40*, and harbors dominant pacemaker activity. As soon as the SV is formed, a *Tbx3*-positive domain is observed in the right-sided part, which likely represents the primordium of the SAN. During the fetal period, *Hcn4* expression becomes restricted to the *Tbx3*-positive SAN, whereas the remainder of the SV acquires a working myocardial phenotype [24, 25]. Within the same period, dominant pacemaker activity becomes confined to the actual SAN structure.

Genetic lineage tracing using the Cre-Lox system revealed that the SV and SAN originate from *Tbx18*-expressing mesenchymal precursors that do not express *Nkx2-5*, which marks the first and second heart field precursors. Closer inspection of the spatiotemporal expression patterns suggested that *Nkx2-5* was briefly expressed in the precursors but downregulated before differentiation [26–29]. *Tbx18*-deficient mice do not properly form the SV and fail to form the head of the SAN [27].

T-box transcription factor Tbx3 is expressed in the SAN and other components of the CCS, except for the PVCS, and is required to suppress atrial working myocardial genes (*Cx40*, *Cx43*, *Scn5a*) in the SAN [20, 30]. Tbx3 confers the pacemaker phenotype to cardiomyocytes, as ectopic expression in the atria results in the expression of pacemaker genes (e.g., *Hcn4*) and spontaneous activity in the atria [20]. Mice with hypomorphic alleles of *Tbx3* showed bradycardia and sinus pauses indicative of SAN dysfunction [31]. In *Nkx2-5*-deficient embryos, *Tbx3* and *Hcn4* are ectopically expressed in the heart tube, indicating that Nkx2-5 suppresses the SV/SAN program in the heart tube [24]. Short stature homeobox 2 gene (*Shox2)* is specifically expressed in the SV and represses Nkx2-5. The absence of *Shox2* results in a hypoplastic SV and upregulation of *Nkx2-5* and downregulation of *Hcn4* and *Tbx3* in the SAN primordium. Consistent with the loss of the pacemaker program, *Shox2*-deficient mice show bradycardia [32, 33]. Analysis of *Shox2*-deficient hearts identified LIM homeodomain transcription factor Islet1 (Isl1) as a downstream target. In a zebrafish model, it was shown that *Shox2*-deficiency-caused bradycardia which was rescued by islet1 (*Isl1)* overexpression [34]. *Isl1* marks the pacemaker cells of the zebrafish heart and is required for its development [35]. Furthermore, lowering *Tbx5* expression by a hypomorphic or knockout allele results in a reduction of *Shox2* and *Tbx3*, showing that Tbx5 acts upstream of them [36, 37]. Interestedly, a slight reduction in Tbx5 levels, in mice heterozygous for a hypomorphic *Tbx5* allele, results in twofold reduction in *Shox2* and *Tbx3* expression, suggesting that they are very sensitive to Tbx5 dosage [36]. The right sidedness of the SAN is controlled by the transcription factor paired-like homeodomain 2 (Pitx2), expressed in the left side of the atria and SV. Deficiency in *Pitxc2* results in two indistinguishable SANs at both sides of the sinoatrial junction, suggesting that this factor is necessary to suppress SAN formation in the left SV [38].

8.4 Development of the Atrioventricular Node and Atrioventricular Junction

The AVJ includes the AVN, a complex and heterogeneous structure consisting of different cell types with distinct gene expression profiles. The AVJ also encompasses the left and right AV ring bundles that contact anteriorly at the "retroartic node," thus forming a ring [1, 30, 39, 40]. The AVN and AVJ cells are more primitive than working cardiomyocytes. Their expression profile reflects their pacemaking and slow-conducting properties. The mouse AVN and AVJ express Cx45, Cx30.2, Cav3.1, Hcn4, and Tbx3, whereas Cx40, Cx43, and Scn5a are virtually absent. The anatomical position, expression profile, and slow-conducting property of the AVJ resemble that of the embryonic AVC, suggesting that the AVJ is derived from the AVC [41, 42]. The AVC, first visible around E9-9.5, retains a primitive phenotype when the working myocardial gene program is activated in the future atria and ventricles. In contrast to the fast-conducting working myocardium, the AVC conducts slowly, resembling the function of the AVN in the formed heart [4].

Retrospective clonal analyses revealed a common origin of AV conduction system cells with adjacent working myocardial cardiomyocytes, revealing a cardiogenic origin of the avian CCS [43, 44]. Genetic lineage analysis of *Tbx2*-positive AVC myocardium revealed that the embryonic AVC forms the AVN, left and right AV ring bundle, and a large part of the free wall of the LV, but not the AVB and BBs [7]. The neural crest does not contribute to the myocardial part of the AVN cardiomyocytes [45].

Tbx3 and Tbx2 are expressed within the AVC and act as repressors with overlapping functions [30, 46, 47]. *Tbx2* expression in the heart is lost during the late fetal period, whereas *Tbx3* expression is maintained. The retention of the primitive phenotype in the AVC largely depends on the repressive function of Tbx2 and Tbx3. Both proteins compete with Tbx5 for binding of T-box elements in enhancers of T-box target genes (e.g., *Nppa* and *Cx40*) and interaction with Nkx2-5 [48]. The loss of both proteins results in loss of the AVC phenotype shown by the expansion of the chamber program (*Nppa* and *Cx40*) expression in this region and failure to form AV cushions [47]. *Tbx3*-deficient embryos have no AVC abnormalities but do show defects in the *Tbx2*-negative SAN and AVB [49]. Postnatally, correct function of the AVN is dependent on Tbx3 in a dose-dependent manner. Lowering or deleting Tbx3 causes AV conduction abnormalities [31].

The excreted bone morphogenetic protein (Bmp) 2 is expressed specifically in the embryonic AVC and required for AVC specification through activation of the bone morphogenetic protein receptor type IA (Bmpr1A/Alk3) and downstream Smad effectors. Inactivation of Bmp2 in the mouse embryo resulted in loss of *Tbx2* expression and failure to form the AVC [50]. In chicken, Bmp2 is sufficient to activate expression of *Tbx2* and *Tbx3* [46, 50]. Abrogation of BMP signaling by inactivation of the type IA receptor *Bmpr1a/Alk3* in the AVC results in inadequate formation of the annulus fibrosus and causes preexcitation [51, 52].

Wnt signaling is fundamental to correct AVC formation. Wnt signaling can be subdivided in canonical (via β-catenin) and noncanonical signaling pathways. A study in zebrafish demonstrated that the canonical signaling pathway is necessary and sufficient to induce *Bmp4* and *Tbx2b* expression in the AVC [53].

The transcription factors Tbx5, Gata4, and Nkx2-5 are expressed broadly in the heart and required for chamber formation. Mutations in *TBX5* and *NKX2-5* cause congenital heart defects and AV conduction defects [54–56]. Mice haploinsufficient for *Nkx2-5* have a hypoplastic AVN, AVB, and BBs. In *Tbx5* mutant embryos, the expression of *Tbx3* and *Cx30.2* are reduced. Postnatally, maturation of the AVN is hampered [57–59]. *Gata4* heterozygous embryos show a reduction in *Cx30.2* expression. In adult mice, *Gata4* haploinsufficiency results in shortened PR intervals [56].

Notch signaling and downstream transcription factors Hey (hes-related family bHLH transcription factor with YRPW motif) 1 and Hey2 sharpen the boundary between AVC and the chambers. In chicken, Notch2 activates Hey1 and Hey2 in the chambers, which in turn repress *Bmp2* and *Tbx2*. In the AVC, Tbx2 represses *Hey1* and *Hey2*, thereby constituting a feedback loop that delimits the AVC [60, 61]. *Tbx20*-deficiency leads to ectopic expression of *Tbx2* in the entire heart tube [62,

63]. Tbx20 represses *Tbx2* expression in the chambers by interfering with Bmp-signaling-mediated activation of *Tbx2* through interacting with Smad1/5 [62, 63].

The annulus fibrosus and central fibrous body electrically insulate the atria from the ventricles. The only myocardial electrical connection between the atria/AVN and ventricles is the AVB. The annulus fibrosus forms by the invasion of subepicardial mesenchyme of the AV groove in-between the atrial/AVJ myocardium and ventricular myocardium [42]. This latter process is initiated in the early fetal period and continues postnatally [64]. The presence of ectopic fast-conducting myocardial connections between the atria and ventricles involves abnormalities in the invasion of the connective tissue and the normally slow-conducting AVC myocardium acquiring fast-conducting properties [65, 66].

Several genes have been implicated in the formation of accessory pathways and ventricular preexcitation. Mutations in *PRKAG2* encoding the protein kinase, AMP-activated, and gamma 2 non-catalytic subunit have been associated with ventricular preexcitation due to disruption of the annulus fibrosus [67]. In addition, a 20p12.3 microdeletion that includes *BMP2* was found to predispose to Wolff-Parkinson-White syndrome [68]. In mice, the loss of *Bmp2* receptor Alk3 causes additional myocardial strands. Inactivation of *Tbx2* in the developing heart results in a *Cx40*-, *Cx43*-, and *Scn5a*-positive and presumably fast-conducting accessory pathway on the left dorsal side of the AVC [65]. Activation of Notch signaling by overexpression of the Notch1 intracellular domain in the heart results in accessory pathway formation and preexcitation [69]. Altogether, these data reveal a Bmp-signaling-Tbx2-Notch pathway that is involved in transcriptional control of correct development of the AVC and annulus fibrosus [51, 52, 65, 68].

8.5 Origin and Development of the Ventricular Conduction System

The ventricular conduction system (VCS) comprises the AVB, left and right BB, and PVCS. Cardiomyocytes in these structures have a poorly developed contractile apparatus and T-tubular system. Gap junctions in the adult VCS are well developed and consist of Cx40 and Cx43 subunits resulting in fast conduction. Gap junction subunit Cx40 is a specific marker for the complete VCS [70, 71]. As soon as the LV and RV emerge in the heart tube (E9-9.5), a *G1N2/Tbx3*-positive "primary ring" [30, 72] is present in-between the LV and RV. As the interventricular septum is being formed at this location, its crest differentiates into the future AVB [73]. The BBs are gradually formed subendorcardially during growth of the interventricular septum. *Tbx3* is expressed in the BBs in a diminishing gradient towards the apex [49]. *Cx43*, *Tbx18*, and *Tbx20* are also expressed in the IVS but absent from the Tbx3-positive developing AVB and BBs and are therefore useful as negative markers of the AVB and BBs. Tbx3-deficiency results in ectopic expression of these genes, but also of *Nppa* and *Cx40*, within the crest of the septum. This again shows that Tbx3 is required for specification by repressing the ventricular working myocardial phenotype. Although *Cx40* initially is suppressed in the AVB by Tbx3, this gene is upregulated after E12 to become highly expressed in the AVB [49].

Tbx5 and Nkx2-5 are expressed in the AVB and BBs and were shown to be important for its correct patterning and specification. Mice haploinsufficient for *Tbx5* and *Nkx2-5* do not establish an AVB and develop conduction block after birth. This defect is caused partly by the failure to activate inhibitor of differentiation protein 2 (Id2) in the AVB [57]. Furthermore, Tbx5 is required to activate both *Cx40* and *Scn5a* in the developing and mature AVB [74]. We speculate that the relative level of Tbx5 to Tbx3 increases in the AVB, leading to the activation of *Cx40*. In *Tbx3*-deficient embryos, inhibitor of DNA binding 2 (*Id2)* expression is not affected showing that activation of the *Id2* locus is independent of Tbx3 and suggesting the presence of independent pathways in the formation of the AV bundle and BBs [49]. Iroquois homeobox 3 (*Irx3)* expression confines to the AVB, BB, and PVCS during development. *Irx3* knockout mice display prolonged ventricular activation due to asynchronous activation of the left and right ventricles. Irx3 represses *Cx43* and indirectly activates the expression of *Cx40* in the AVB and BB [75].

The embryonic ventricular chambers mainly consist of trabecules and a thin outer layer. The impulse preferentially conducts over the trabecules [76]. Early on, the LV is entirely *Cx40*-positive, whereas the RV is partially *Cx40*-positive [71], a pattern reflecting that of its essential activator Tbx5 [77]. With further development, the compact walls form (>E11), and expression of Cx40 becomes confined to the trabecular zone. After birth, *Cx40* expression is confined further to the subendocardial VCS. This pattern suggests that the *Cx40*-positive embryonic trabecular myocardium gives rise to both the *Cx40*-positive VCS and the *Cx40*-negative compact wall [41]. Prospective and retrospective genetic labeling experiments substantiated the hypothesis derived from the *Cx40* (and *Nppa*) expression pattern that the embryonic trabecules give rise to the VCS and that the compact wall myocardium lineage gradually diverges from the initially *Cx40*-positive embryonic ventricular walls [71].

In the chick, endothelin 1 (Et1) is an inductive signal secreted from the endocardium and coronary arterial endothelium. Et1 and its receptors were found to be involved in the functional maturation of the VCS and in the induction of expression *Cx40* (in chick a subendocardial and periarterial VCS is present) [78]. In mice, neuregulin-1 (Nrg-1) influences patterning of the PVCS, probably through its role in trabecular development [79]. In addition, *Notch1* mutants display defective trabeculation mediated by reduced expression of *Nrg-1* and *Bmp10* [80]. Bmp10 is known to be important for cardiomyocyte proliferation [81]. Recently, it was shown that Notch signaling plays an important role in specification of prenatal ventricular cardiomyocytes towards a conduction phenotype that resembles the PVCS [82].

Conclusion

Our basic understanding of the development and correct functioning of the CCS has been greatly enhanced over the recent years. Genetic lineage tracing uncovered the progenitor pools of the heart and the CCS and a multitude of factors organized in transcriptional circuits have been identified [83, 84]. Genome-wide analyses of tissue-specific transcription factor-DNA interactions (ChIP-seq) and transcriptional profiling (RNA-seq) reveal how these circuits orchestrate gene

expression patterns within the heart. Regulatory DNA sequences have been discovered and the 3D conformation of genomic loci unraveled [85]. Recently, the role of histone modifications, chromatin remodeling complexes, and other epigenetic factors has been implicated in CCS development [86, 87]. Together, these data provide new insights and a better understanding into the mechanisms underlying the establishment and maintenance of the CCS.

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Hemodynamics During Development and Postnatal Life

9

David Sedmera

Contents

Abstract

Unlike other embryonic organs, the developing heart must support, through its proper function, the developing embryo from the time diffusion becomes limiting. Hemodynamics is thus not only a key to remodeling of the developing vasculature but also a powerful stimulus for cardiac growth and differentiation. In this chapter are discussed prenatal models of hemodynamic perturbation that help clarifying the role of blood flow in embryogenesis.

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9.1 Introduction

The role of hemodynamics during embryonic development has been recognized for a long time [1], for example, its importance in molding the endocardial cushions into cardiac septa. Most of the experimental work was performed in the chick embryonic model, mostly because of its simplicity and the feasibility of experimental manipulations. However, other models, such as developing zebrafish, have been used to study the earliest phases of circulatory system formation, as the larvae are able to survive without a functional cardiovascular system for up to 1 week, allowing one to study otherwise lethal phenotypes. To better approximate the human situation, several groups have developed various mammalian models to answer specific questions. The lamb model has been most frequently used, mainly because of the specific ability of sheep uterus to tolerate surgery without induction of premature labor. We organize this chapter according to various animal models, ending with the examples from clinical cases in humans.

9.2 Studies in Developing Zebrafish

Three-dimensional studies of the tubular zebrafish heart enabled detailed visualization during different phases of the cardiac cycle [2] and changed our idea about the "peristaltic" contractions of the cardiac tube into a more appropriate model of a suction pump. Perturbation of the heart at such early stages via implantation of microbeads [3] altered normal blood flow through the heart and resulted in hearts with an extra third chamber, diminished looping, and abnormal valve formation, the last also known from human examples of congenital heart disease with blood flow alterations. This underscores the critical role of hemodynamics in cardiovascular morphogenesis.

Silent heart phenotype in zebrafish results in electrically normally active hearts, which however lack contraction because of a mutation in cardiac troponin T [4]. In this model, lack of endocardial cushion formation was observed, confirming the role of hemodynamic stress on the process of endocardial endothelial to mesenchymal transition. Since this hypothesis was strengthened by pharmacological inhibition of cardiac contractions, the authors postulated that abnormal function is translated into abnormal cardiac morphogenesis and could be an explanation for some forms of human cardiac congenital anomalies.

9.3 Chick Embryonic Model

The chick embryo has frequently been used to perform hemodynamic interventions, as both the visualization of the beating heart by videomicroscopy and more sophisticated functional evaluation (pressure monitoring, ultrasound biomicroscopy and Doppler, optical coherence tomography) are relatively straightforward. Hogers et al. introduced a venous clip model that mimics placental infarction and observed

resulting changes in intracardiac blood flow patterns [5]. These led frequently to abnormal morphogenesis translating in incomplete ventricular septation (VSD), valve anomalies, and pharyngeal arch artery malformations.

Hemodynamically induced shear stress is correlated with blood flow. To test the hypothesis that shear stress is also involved in cardiac development, Hogers et al. investigated the expression patterns of hemodynamically induced genes endothelin 1 (ET1), nitric oxide synthase 3 (NOS3), and Kruppel-like factor 2 (KLF2) at the mRNA level in a series of developmental stages of the chicken embryo. The authors found areas with mutually exclusive expression of ET1 and KLF2/NOS3, with KLF2 being expressed in the regions of highest shear stress. Visualization of stresses and strains in two altered hemodynamic models was performed by Buffinton et al. [6]. These investigators showed among other things the importance of realistic tissue geometry for determining the strains in mathematical models. The highest levels were found at the tips of ventricular trabeculae; ignoring this geometry and modeling the ventricle as a thick-walled shell, one would underestimate the values by up to two orders of magnitude.

Constriction of the outflow tract (conotruncal banding) was used as a model of increased pressure load of the embryonic ventricle [7]. The procedure itself is rather simple, as it is performed through a still avascular chest wall (Fig. 9.1). It has, in addition to increasing pressure load, pronounced effects on cardiac morphogenesis, producing lethality and defects in survivors [7, 8]. These include a VSD, with either double outlet right ventricle or persistent truncus arteriosus. This could be due, apart from considering mechanical effects of the suture, to hypoplasia of the conotruncal ridges as a result of altered intracardiac bloodstream patterns [9]. There are also pronounced effects on ventricular morphology. There is a change in shape due to dilation with increased proportion of the compact myocardium and spiraling of the trabeculae in the left ventricle. This is an interesting feature, since it resembles the definitive orientation of trabeculation in this location, and is similar to the course of muscle fibers in the compact layer [10]. This spiraling likely reflects adaptation to gradually increasing functional demands [11]. Increased thickness of the compact myocardium occurs within 48 h [9] and together with anomalies in the development of the coronary arteries [12] could be responsible for a decline in survival occurring around day 8 when the coronary circulation normally becomes functional. Interestingly, the growth of capillaries matches that of the myocytes, so the ratio is kept constant. Increased pressure loading is at this stage also a powerful stimulus for active myocardial growth based on cell proliferation [7, 13, 14].

The hemodynamically induced changes in myocardial architecture in this model also could be a substrate of altered electrical pathways. These were investigated as well using optical mapping on isolated hearts [15, 16]. It was found that increased pressure loading accelerated maturation of the ventricular conduction system. At the molecular level, these changes were paralleled by upregulation of conduction system differentiation markers endothelin-converting enzyme 1 (ECE1) and connexin 40 (GJA4).

Another popular model with a long history is left atrial clipping [17, 18] or ligation (LAL [9]) that mimics human hypoplastic left heart syndrome (HLHS). The

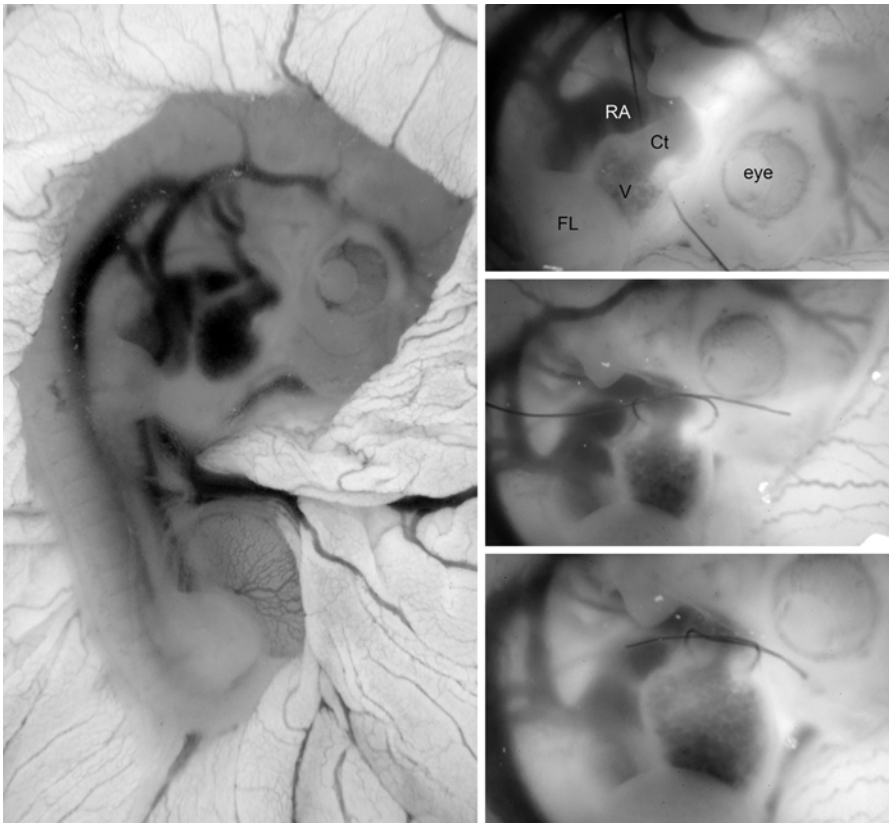

Fig. 9.1 Pressure overload model in the chick embryo. The embryo at day 4 of incubation (*left panel*) is exposed by amniotomy, and through the developing chest wall, a piece of 10-0 nylon thread is passed under the outflow tract (*top right*), tied in an overhand knot (*middle*), and cropped (*bottom*) to prevent piercing of the fragile surrounding vessels by its sharp ends. *Ct* conotruncus, *FL* fore limb bud, *RA* right atrium, *V* ventricle

phenotype of LAL hearts (Fig. 9.2) shows considerable variability from almost normal to extreme involution of the left ventricle with apex-forming right ventricle [19]. Occasional occurrence of VSD in about 25 % of the survivors is similar to results obtained in the left vitelline venous clip model [5] and likewise could be attributed to changes of intracardiac bloodstream patterns [20]. In severe cases, the right atrioventricular valve loses its typical muscular flap-like morphology and resembles a bicuspid fibrous valve. This suggests that hemodynamic stress is an important determinant of morphology of the developing valvular structures and influences differentiation of the connective tissue. In addition, there is a molecular phenotype [21] suggestive of heart failure, and a delay of about 2 days in expression of myocardial differentiation genes (contractile protein isoforms, energetic metabolism enzymes) was detected by microarray analysis in both ventricles. Adaptation of the right ventricle to gradually increasing volume load is progressive. First, there

is dilatation with in extreme cases alterations of trabecular orientation from radial to circumferential [9]. Second, there is an increased proliferation within the trabeculae [14] followed by eventual thickening of the compact myocardium, a finding that could be regarded as an acceleration of the normal course of development [22]. These changes in myocardial architecture and proliferative activity could be reversed by subsequent prenatal interventions. Surgical clipping of the right atrial appendage [20] normalized the hemodynamics and rapidly resulted in increased myocyte proliferation in the left ventricle and a tendency toward normalization of reduced left ventricular myocardial volume. This observation demonstrates the feasibility of fetal interventions aimed at mitigating the severity of a number of human congenital cardiac malformations [23, 24].

9.4 Prenatal Lamb Model

However simple and self-contained the chick model is, its relevance for human pathology has sometimes been questioned, and fetal mammalian models were thus developed. The lamb is the standard large animal model for investigating intrauterine development of the cardiopulmonary system and testing the effects of various prenatal interventions. In this model it is possible to reproduce typical anatomical and pathophysiological lesions and develop of procedures for their repair. Pioneering surgical studies were performed and summarized by Rudolph [25]. Early investigations demonstrated the suitability of this model for examination of both the pathophysiological consequences of cardiac lesions and the technical methods of intrauterine surgery [26]. With prenatal identification of cardiac lesions, intrauterine cardiothoracic surgery is possible [27, 28]. While a study comparing the effects of early vs. late pulmonary stenosis repair did not show any difference in transverse myocyte diameter even in non-repaired hearts [27], a study comparing the effects of pulmonary stenosis and atresia [28] showed an increase in myocyte width dependent on the level of overload and myocardial weight increase, suggesting a mix of both hypertrophy and hyperplasia. There are thus multiple factors, such as differences in methodological approach, the species used, timing of surgery, and the level of the overload, which influence the nature of myocardial response to prenatal hemodynamic challenge. It appears that even the adult heart, traditionally, and according to the latest evidence, rather incorrectly [29] regarded as a postmitotic organ, is capable of considerable proliferation in response to a long-standing pressure overload [30].

Both the pulmonary artery and aorta were banded in work by McAuliffe and Robbins [31]. This study revealed that only one dominant isoform of cardiac troponin is expressed during fetal and adult development and that expression levels do not change in response to pressure overload. Thus, changes in contractile protein isoform expression ("fetal gene program") known from adult models of heart overload [32] could not be recapitulated here. This model was used to study the changes in calcium-handling proteins, which could be interpreted as acceleration of the normal course of developmental [33]. At the cellular level, hypertrophy of cardiomyocytes was followed by hyperplasia and decreased proportion of binucleated cells.

This is an interesting observation, showing differences from the embryonic chick model as well as the ability of the fetal mammalian heart to behave similarly to the adult heart. As in the case of the embryonic chick pressure overload model, Flanagan and colleagues [34] found that capillary density was maintained in the overloaded group and that the functional flow parameters and coronary resistance were also within the normal range. This shows that the fetal heart adapts its structure in response to pressure challenge (within limits) in a coordinated manner and that the myocardium thus created is normal and well perfused.

Fishman et al. [35] created a prenatal sheep model of the hypoplastic left heart syndrome by obstructing the left ventricular inflow or outflow with a balloon catheter. With the inflow obstruction, within a week, the left to right ventricular weight ratio decreased to 70 % of control, and the mean chamber volume ratio decreased to less than 50 %. The outflow obstruction resulted in less pronounced left ventricular output decrease. Over the long term, the left to right ventricular weight ratio decreased even further, and the left ventricular chamber was nearly obliterated, simulating very severe congenital aortic stenosis. These experiments confirmed the existence of two possible hemodynamic pathways of pathogenesis of hypoplastic left heart syndrome: either through decreased preload or increased afterload of the left ventricle, as seen in fetal mitral or aortic stenosis, respectively.

9.5 Fetal Studies in Rodents

9.5.1 Guinea Pig

An elegant fetal surgical study in small animal models was performed in guinea pigs [36]. Fetal guinea pigs in the third trimester were accessed by hysterotomy, and their ascending aorta was constricted to 50 % of its original diameter. The heart to body weight ratio and left ventricular wall thickness increased significantly in the banded group. There was also a significant increase in percentage of marker of proliferation Ki-67-positive (i.e., proliferating) cells in both ventricles with no changes in apoptosis, suggesting the existence of an adaptation mechanism similar to that observed in the other prenatal models.

9.5.2 Mouse

Mouse embryo is not a frequent subject of hemodynamic studies, mainly because of its small size, the presence of multiple embryos in the same dam making individual longitudinal studies difficult, and general constraints of mammalian model. However, there is wealth of information that could be learned due to availability of transgenic animals mimicking sometimes human cardiac malformations. Basic study of mouse embryonic cardiovascular function and dimensions was performed by Keller et al. [37], replicating similar studies performed previously in chick and rat embryos. Experimental pacing study in murine embryos demonstrated feasibility of

Doppler measurements of flows and velocities in this model and confirmed deleterious effects of tachycardia on embryonic cardiac performance [38]. Later studies by this group revealed patterns of response of the embryonic heart rate to hypoxia, which varied according to developmental stage. The initial response to maternal hypoxia was bradycardia, followed at reoxygenation by either overshoot prior to return to baseline (paralleling the maternal response), continued bradycardia, or simple return to normal rate [39]. Pharmacological study of effects of moderate doses of caffeine [40] showed a slight but significant deleterious effect on embryonic growth and cardiac performance, possibly mediated via adenosine receptor blockade.

Phoon and colleagues investigated with ultrasound biomicroscopy mice with NFATc1 deletion [41]. These mice lack the outflow valves and die rather suddenly at mid-gestation. It was shown that the decline is indeed rapid, with bradycardia preceding the embryonic demise while the myocardial contractile function remained preserved despite regurgitant blood flow propagating back to the placental circulation.

9.5.3 Rat

Rat embryos were also used as models for pharmacological testing (recently reviewed by Sedmera et al. [42] from the perspective of fetal arrhythmias). Of course, significant arrhythmias often result in hemodynamic instability and could lead to fetal complications. Another recent report from rat model was investigating the pathogenesis of preeclampsia induced by inflammatory reaction after lipopolysaccharide injection [43].

9.6 Fetal Hemodynamics in Humans

The ultimate goal of studies performed in various animal models (Fig. 9.3) is to gain better understanding into working of prenatal human circulation, especially at stages which are not easily studied in depth (particularly the first trimester).

As mentioned above, the fetal hemodynamics is dependent upon normal and regular heart rate [42]. Alterations in hemodynamics are believed to be one of the causes for cardiac malformations such as hypoplastic left heart syndrome [19] or valvar atresias (Fig. 9.2). Abnormalities in cardiac performance can bear influence on all the systems in the developing fetus, including the central nervous system [44]. Experimental interventions aimed at normalizing hemodynamics and preventing irreversible changes include balloon aortic or pulmonary valvuloplasty [24, 45]. However, a recent American Heart Association statement [46] lists these treatment options as experimental, as not all of them might translate into desirable outcome despite technical success [47]. This highlights the need of further basic as well as translational research into mechanisms of interactions between the structure and function of the developing heart.

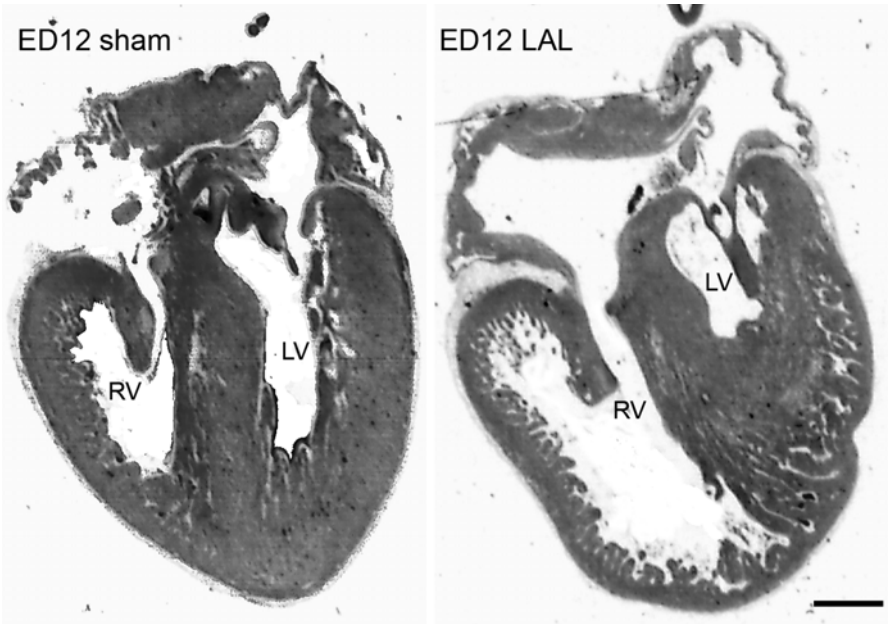

Fig. 9.2 Phenotype mimicking human hypoplastic left heart syndrome induced in the embryonic day 4 chick embryo by partial ligation of the left atrial appendage (*LAL*). Note the apex-forming right ventricle in the ligated embryo. Scale bar 1 mm

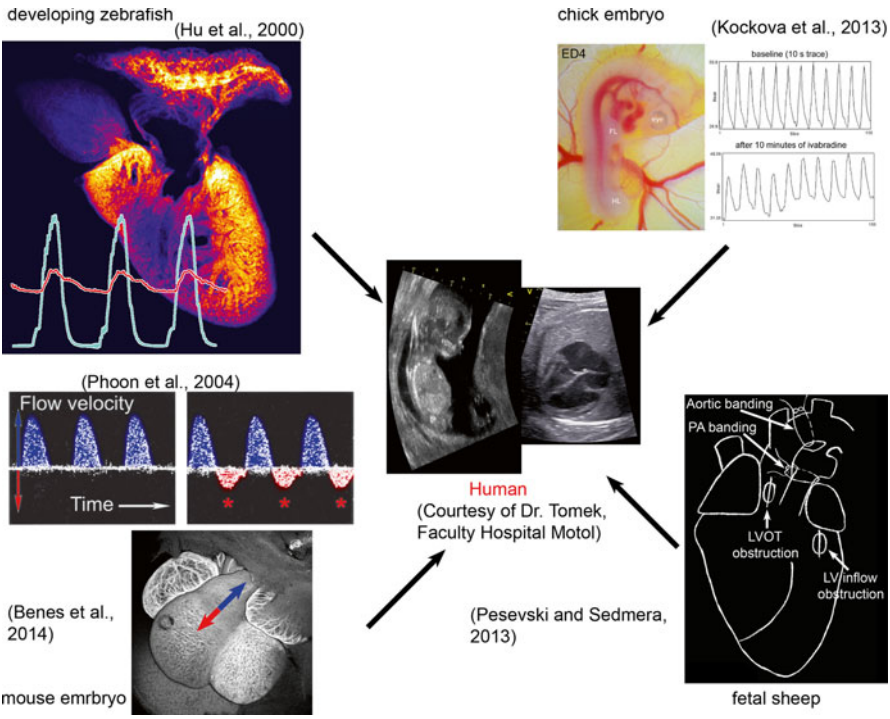

Fig. 9.3 Overview of various hemodynamic models discussed in this chapter [49–51]

9.7 Postnatal Hemodynamics

Abrupt changes in circulation occurring after birth have been the subject of numerous studies, and there is considerable variation among species in, e.g., mechanisms of closure of the foramen ovale (cell proliferation closing multiple foramina in birds or flap-like valve sealing the double septum in mammals). The neonatal heart temporarily retains some of the characteristics of the fetal heart, notably the proliferative activity that is used to compensate altered functional demands [48]. As we have recently reviewed this issue in depth [33], we may refer the readers to this book.

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Evolutionary Aspects of Cardiac Development

10

Bjarke Jensen and Antoon F.M. Moorman

Contents

Abstract

The formed hearts of vertebrates are widely different in anatomy and performance, yet their embryonic hearts are surprisingly similar. Developmental and molecular biology are making great advances in reconciling these differences by revealing an evolutionarily conserved building plan to the vertebrate heart. This suggests that perspectives from evolution may improve our understanding of the formation of the human heart. Here we exemplify this approach by discussing ventricular septation, remodelling of the atrioventricular junction and formation of the atrioventricular insulating plane.

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10.1 Introduction

With an output of 5 l per minute at rest and a systolic blood pressure of 120 mmHg, the formed human left ventricle clearly outperforms the slow-beating and low-pressure ventricles of ectothermic or cold-blooded vertebrates [1]. Architecturally, the human heart is different from the heart of a shark, where pulmonary circulation is absent and four chambers are aligned in series from the sinus venosus, atrium and ventricle to the myocardial outflow tract. Yet the embryos of all vertebrates form an early heart with a single atrium and a single trabeculated ventricle. Further, the electrocardiograms (ECG) of embryonic and adult vertebrate hearts are strikingly similar, all with distinct P, QRS and T waves [2, 3]. The question then arises whether the vast differences in cardiac performance and structure across adult vertebrates are simply variations of an evolutionarily conserved design. We will argue that this is the case and we will exemplify how an evolutionary perspective is informative for our understanding of congenital malformations of the human heart with regards to late embryonic events of ventricular septation, remodelling of the atrioventricular junction and formation of the insulating atrioventricular plane. Our understanding of the evolution of the forming heart tube from distinct populations of mesodermal cells, the so-called heart fields, has recently been greatly advanced, and for this discussion we refer to Chap. 3.

10.2 An Evolutionarily Conserved Building Plan of the Developing Heart

During development all vertebrates form a heart tube with no anatomically distinct pacemaker. Instead, a gradient of automaticity is present from the intake to the outflow so that the heartbeat initiates at the venous pole [4]. Soon thereafter, the heart tube loops and acquires an atrium and a ventricle by ballooning at the outer curvatures (Fig. 10.1a, b). The flanking regions remain tube like; these are the sinu-atrial border, the floor of the atrium, the atrioventricular canal, the inner curvature of the ventricle and the myocardial outflow tract (Fig. 10.1b). The ballooning atrium and ventricle consist of ‘working myocardium’ and have faster electrical propagation and contraction than the remaining heart tube myocardium [5]. There are no valves, but only cushions in the embryonic heart. Therefore, the slow and sustained contractions of the heart tube myocardium of the atrioventricular canal and the myocardial outflow tract are important to maintain unidirectional flow [6]. This ‘electrophysiological patterning’ of the heart is known to reflect in the working myocardium the rich expression of genes involved in propagation of the depolarizing impulse (calcium and sodium channels with voltage sensitivity), conduction (gap junction proteins) and excitation-contraction coupling (sarcomeric and calcium-handling proteins) (Fig. 10.1b) [7, 8].

The division of the embryonic heart into fast and slow propagating chambers and junctions, respectively, can give rise to an almost adult-like ECG with distinct P,

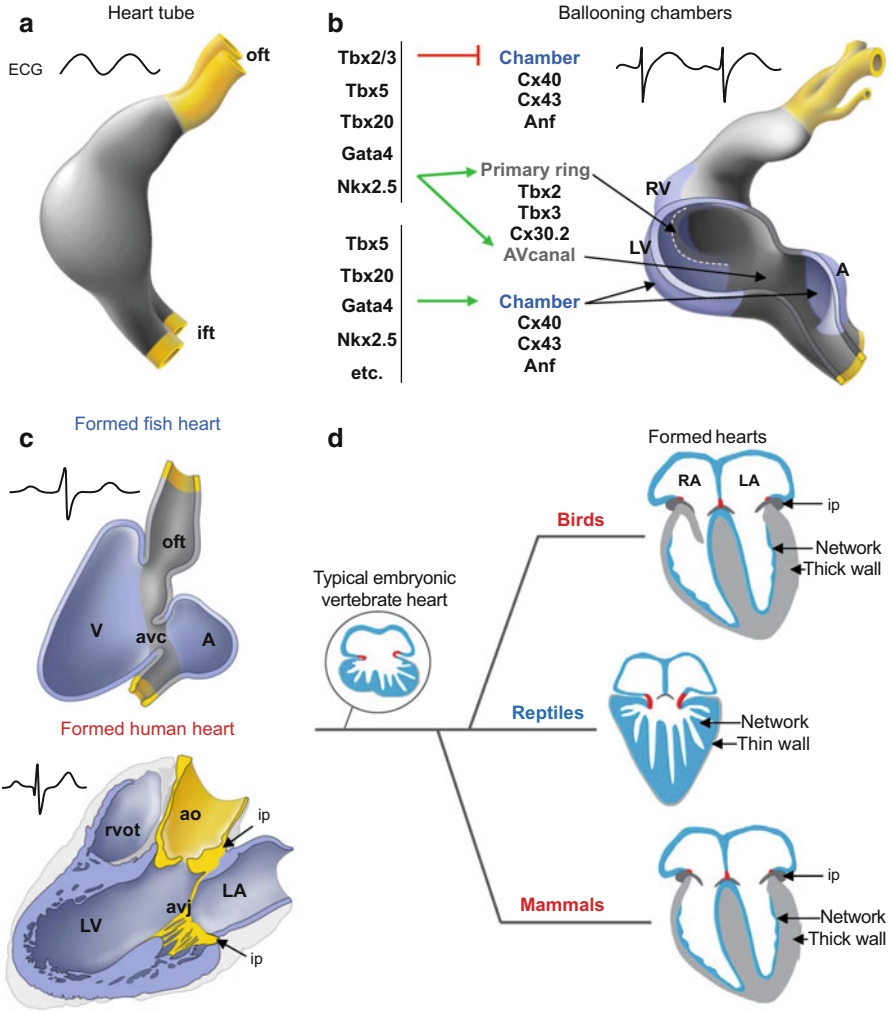


Fig. 10.1 The evolutionarily conserved building plan of the heart. (**a**) All vertebrate embryos form a heart tube with slow propagating peristaltic contractions reflected in a sinusoid ECG. (**b**) Atrial and ventricular compartments with a distinct molecular phenotype balloon out (*blue*) and due to faster propagation in these compartments the electrocardiogram (ECG) acquires an adult-like profile. Full ventricular septation in mammals and birds coincides with a primary ring that expresses the Bmp2/Tbx3 pathway. (**c**) The configuration of **b** is essentially the scaffold of the adult fish heart, except there is no primary ring, and even resembles the systemic side of the formed human heart. (**d**) The atrioventricular conduction axis is established in the embryo with a slow propagating atrioventricular canal (*red*) and a fast propagating trabeculated ventricle (*blue*) with a thin compact wall (*light grey*). This design is maintained in ectothermic vertebrates and modified in birds and mammals by the presence of the ventricular septum, thickened ventricular walls and an insulating plane (*ip*) (Modified from Jensen et al. [5])

QRS and T waves [6]. Similar regions are present in the adult hearts of fishes and amphibians, except the most basal vertebrates (hagfishes) where a myocardial outflow tract is absent and most teleost bony fishes, e.g. the zebrafish, where the myocardial outflow tract is feebly developed and the cardiac inflow is almost devoid of myocardium [9].

Differentiation of the working myocardium relies on broadly expressed transcription factors, like GATA binding protein 4 (*Gata4*) and NK2 homeobox 5 transcription factor (*Nkx2-5*), and T-box transcription factors *Tbx5* and *Tbx20* that are expressed in gradients from inflow to outflow and outflow to inflow, respectively [10]. The remaining heart tube myocardium escapes differentiation into working myocardium due to transcriptional repression. The repressive action of the pathway of bone morphogenetic protein 2/4 (*Bmp2/4*)-*Tbx2/3* appears very important [11] and is regulated upstream by the signalling pathways of Notch [12] and Wnt/β-catenin [13] and activated by co-localization with Gata4 [14] (Fig. 10.1b). This model of transcriptional regulation of chamber formation has largely been unravelled in mechanistic studies in mice, but we and others have shown a similar pattern of expression of transcription factors in all vertebrate classes investigated, ranging from lampreys, bony fishes, amphibians, reptiles, birds to mammals [15, 16]. During further development of mammals and birds, the non-chamber regions may acquire the phenotypes of the cardiac conduction system, and *Tbx3* is expressed in the sinus node, the atrioventricular node and the His bundle and its bundle branches [17] and functions in a very dosage-sensitive manner [18]. The Purkinje fibres, the terminal branches of the ventricular conduction system, share the molecular phenotype of the trabeculations of the embryonic ventricle and the fully formed ventricle of the ectotherms (Fig. 10.1d) [16]. The cardiac conduction system, thus, is built on the old scaffold of the vertebrate heart, and this may explain the great similarity in the ECGs from very different vertebrates and developmental stages (see Chap. 8).

10.3 Evolution of Cardiac Septation

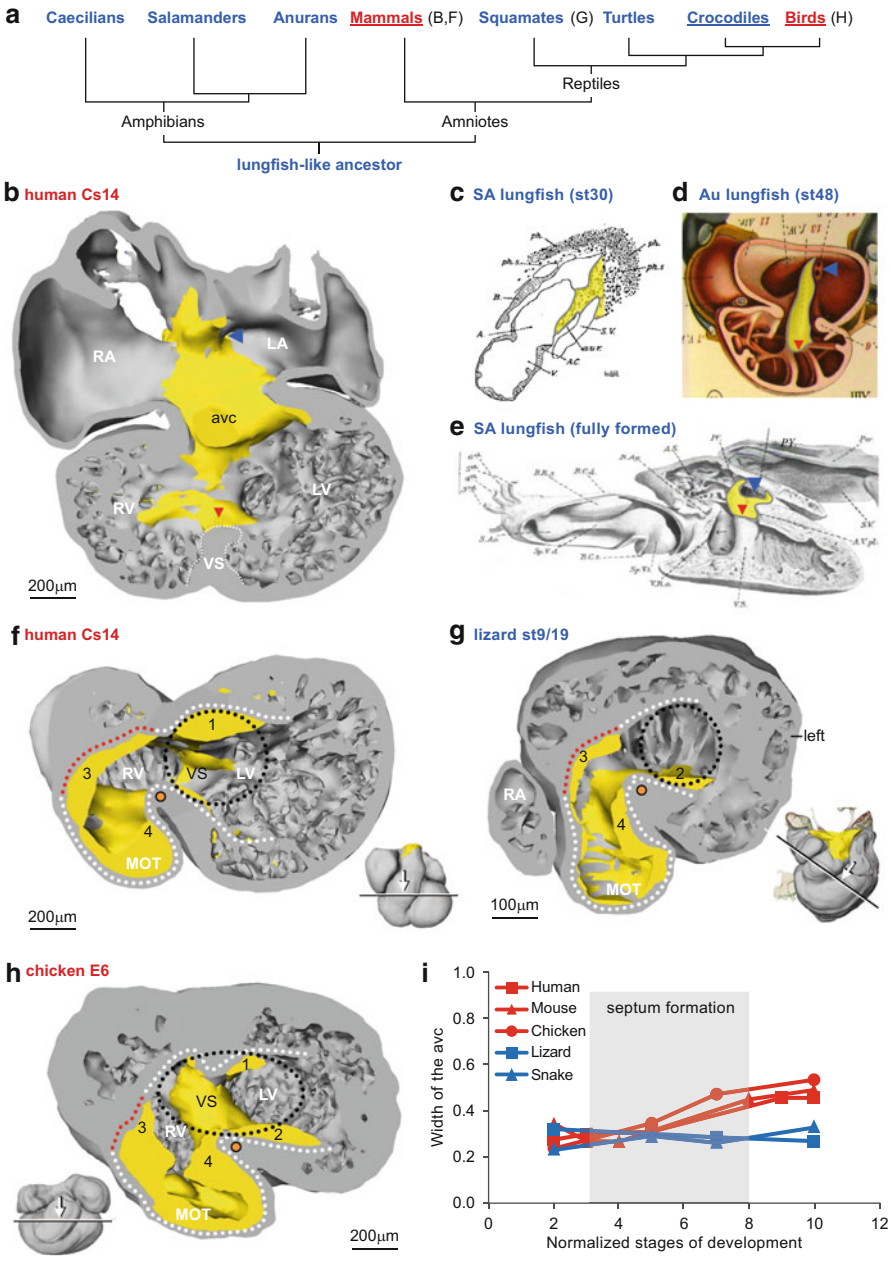
In mammals and birds, the early atrial and ventricular septa have a mesenchymal cap that expresses the *Bmp2/Tbx3* pathway [17]. This mesenchyme is continuous with the mesenchymal cushions of the atrioventricular canal and the so-called dorsal mesenchymal protrusion (or spina vestibuli of His the elder) that harbours the orifice of the pulmonary vein and contributes to the completion of atrial septation (Fig. 10.2b) [19, 20] (see Part VI). It is remarkable that a very similar mesenchymal configuration can be found in the heart of lungfishes (Fig. 10.2c–e). Benninghoff [21] called this mesenchyme a ‘Leitbahn’, or guide, for septation. Lungfishes are the extant vertebrates that resemble the tetrapod ancestors the most. They are also the only fishes where the heart displays partial septation of the atrium, ventricle and outflow. Studies on lungfish cardiogenesis, however, are experiencing a century-long hiatus and have to be extended with modern approaches [22–24]. A prominent ventricular septum of trabeculated myocardium develops in the lungfishes of Africa

and South America (Fig. 10.2e), but not in the heart of the Australian lungfish, which nonetheless has a pronounced mesenchymal guide in development (Fig. 10.2d). A complete ventricular septum, including a membranous septum, is found only in mammals, crocodilians and birds (Fig. 10.2a) [25]. Comparing these groups, it appears that ventricular septum formation relies on firstly ballooning and secondly condensation of trabeculations (see Part V). In placental mammals complete atrial septation necessitates the fusion of the first and second atrial septum (see Part IV), whereas reptiles and birds simply develop a single perforated atrial septum that closes around hatching [25].

When ventricular septum formation commences in mammals and birds, the atrioventricular canal is positioned on the left of the body midline as in reptiles (Fig. 10.2f–g). Soon after, the atrioventricular canal expands and shifts rightwards so that the right atrium remains in communication with the right ventricle (Fig. 10.2h). This process does not occur in ectothermic vertebrates (crocodilians excluded) [25]. Accordingly, the width of the atrioventricular canal almost doubles in mammals and birds, but not in the incompletely septated reptile hearts (Fig. 10.2i). Congenital malformations of the human heart like double-inlet left ventricle and tricuspid dysplasia and atresia may be considered as an incomplete rightward shift and expansion (see also Part XIV). Indeed, tricuspid atresia is not confined to man and has been reported in multiple mammalian species [26]. It is therefore crucial to know the mechanisms and forces that underlie the rightward shift and expansion of the atrioventricular canal in animals with complete ventricular septation. In formed crocodiles and birds, the mural leaflet of the right atrioventricular junction is largely myocardial and in mammals it is only late in gestation that the mural leaflet loses its myocardium and is exclusively composed of connective tissue. In Ebstein's anomaly, which has also been reported for multiple mammalian species [26], the mural leaflet has persisting myocardium [27] (see Part XV).

10.4 Evolutionary Perspectives on the Atrioventricular Insulating Plane

After the onset of ventricular septation, an insulating plane of fibro-fatty tissue will form in between the atria and ventricles. Only mammals, crocodilians and birds develop an insulating plane [25, 28], and only they have a complete ventricular septum and an expanded atrioventricular junction with large mural leaflets (Fig. 10.1c, d). Formation of the mural leaflets of the atrioventricular junction largely depends on migrating cells derived from the atrioventricular sulcus [29]. It is then possible to view the insulating plane of fibro-fatty tissue as a settling of the migrating cells of the atrioventricular sulcus. In evolution, there may have been a causal chain from septum formation, to formation of large mural leaflets and then formation of the insulating plane. If so, the insulating plane may be of little significance for electrical insulation. Indeed, ectothermic vertebrates have no insulating plane and have an ECG that is comparable to the human ECG, including a several


a
Caecilians
Salamanders
Anurans
Mammals (B,F)
Squamates (G)
Turtles
Crocodiles
Birds (H)
Reptiles
Amphibians
Amniotes
lungfish-like ancestor
b human Cs14
RA
LA
avc
RV
LV
VS
200μm
c SA lungfish (st30)
d Au lungfish (st48)
e SA lungfish (fully formed)
f human Cs14
1
2
3
4
RV
VS
LV
MOT
200μm
g lizard st9/19
left
RA
MOT
100μm
h chicken E6
1
2
3
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VS
LV
RV
MOT
200μm
i
Human
Mouse
Chicken
Lizard
Snake
septum formation
Width of the avc
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
12
Normalized stages of development

hundred millisecond delay between atrial and ventricular activations [3]. Further, studies in mice strongly suggest that the key to proper atrioventricular insulation involves evolutionarily conserved molecular patterning of the atrioventricular myocardium by *Tbx2* to repress formation of working myocardium [12, 30]. The insulating plane may therefore not be necessary for insulation, albeit it may provide another layer of safety. Alternatively, it may be important to anchor the atrioventricular valves in the presence of large pressure gradients. The high-pressure ventricle of tuna fish (ca. 100 mmHg) seemingly negates this alternative, because there is no insulating plane and the atrioventricular valves are imbedded in the atrioventricular myocardium [31, 32]. It is not clear whether the cells of the atrioventricular valves of ectothermic vertebrates are coupled to cardiomyocytes or are supported by the interstitial fibroblasts between the cardiomyocytes of the atrioventricular canal.

Fig. 10.2 Mesenchyme reflects the embryonic cardiac building plan. (**a**) Phylogeny of tetrapods with ectotherms in *blue* and endotherms in *red*, and underlined animals have complete ventricular septation. (**b**) The dorsal half of the embryonic human heart with mesenchyme (*yellow*) associated with the orifice of the pulmonary vein (*blue arrowhead*), atrial septum, cushions of the atrioventricular canal (avc) and the crest of the ventricular septum (*red arrowhead*). (**c**) In the embryonic South American lungfish, a dorsal mesenchymal protrusion harbouring the pulmonary vein precursors enters the atrium. (**d**) The cardiac mesenchyme in the embryo of the basal Australian lungfish associates with the orifice of the pulmonary vein, atrial septum, cushion of the atrioventricular canal and ventricular trabeculations (*red arrowhead*). (**e**) The right half of the fully formed heart of the South American lungfish showing the guiding mesenchyme associated with the orifice of the pulmonary vein, the perforated atrial septum (AS) and the crest of the ventricular septum (VS). (**f**–**g**) In early embryos of all amniote vertebrates, the atrioventricular canal (outlined by *black dots*) is to the left of the bulboventricular fold (*orange dot*) as exemplified by the human (**f**) and lizard (**g**). (**h**) In later embryonic stages of species with complete ventricular septation, exemplified by the chicken, the atrioventricular canal will expand to the right of the bulboventricular fold. (**i**) Quantification of the width of the atrioventricular canal. Each point is one measurement from a 'four-chamber' section of the maximal width (diameter) of the atrioventricular canal and ventricle. Stage 2 is at the onset of ventricular trabeculation; stages 3–8 encompass initiation and completion of ventricular septation in the endotherms (**c**, **e** are modified from Robertson [22], and **d** is modified from Greil [23])

Conclusion

There are vast differences in the performance and anatomy of the hearts of endothermic and ectothermic vertebrates. Conversely, there are striking similarities between electrocardiograms and cardiac embryology of all vertebrates. Recent molecular studies are harmonizing these discrepancies by revealing an evolutionarily conserved building plan to the heart. Such advances challenge common notions and may enable us to better answer not only how, but why, the human heart acquired the particular features that are necessary for a healthy life and are prone to diseases.

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Part III

Central Molecular Pathways

Inter- and Intracellular Signaling Pathways

11

Jörg Heineke

Contents

Abstract

Congenital heart disease arises from defects during prenatal heart development. This process is coordinated through a complicated web of intercellular communication between the epicardium, the endocardium, and the myocardium. In the postnatal heart, similar crosstalk between cardiomyocytes, endothelial cells, and fibroblasts exists during pathological hemodynamic overload that emerges as a consequence of a congenital heart defect. Ultimately, communication between

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cells triggers the activation of select intracellular signaling circuits to mediate hypertrophy in cardiac myocytes. Here, I review the inter- and intracellular signaling mechanisms in the heart as they were discovered mainly in genetically modified mice. The hope is that in the future, targeted therapy of specific molecules or cascades will allow correction of defects that lead to the development of congenital heart disease or pathological hypertrophy.

11.1 Introduction

Signaling between different cell types of the heart coordinates the complex events of cardiac development that finally result in the functioning four-chambered heart. Specific growth factors are released from a particular cell type at a given time point in development to direct and control the differentiation, proliferation, or migration of one or multiple neighboring cell types. In this manner, endocardial cells release, for example, neuregulin1 to induce trabeculation and proliferation of the adjacent myocardium. Similar signaling circuits originate from cardiomyocytes to impact endocardial cells and also exist between epicardial cells and the myocardium. Defects in these events (e.g., as a consequence of an inherited gene mutation) are a common reason for congenital heart disease. On the other hand, the existence of congenital heart disease puts an extra burden (i.e., pressure or volume overload) on the right or left ventricle (depending on the type of defect), which – in order to reduce wall stress – mounts a pathological hypertrophic response of the myocardium. Pathological hypertrophy, although compensatory in the short term, constitutes a common and important risk factor for the development of chronic heart failure. Heart failure is a serious condition, in which the heart, due to decreased pump function, is unable to pump enough blood into the circulation to meet systemic demands and is associated with high morbidity and mortality. The severity and clinical implications of hypertrophy or heart failure depend, to a large degree, on the type and severity of the underlying cardiac defect. Pathological myocardial growth is associated with activation and proliferation of fibroblasts, which secrete extracellular matrix. Similarly, endothelial cells initially proliferate, and angiogenesis is increased to deliver sufficient oxygen and nutrients to the growing cardiac myocytes, although in end-stage heart failure capillary rarefaction exists. Recently it has emerged that fibroblasts as well as endothelial cells serve an important regulatory role in the myocardium during chronic stress exposure in the adult – similar to their role in heart development. Growth factors and extracellular matrix act on cardiac myocytes and trigger intracellular signaling during intrauterine or postnatal physiological growth as well as under conditions of pathological hypertrophy and heart failure. Although signaling circuits are inherently complex and abundant, this chapter will highlight the central inter- and intracellular pathways and effectors that mediate cardiac development and the growth response in cardiomyocytes.

11.2 Intercellular Signaling in Cardiac Development and Physiological Growth

11.2.1 Endocardial-Myocardial Signaling

Endocardial cells constitute a cell sheet that lines the chambers of the heart and that is present directly adjacent to the myocardium, separating it from the chamber lumen. Endocardium is derived from the early cardiac mesoderm and already exists in the linear heart tube, which consists of two epithelial layers: the outer one being the myocardium (i.e., cardiomyocytes) and the inner one the endocardium. Both layers are separated by extracellular matrix, termed the cardiac jelly. The endocardium exerts a crucial influence on development of the myocardium. Lack of the endocardium results in failed myocardial maturation, with reduced cardiomyocyte proliferation and lack of myocardial trabeculation that together leads to embryonic heart failure and death. Trabeculae are folds of the myocardium that help to direct blood flow in the ventricular chambers and increase contractility. The trabeculae later contribute to the formation of papillary muscle, interventricular septum, and the cardiac conduction system. Increased or persistent trabeculation (hypertrabeculation) is associated with left ventricular noncompaction, a class of inherited cardiomyopathy [1]. Mainly mouse-based research has revealed that the endocardium releases growth factors that potently act on adjacent cardiomyocytes.

Neuregulin1 (NRG1) belongs to the epidermal growth factor family and is secreted specifically by endocardial cells during midgestation (Fig. 11.1). The tyrosine kinase receptors v-erb-b2 avian erythroblastic leukemia viral oncogene homolog (ErbB)2 and ErbB4 on cardiomyocytes act as neuregulin1 receptors (hetero-) dimerize upon ligand binding and induce ERK1/2 MAP kinase or protein kinase B/

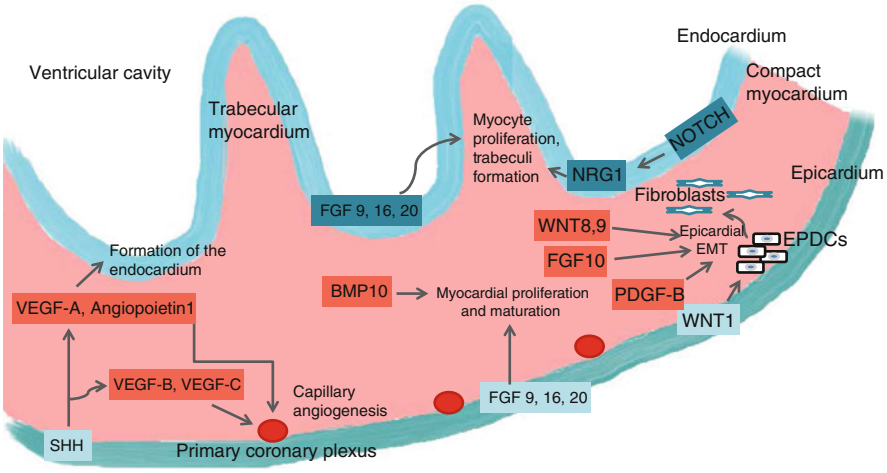

Fig. 11.1 Growth factor signaling between epicardium, endocardium, and myocardium during midgestation. Details are described in the text

Akt-dependent signaling in these cells (see below) [2]. Neuregulin1 directly induces cell survival, hypertrophy, and proliferation in isolated cardiomyocytes. NRG1-knockout mice die in the middle of embryogenesis (E10.5) due to lack of normal trabeculation of the ventricles and ErbB2- and ErbB4 null mice also lack ventricular trabeculation [2, 3]. Conversely, NRG1 injection into the developing heart leads to hypertrabeculation of the ventricles [4]. Thus, endocardial-derived NRG1 induces trabeculation of the myocardium. In addition, it is important for the maintenance of myocardial-specific gene expression in the trabecular as well as non-trabecular myocardium. Insulin-like growth factor 1 (IGF1) is also secreted by the endocardium and acts together with NRG1. Neuregulin1 expression is regulated at least in part by endocardial Notch signaling [5]: Notch is an evolutionary conserved transmembrane receptor family (Notch1-4). Upon ligand binding, the intracellular domain of the Notch receptor translocates to the nucleus to act as cofactor of the transcription factor recombination signal binding protein for immunoglobulin kappa J region (RBPJK). The membrane-bound proteins of the Delta or Jagged family are ligands of Notch. The expression of Delta4, Notch1, and Notch4 has been detected in the endocardium. Ablation of Notch signaling in the endocardium leads to hypotrabeculation as a result of reduced ephrinB2 expression (direct target of Notch), which in turn leads to reduced NRG1 expression. Through a still unknown factor, endocardial Notch activation leads to expression of BMP10 in the myocardium, which induces proliferation of cardiomyocytes in the trabeculae. In accordance with these data, over-activation of endocardial Notch leads to myocardial hypertrabeculation [6]. Fibroblast growth factors (FGF) 9, 16, and 20 are expressed in the endocardium (and in the epicardium, see below), but not in the myocardium during midgestation. They act on the myocardial FGF receptors FGFR1 and FGFR2c, to induce cardiomyocyte proliferation and differentiation [7].

11.2.2 Myocardial-Endocardial Signaling

The myocardium plays a strong paracrine and autocrine role in the regulation of cardiac development and growth and has an important impact on the endocardium as well as on endothelial cells (e.g., from capillaries). It was demonstrated, for example, that cardiomyocytes are the main source of vascular endothelial growth factor A (VEGF-A), which is one of the most potent angiogenesis-inducing factors of the body [8]. Cardiomyocyte-specific VEGF-A knockout reduces the level of VEGF-A mRNA to <15 % of the wild-type level and leads to significant embryonic mortality and hypovascular, thin-walled hearts [8]. VEGF-A mainly binds and activates the receptor tyrosine kinase VEGF receptor2 (VEGFR2, also called Flk1), which is the major mediator of VEGF-A-dependent mitogenic, angiogenic, and permeability-enhancing effects. VEGFR2 is expressed in endothelial cells including the endocardium. VEGFR2 knockout mice die at embryonic day E8.5 – E9.5 and completely lack the endocardium, blood vessels, and cardiac trabeculation [9]. Thus, myocardial-derived VEGF-A is functionally important for the formation of the endocardium, which in turn provides crucial signals for myocardial development

(Fig. 11.1). In a similar manner, cardiomyocyte-derived angiopoietin-1 binds to its receptor Tie2 (expressed on endothelial and endocardial cells) to induce the formation of the endocardium [3].

An important area of signaling between the endocardium and myocardium is found in the development of endocardial cushions in the outflow tract (OFT) and the atrioventricular canal (AVC) that later form the cardiac valves [10]. The endocardial cushions are expansions of the cardiac jelly and become visible at embryonic day E9 of mouse development. Endocardial cells lining these cushions become mesenchymal cells and move into the cushions to form the future valve interstitial cells. This process is called epithelial-to-mesenchymal transition (EMT) and is triggered by myocardial signals, for example, by bone morphogenic protein (BMP)2, which is expressed first in the myocardium surrounding the AVC [10]. Its importance for endocardial EMT is shown in cardiomyocyte-specific BMP2 deleted mice, which exerts disturbed AV cushion formation [11]. BMPs bind to type I (in the myocardium ALK2/3) and type II receptors (in the myocardium encoded by *Bmpr2*) at the surface of its target cell and trigger SMAD1/5/8-dependent signaling. Endocardial-specific ablation of *Alk2*, *Alk3*, or *Bmp2r* results in strongly impaired EMT [10]. Cardiomyocyte-derived transforming growth factor (TGF) β2 and VEGF-A are similarly involved in the regulation of endocardial EMT, whereby it has been suggested that precise levels of VEGF-A are essential for this process, since both overexpression and inhibition of VEGF-A lead to impaired EMT [10]. In this regard, deregulated myocardial VEGF-A levels were proposed to account for endocardial cushion defects due to environmental stresses or Down syndrome. Some cardiomyocyte derived growth factors also act in an autocrine fashion to regulate formation of the ventricular myocardium. Cardiomyocyte-derived BMP10, for instance, induces cardiomyocyte proliferation and maintains a normal expression level of key cardiogenic factors like NK2 homeobox 5 (NKX2-5) and myocyte enhancer factor 2c (MEF2c); lack of BMP10 in mice results in embryonic mortality between E9.5 and E10.5 with a hypoplastic myocardium [12].

11.2.3 Epicardial-Myocardial Signaling

During midgestation, around E10.5, the epicardium is beginning to form as the outmost epithelial layer that ensheathes the heart in parallel to growth of the myocardium and formation of the coronary vasculature [10]. Before that time point, the linear and looping heart tube consists only of two layers, the outer myocardium and an inner layer of endothelial cells (endocardium). The developing epicardium promotes cardiomyocyte proliferation and thereby the proper development of the myocardial compact layer, which is extremely thin after experimental removal of the epicardium in chick embryos. This regulatory role is mainly mediated by the release of paracrine factors: FGF 9, FGF16, and FGF20 are produced in the epicardium and act on FGFR1/FGFR2 on cardiomyocytes in the adjacent compact layer to induce cellular proliferation and modulate differentiation (Fig. 11.1) [3]. FGF effects in the myocardium also trigger activation of hedgehog (HH) signaling [13]; this circuit is

comprised of three ligands, sonic (S)HH, indian (I)HH, and desert D(HH), which are all expressed in the embryonic and adult heart, SHH being the most abundant. At midgestation, SHH is expressed in the epicardium, but not in the myocardium. HH ligands bind to the cell surface receptor patched (PTC). In the absence of ligand, PTC inhibits the activation of the multi-transmembrane protein Smoothend (Smo). Upon HH ligand binding, Smo triggers downstream signaling that culminates in activation of GLI transcription factors. Epicardial-derived SHH binds to PTC, which is expressed on cardiomyoblasts and perivascular mesenchymal cells in the myocardium and induces the expression and release of multiple proangiogenic molecules, especially VEGF-A, VEGF-B, VEGF-C, and angiopoietin2 from its target cells. These factors, in turn, promote coronary vascular development, which starts in the subepicardial space with the formation of the primary vascular plexus (between E11.5 and E13.5) that is later remodeled and expands into the myocardium to form the mature coronary tree. Inhibition of HH signaling in whole heart cultures with the specific inhibitor cyclopamine completely blunts coronary plexus formation, while transgenic overexpression of the HH transcription factor GLI2 in the myocardium at E13.5 increased the vessel density in the subepicardium [14]. Interestingly, HH signaling is still important for maintenance of the coronary vasculature in adult mice, as induced genetic deletion of PTC in cardiomyocytes leads to diminution of cardiac capillarization, aggravation of cardiac hypoxia, cardiomyocyte cell death, subsequent heart failure, and death. Inhibition of HH signaling after myocardial infarction (MI) triggers increased scarring, heart failure, and lethality. Of note, the ligand SHH is expressed in perivascular and interstitial fibroblasts in the myocardium of adult mice, which are cells that are originally mainly derived from the epicardium. In addition, the epicardium itself becomes reactivated and expands after MI and starts to release paracrine factors like WNT1, VEGF-A, FGF, TGFβ2, stromal cell-derived factor 1 (SDF1), and monocyte chemoattractant protein 1 (MCP1) [15, 16]. These factors play a protective role when administered to the myocardium after MI [16].

11.2.4 Myocardial-Epicardial Signaling

During embryonic development, the epicardium does not only play a prominent regulatory role via the release of paracrine factors but also contributes to different cell types (mainly fibroblasts and vascular smooth muscle cells) in the heart through EMT. Epicardial cells start to undergo EMT (starting at around E11.5 in mice) to form mesenchymal cells known as epicardium-derived cells (EPDCs, Fig. 11.1) [10, 13]. EPDCs reside in the subepicardial space and also migrate into the myocardium to form a subset of cardiac fibroblasts and vascular smooth muscle cells. Although previously it was thought that all capillary endothelial cells also arise from EPDCs, recent data revealed that only a small fraction of these cells originate from the epicardium [13]. The majority of vascular endothelial cells are derived from the endocardium and the sinus venosus. Epicardial cell proliferation and activation as well as EMT occur during myocardial injury such as MI also in

adult mice. This leads to a thickened epicardial layer on the cardiac surface, but in contrast to embryonic development, not to an invasion of the subjacent myocardium. Epicardial EMT mainly is triggered by myocardial-derived growth factors. In this regard, Wnt ligands (WNTs) from the myocardium (e.g., WNT8a or WNT9) but also autocrine epicardial WNT1 act on the epicardium [3]. In their canonical signaling mode, WNTs bind to a co-receptor complex consisting of frizzled (Fzd) family seven-pass transmembrane proteins and the lipoprotein receptor related 5/6 (LRP5/6), which ultimately leads to stabilization and nuclear translocation of β-catenin. β-Catenin forms a complex with LEF/TCF family DNA binding proteins to activate the transcription of WNT target genes. Elimination of β-catenin from the epicardium with a Gata5-Cre leads to embryonic mortality in mice between embryonic day 15 and birth, whereby the mutant mice display a defective coronary artery development, but normal veins and microvasculature. This phenotype is the consequence of failed expansion of the subepicardial space and impaired differentiation of epicardium-derived coronary smooth muscle cells, indicating a defective EMT in response to ablation of WNT signaling. In a similar manner, FGF10 from the myocardium might act on FGF receptors-1/2 on epicardial cells. Elimination of this signaling axis leads to myocardial hypoplasia and a defect in cardiac fibroblast formation, although this was disputed in one study [17, 18]. In a third important pathway, platelet-derived growth factor (PDGF) signaling from the myocardium regulates epicardial EMT. When both epicardial PDGF receptors α and β are eliminated from epicardial cells, the process of EMT is blocked with a complete lack of EPDCs. However, as revealed by a selective knockout of the α- and β- form of the receptor in the epicardium, both receptors also have independent functions: the PDGF receptor α promotes the development of myocardial fibroblasts, while the PDGF receptor β promotes the formation of coronary vascular smooth muscle cells.

11.3 Intercellular Signaling During Pathological Cardiac Growth

While intercellular coordination during the embryonic phase, when the developing heart is still small, depends mainly on signals from the epicardium and endocardium, in the adult heart – due to larger distances – interspersed cells such as fibroblasts and capillary endothelial cells become a more important source of regulatory growth factors that impact cardiac myocytes.

11.3.1 Communication Between Cardiomyocytes

Although in terms of cell number cardiomyocytes represent only 30–45 % (depending on species) of all heart cells, because of their size they contribute to more than 90 % of the heart's volume. Rich communication takes place among cardiomyocytes. Importantly, cardiac myocytes are directly coupled via gap junction at the intercalated disk (Fig. 11.2) [19]. Mainly ions (Ca^{++}) and small solutes pass through

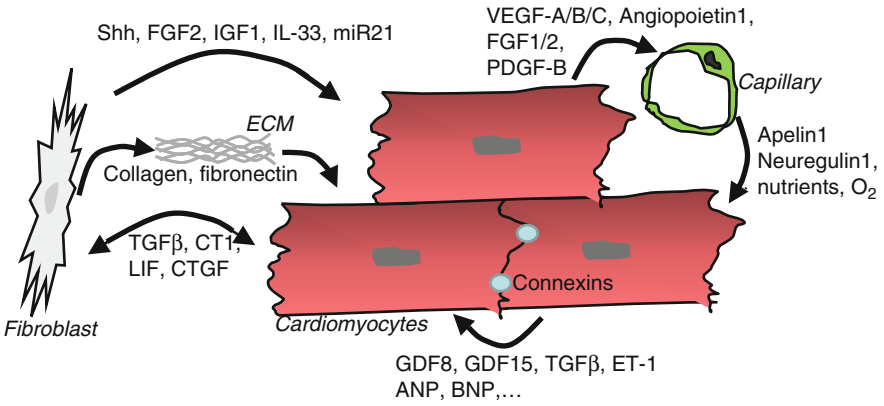

Fig. 11.2 Intercellular communication in the adult myocardium as it occurs during hemodynamic overload. Details are described in the text

gap junction to promote impulse conduction in the cardiac conduction system and the working myocardium. The gap junctions in adult mice are constituted by connexin40 in myocytes of the conduction system, but by connexin43 in the working myocardium. Besides impulse conduction, the connexins are important for cardiac morphogenesis, since heterozygous and homozygous deletion of connexin40 leads to developmental abnormalities of the heart, including double-outlet right ventricle, tetralogy of Fallot, and endocardial cushion defects [20]. Cardiomyocyte-specific deletion of connexin43 results in outflow tract abnormalities and cardiac hypertrophy after birth.

Cardiomyocytes also secrete various growth factors, which either act in an autocrine fashion on the secreting cell, a neighboring cardiomyocyte or on non-cardiomyocytes. Among the myocyte-derived factors with functional autocrine effects are, for example, endothelin 1, ANP, and BNP as well as multiple TGFβ family members, including TGFβ, growth differentiation factor (GDF) 15, and myostatin (GDF8). TGFβ is released from cardiac fibroblasts, but also from cardiomyocytes. Ablation of the TGFβ receptor (R)2 specifically in cardiac myocytes strongly reduced cardiac hypertrophy, fibrosis, and improved cardiac function during experimental pressure overload in mice by transverse aortic constriction (TAC). In this procedure, which often is used as a model of human disease in mice, a ligature is placed between the left common carotid artery and the right innominate artery around the ascending aortic arch. This leads to a robust mechanical pressure overloading of the left ventricle with cardiac hypertrophy (+30–60 % in heart weight) within 2 weeks and reduced cardiac function within 4–8 weeks after surgery. The data from cardiomyocyte-specific Tgfβr2 knockout mice indicate that intrinsic cardiomyocyte TGFβ signaling is a strong promoter of pathological hypertrophy and dysfunction under these circumstances. In contrast, cardiomyocyte GDF15 acts to inhibit hypertrophy and death in these cells. Myocardial myostatin is crucial for the maintenance of cardiac homeostasis, as its selective-induced genetic ablation under baseline conditions resulted in

hypertrophy, heart failure, and death associated with metabolic imbalance and over-activation of the AMP-activated kinase (AMPK) [21].

11.3.2 Endothelial-Cardiomyocyte Crosstalk

As an organ highly dependent on oxidative energy production, the capillary density in the heart is high, and each cardiomyocyte is supplied roughly by one capillary [22]. Capillary endothelial cells are closely associated with cardiomyocytes in an ideal diffusion range for capillary-derived nutrients and oxygen but also for reciprocal paracrine signals between these cells. It has been demonstrated that cardiomyocytes regulate the formation and adaptation of the myocardial capillary network and that angiogenesis (i.e., the formation of capillaries from preexisting endothelial cells) is enhanced during increased hemodynamic load and cardiac hypertrophy in multiple different species (mice, sheep, humans). In fact, this increase in myocardial capillaries (by about 30–50 %) is important for the preservation of cardiac function during hypertrophy. How is myocardial angiogenesis regulated, especially during pathological overload? As stated above, around 85 % of the VEGF-A within the heart is produced by cardiac myocytes [8]. Similarly, expression of proangiogenic growth factors like VEGF-B, VEGF-C, angiopoietin1, FGF1, FGF2, EGF, matrix metalloproteinase (MMP) 9, as well as PDGF-B has been reported in cardiac myocytes (Fig. 11.2). Expression of these molecules is triggered in cardiomyocytes by signaling molecules, transcription factors, and transcriptional co-regulators. In this regard, the transcription factor GATA4, which is activated by mechanical overload in cardiomyocytes, directly binds, and activates the *Vegfa* promoter [23]. Consequently, cardiomyocyte-specific GATA4 overexpression induces VEGF-A and capillary angiogenesis in the myocardium of mice, while in turn, genetic deletion of GATA4 in cardiac myocytes reduces myocardial angiogenesis and also leads to heart failure. In parallel, the hypoxia-sensitive transcription factor HIF1-α, which is a known direct regulator of VEGF-A and other angiogenic growth factors, becomes activated early in the course of cardiac pressure overload, when cardiomyocyte growth exceeds the ability of the existing capillary network to deliver enough oxygen for the muscle cells and hypoxia emerges [24]. Other cardiomyocyte-based regulators with positive effects on myocardial capillary growth include the transcriptional co-regulator peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (PGC1α), the transcription factor signal transducer, and activator of transcription 3 (STAT3) and protein kinase B/Akt. Although capillary growth in the heart is enhanced during the initial compensatory phase of cardiac overload, capillary density decreases with disease progression, and capillary rarefaction is ultimately present in terminal heart failure. This might, at least in part, be due to the fact that cardiomyocyte GATA4 activation decreases and Hif1-α becomes inhibited by p53 in persisting pressure overload. Restitution of capillary density under these conditions, for example, by the delivery of angiogenic growth factors like VEGF-A and angiopoietin1, improves cardiac function, indicating that sufficient angiogenesis is crucial for the maintenance of heart function under pathological stress.

How do endothelial cells influence cardiomyocytes to maintain their function? First and foremost, they deliver oxygen and nutrients (i.e., amino acids, glucose, and fatty acids) to enable the production of ATP by myocytes. Second, paracrine factors released by the endothelial cells play an important role for cardiac homeostasis and survival. In a co-culture system of cardiomyocytes and endothelial cells, in which oxygen and nutrient delivery by capillaries naturally do not play a role, endothelial cells are essential for the survival of myocytes and also trigger their spatial organization and rhythmic contraction. As endothelial-derived paracrine factors, neuregulin1, which acts on ErbB2 and ErbB4 receptors on cardiomyocytes and which promotes myocyte survival and hypertrophy, as well as apelin1, which induces a strong positive inotropic response via its G-protein coupled receptor APJ, have been identified [2, 19]. Interestingly, both neuregulin1 and apelin1 are being evaluated as therapy in patients with heart failure.

11.3.3 Fibroblast-Cardiomyocyte Crosstalk

Cardiac fibroblasts are spindle-shaped cells that specifically express the PDGF receptor α as well as vimentin. They arise through EMT from the epicardium as well as the endocardium during embryonic development [25]. The primary function of cardiac fibroblasts is to synthesize (and degrade) extracellular matrix, which forms a three-dimensional structural network that supports cohesion of myocardial cells, cardiac shape, and function. Extracellular matrix (ECM) in the heart consists mainly of collagen I and III, fibronectin, proteoglycans, and glycoproteins [26]. In addition, fibroblasts communicate with cardiomyocytes via the release of specific growth factors, through extracellular matrix and even more directly by forming connexin containing gap-junction between these two different cell types.

In the embryonic heart, release of fibronectin, EGF-like growth factor and collagen by fibroblasts, promotes cardiac myocyte proliferation, by stimulating β1-integrin-dependent signaling in these cells [27]. In the healthy adult heart, fibroblasts are mainly quiescent, but become activated in response to mechanical overload and profibrotic molecules like TGFβ and connective tissue growth factor (CTGF), which are expressed in fibroblasts as well as cardiomyocytes (Fig. 11.2). Activation leads to a dramatic increase in fibroblast proliferation, secretion of extracellular matrix proteins, and growth factors. In addition, a fraction of fibroblasts (around 15 % in murine pressure overload) become myofibroblasts, which is a contractile cell type, characterized by the expression of α-smooth-muscle actin.

Co-culture with adult heart fibroblasts leads to hypertrophy in cardiomyocytes [27]. One of the growth factor responsible for this effect could be TGFβ1, which is released abundantly from myocytes as well as fibroblasts and which induces hypertrophy and dysfunction in cardiomyocytes and extracellular matrix production in fibroblasts. Interestingly, the endogenous prohypertrophic agonist angiotensin II primarily acts on the angiotensin type 1 receptor on cardiac fibroblasts and triggers cardiomyocyte growth indirectly through the induction of TGFβ1 and FGF2. FGF2 is mainly produced in myocardial fibroblasts and induces hypertrophy of adjacent

cardiomyocytes. In support of this, FGF2 knockout mice showed reduced hypertrophy during pressure overload. Cardiac IGF1 is predominantly derived from fibroblasts, where its expression is induced by the transcription factor Krüppel-like-factor 5 (KLF5) [28]. Fibroblast IGF1 promotes cardiomyocyte hypertrophy and myocardial fibrosis, but is also essential for preventing heart failure and mortality during pressure overload in mice. Members of the IL-6 family like cardiotrophin-1 and leukemia inhibitory factor (LIF) are synthesized by cardiac fibroblasts and myocytes and signal through the transmembrane gp130 receptor to induce cardiomyocyte hypertrophy. In addition, CT-1 also promotes fibroblast migration, while LIF inhibits myofibroblast transition and collagen synthesis.

Fibroblasts do not only release factors that induce growth in cardiac myocytes. Interleukin-33 (IL-33), which is expressed by cardiac fibroblast in response to mechanical load, inhibits cardiomyocyte hypertrophy in a paracrine and dose-dependent manner by binding to its receptor STL2.

It is currently emerging that cardiomyocyte-fibroblast crosstalk is not only regulated by proteins but also by non-coding RNA molecules such as microRNAs (miRs) [29]. MiRs are short (17–25 nucleotides) non-coding RNAs that function mainly to inhibit gene expression of specific mRNAs. They are processed from 60 to 70 nucleotides pre-miRs, which are exported to the cytosol where they are cleaved by the enzyme dicer to produce the mature duplex miR, consisting of a guide strand and a passenger strand. Mainly the guide strand targets cellular mRNA for silencing. miR133a is predominantly expressed in cardiomyocytes, where it blocks the expression of the profibrotic factor CTGF. As a result, miR133 knockout mice develop heart failure with massive myocardial fibrosis, while cardiomyocyte-specific overexpression protected the mice from cardiac fibrosis after pressure overload. miR21, in contrast, was found to be specifically upregulated in fibroblasts of failing hearts, where it targets intracellular signaling and the paracrine influence on cardiomyocytes. Inhibition of miR21 by an antagomiR (small, chemically modified RNA) reduces cardiac fibrosis and hypertrophy after pressure overload. Fibroblasts also release the passenger strand of miR21 in exosomes (small vesicles), which are taken up by cardiomyocytes, and thereby induce hypertrophy in these cells.

11.4 Cardiomyocyte Intracellular Signaling During Pathological and Physiological Heart Growth

Cardiomyocytes have a very low ability to divide after birth, and myocardial growth then occurs through enlargement (i.e., hypertrophy) of these cells [30]. Indeed, physiological hypertrophy drives the normal growth of the heart from birth to early adulthood and during professional exercise and pregnancy. Physiological hypertrophy is characterized by a balanced growth of chambers and ventricular walls and is not associated with dysfunction or fibrosis. In contrast, pathological hypertrophy occurs in response to disease associated stimuli like pressure overload or volume overload. During pressure overload, cardiomyocytes become mainly thicker and concentric hypertrophy (thick ventricular walls and small chambers) develops.

Eccentric hypertrophy (with dilated chambers and thin walls) is the consequence of volume overload and cardiomyocyte elongation. All forms of pathological hypertrophy are only partially reversible, associated with ventricular dysfunction, exert interstitial, and perivascular fibrosis and often lead to heart failure.

11.4.1 Signaling in Physiological Heart Growth

Insulin, growth hormone, and IGF1 are prominent inducers of physiological cardiac growth (Fig. 11.3) [31, 32]. Insulin binds and activates the tyrosine kinase insulin receptor, which leads to the recruitment and phosphorylation of the adaptor proteins insulin receptor substrate 1 (IRS1) and IRS2 that in turn activate phosphoinositide 3-kinase (PI3K)α/AKT (v-akt murine thymoma viral oncogene homolog) signaling (see below) at the plasma membrane. IGF1 binds to the insulin receptor and the IGF1 receptor, which also leads to the activation of PI3Kα. PI3K converts the plasma membrane lipid phosphatidylinositol-4,5-bisphosphate to phosphatidylinositol-3,4,5-trisphosphate. PI3Kα is a heterodimer that consists of a p85 regulatory subunit and a p110α subunit. It is functionally important, as cardiomyocyte-specific overexpression of a dominant negative form of p110α inhibited physiological cardiac hypertrophy during postnatal growth and during swimming exercise, while overexpression of a constitutive active p110α produced physiological cardiac hypertrophy. PI3K activation results in the sarcolemmal recruitment of the kinases AKT/PKB

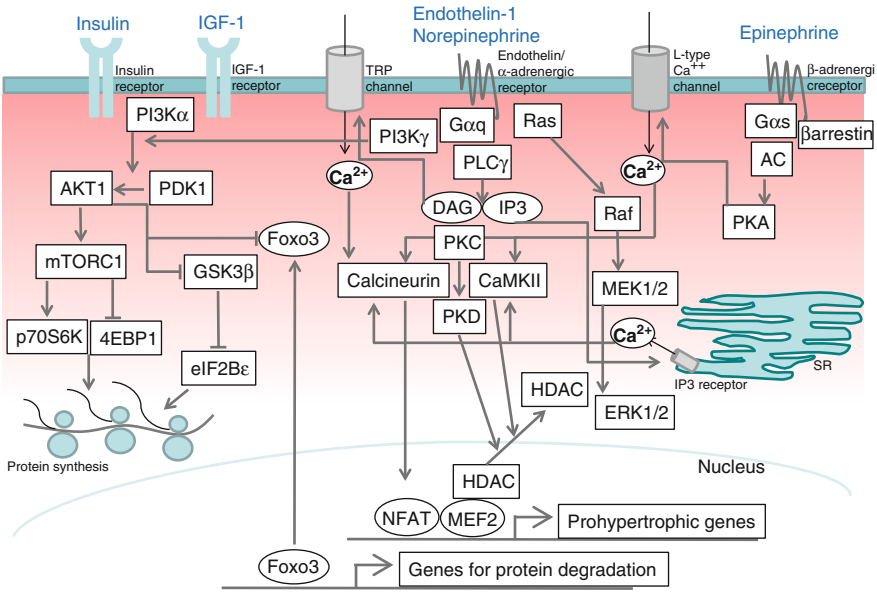

Fig. 11.3 Prohypertrophic intracellular signal transduction in cardiac myocytes. Details are described in the text. Abbreviations not mentioned in the text: *SR* sarcoplasmatic reticulum, *AC* adenylate cyclase, *p70S6K* p70 S6 kinase

and phosphoinositide-dependent kinase1 (PDK1) through their pleckstrin-homology domains. When PDK1 and AKT are brought into close proximity, PDK1 phosphorylates and activates AKT. AKT1 and AKT2 are expressed in the heart. AKT1 is necessary for the induction of physiological hypertrophy, as *Akt1* knockout mice were defective in exercise-induced hypertrophy. In response to pathological pressure overload, AKT1 KO mice displayed cardiac dilation and dysfunction, indicating that AKT1 promotes cardiac compensation in this setting. Short-term (2 weeks) transgenic overexpression of AKT in the heart leads to physiological cardiac hypertrophy, while this hypertrophy acquires pathological features when it persists for 6 weeks, indicating that AKT in principle can induce pathological as well as physiological hypertrophy depending on the duration of the stimulus. Mechanistically, AKT1 directly promotes protein translation, in part by inhibiting constitutive GSK3β activity, which negatively regulates eukaryotic translation initiation factor 2Bε (eIF2Bε), as well as by indirectly activating the protein kinase mechanistic target of rapamycin (mTOR). In addition, AKT inactivates the transcription factors Forkhead box protein O3 (FOXO3) and thereby decreases the expression of ubiquitin ligases like atrogin-1. This reduces the rate of cellular protein degradation and therefore favors the net protein accumulation needed for hypertrophy.

mTOR as part of the so-called mTOR complex 1 (mTORC1, together with the proteins RAPTOR and Lst8) can be activated by AKT as well as, for example, by amino acids. Lack of cellular energy during fasting or starvation leads to activation of the AMP-dependent protein kinase which inhibits mTORC1. mTORC1 promotes ribosomal protein production through activation of S6 kinases and by inhibiting the eIF4E-binding protein1 (4EBP1). Pharmacological inhibition of mTOR by rapamycin reverses cardiac hypertrophy induced by AKT overexpression but also pathological hypertrophy in response to pressure overload. Genetic deletion of *Mtor* in cardiomyocytes results in heart failure without an initial phase of hypertrophy.

11.4.2 Signaling in Pathological Heart Growth

During pathological overload of the heart, angiotensin II, endothelin 1, and α-adrenergic catecholamines bind to seven-transmembrane-spanning receptors that are coupled to heterotrimeric G proteins of the Gαq/α11 subclass and activate phospholipase Cβ (PLCβ, Fig. 11.3) [32]. This induces the generation of diacylglycerol (DAG), which functions as an intracellular ligand for protein kinase C (PKC), leading to PKC activation and induction of inositol-1,4,5-triphosphate (IP3). IP3 leads to the mobilization of internal calcium by direct binding to the IP3 receptor at the sarcoplasmatic reticulum or the nuclear envelope. DAG and IP3 also trigger opening of transient receptor potential (TRPC) channels, which leads to influx of sodium and mainly calcium into cardiac myocytes. Increase in this signaling-associated calcium occurs in cellular microdomains associated with specialized lipid rafts (termed caveolae) or in proximity to plasma membrane invaginations, called T-tubules [33]. Signaling-associated calcium is shielded from the highly abundant and mainly sarcoplasmatic reticulum-based calcium that activates cardiomyocyte contraction by

binding to contractile filaments ("contractile calcium"). Signaling calcium binds to calmodulin and subsequently activates prohypertrophic downstream signaling by the calmodulin-dependent kinase (CaMK) II and the phosphatase calcineurin. Gαq-triggered signaling also leads to activation of MAPK (exact mechanisms not known) and AKT through the PI3Kγ. The β-adrenergic receptor also initiates prohypertrophic signaling: it is associated with Gαs-dependent activation of the adenylate cyclase and subsequent activation of protein kinase A (PKA). PKA induces calcium influx from the extracellular space through the L-type calcium channel, thereby also contributing to signaling calcium to activate CaMKII and calcineurin. In addition, the scaffold protein β-arrestin associates with the intracellular portion of β-receptor to activate CaMKII as well as MAP kinase signaling.

CaMKII is induced in expression and becomes activated in response to pressure overload in the myocardium [34]. Among its different isoforms, CaMKIIδ is the most abundant in the heart, although CaMKIIγ is also expressed there. CaMKII is a serine/threonine kinase that is directly activated by calcium/calmodulin binding and by reactive oxygen species. The splice variant CaMKIIδB is localized to the nucleus, while CaMKIIδC is found in the cytosol. Genetic ablation of CaMKIIδ reduced cardiac hypertrophy, fibrosis, and dysfunction in mice. Cardiomyocyte-specific overexpression of CaMKII, in turn, leads to cardiac hypertrophy (in the case of CaMKIIδB) or dilated cardiomyopathy (CaMKIIδC). CaMKII directly phosphorylates the histone deacetylase (HDAC) 4 and thereby promotes binding of the chaperone 14-3-3 to cause export of HDAC4 from the nucleus. Reduction of nuclear HDAC4 leads to enhanced activation of the prohypertrophic transcription factor MEF2, which usually is directly repressed by class II HDACs. Indeed, class IIa HDACs (HDACS 4,5,7, 9) are known to inhibit cardiac hypertrophy and mice lacking HDAC5 or HDAC9 show spontaneous myocardial hypertrophy with aging or exaggerated hypertrophy in response to pressure overload. Nuclear export of class IIa HDACs can also be induced through phosphorylation by protein kinase D. In contrast, class I HDACs like HDAC 1, 2, and 3, which are constitutively present in the nucleus, are not regulated by kinases, and promote cardiac hypertrophy and failure, as demonstrated with specific pharmacological inhibitors like apicidin that improve adverse remodeling in mice.

The calcium-dependent serine/threonine protein-phosphatase calcineurin consists of a catalytic subunit (CnA) and a 19-kDa regulatory subunit (CnB) [32, 33]. The dimeric protein becomes activated in response to increased calcium concentrations through direct binding of calcium-bound calmodulin. Activated calcineurin dephosphorylates the transcription factor nuclear factor of activated T-cells (NFAT) in proximity to the cardiomyocyte plasma membrane, where calcineurin is anchored by the small adaptor protein CIB1. Upon dephosphorylation, NFAT translocates into the nucleus to induce the expression of hypertrophy inducing genes. Myocardial calcineurin/NFAT signaling appears to be selectively activated in pathological hypertrophy and heart failure, but not under conditions of physiological hypertrophy. Genetic ablation of the CnAβ isoform in mice, which is upregulated in the heart during pressure overload, blunts pathological hypertrophy, as does genetic elimination of NFATc3 and NFATc2 [35–37].

Among the MAP kinases mainly the activation of ERK1/2 contributes to the development of cardiac hypertrophy. Activation of ERK kinases occurs downstream of G-protein-coupled receptors, receptor tyrosine kinases (e.g., IGF1 and FGF receptors), receptor serine/threonine kinases (TGFβ receptors), gp130 receptors, as well as integrins in response to mechanical stretch [8]. Downstream of these receptors the small G-protein Ras is activated, which then recruits the MAP kinase (MAP3K) Raf-1 to the plasma membrane. Raf-1 then activates the dual-specificity kinases MEK1 and MEK2 (MAP2K) that finally phosphorylate and activate ERK1/2. Constitutive expression of an activated MEK1 mutant in cardiomyocytes results in a concentric form of hypertrophy with increased myocyte width, but lack of cardiac fibrosis or dysfunction. Genetic elimination of cardiomyocyte ERK1 and ERK2, in turn, leads to spontaneous eccentric cardiac hypertrophy (i.e., cardiac dilation with cardiomyocyte elongation at the microscopic level) and ventricular dysfunction [38]. Thus ERK1/2 promotes a compensated form of concentric cardiac hypertrophy. The other MAP kinases p38 and JNK, which are also activated in response to pathological stress, appear to inhibit cardiomyocyte hypertrophy by rephosphorylating NFAT and thereby promoting its export from the nucleus. Casein kinase 2α, in contrast, is a kinase that promotes cardiac hypertrophy through phosphorylation of the tumor suppressor protein p27, which is degraded in response, as well as through the activation of HDAC2 [39, 40].

Beside NFAT and MEF2, other transcription factors with prohypertrophic effects in cardiomyocyte include GATA4, GATA6, serum response factor (SRF), and nuclear factor (NF)-κB.

Conclusion

Complex signaling circuits between and within cells coordinate not only cardiac development and growth but also the adaptation in response to disease causing stimuli. Perturbation of these pathways, for example, as consequence of inherited gene mutations or environmental stimuli triggers and perpetuates heart failure. In the future, the understanding of intercellular and intracellular signaling needs to be broadened and include novel deep sequencing-based single cell screening approaches as well as addressing the role of non-coding RNAs (miRs as well as long non-coding RNAs) and epigenetic regulators. The hope exists that specific targeting of signaling molecules or circuits, for instance, by small molecules or gene therapy will one day improve the outcome of individuals with congenital heart defects.

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Cardiac Transcription Factors and Regulatory Networks

12

Marcel Grunert, Cornelia Dorn, and Silke Rickert-Sperling

Contents

Abstract

Cardiac development is a fine-tuned process governed by complex transcriptional networks, in which transcription factors (TFs) interact with other regulatory layers. In this chapter, we first introduce the core cardiac TFs including Gata, Hand, Nkx2, Mef2 and Tbx, and Srf. These factors regulate each other's expression and also can act in a combinatorial manner on their downstream targets. Their disruption leads to various cardiac phenotypes in mice, and mutations in humans have been associated with congenital heart defects. In the second part

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of the chapter, we discuss different levels of regulation including *cis*-regulatory elements, chromatin structure, and micro-RNAs, which can interact with transcription factors, modulate their function, or are downstream targets. Finally, examples of disturbances of the cardiac regulatory network leading to congenital heart diseases in human are provided.

12.1 Transcription Factor Networks Driving Heart Development

A multitude of studies has revealed a set of myogenic transcription factors (TFs) that is at the core of an evolutionary conserved regulatory network defining cardiac cell fate, myogenic differentiation, and cardiac morphogenesis. These transcription factors are functionally highly interconnected with each other and operate in concert with upstream signaling pathways and downstream target genes. The transcriptional network involved in heart development is highly conserved across vast phylogenetic distances, and the first heart-like organ is believed to have appeared over 500 million years ago in an ancestral bilaterian (see Chap. 10). So, the emergence of hearts with increasing complexity occurred through modification and expansion of an ancestral network of transcription factors and their *cis*-regulatory elements. During evolution, the heart evolved from a single-layered tube with peristaltic contractility to a powerful multichambered pump. *Drosophila* has provided a model to delineating the architecture of the cardiac regulatory network, due to the lack of functional redundancies in the network. Mutations of cardiac transcription factors in *Drosophila* often result in dramatic phenotypes, whereas mutations of individual paralogs of these genes in mammals affect specific substructures of the heart that do not exist in insects. The core cardiac transcription factor network in *Drosophila* consists of NK-2 (tinman), MEF2, TBX (mid-H15), GATA (Pannier), and HAND and corresponds to the vertebrate paralogs Nkx2-5 (NK2 homeobox 5), Mef2 (myocyte enhancer factor 2), Tbx (T-box) 1/2/3/5/18/20, Gata-4 (GATA binding protein 4) and Hand1/2 (heart and neural crest derivatives expressed 1 and 2) as core factors of the mammalian heart. In an evolutionary context, the linear heart tube gives rise to the left ventricle of the heart, which is the ancestral chordate cardiac component, whereas the right ventricular chamber and outflow tract are later evolutionary additions formed from an adjacent population of progenitor cells, referred to as the second heart field (see Chap. 3) [1]. Along with the addition of the second heart field, a further transcription factor named Isl1 joined this core transcription network; however, Isl1 is not cardiac specific and functionally requires combinatorial mechanisms with other factors [2, 3].

Core cardiac transcription factors cross-regulate and autoregulate their expression, thus activation of one or a few factors within the network may ultimately activate them all, as well as common sets of downstream targets [4]. One of the first large-scale studies on cardiac transcription networks used an integrative approach to

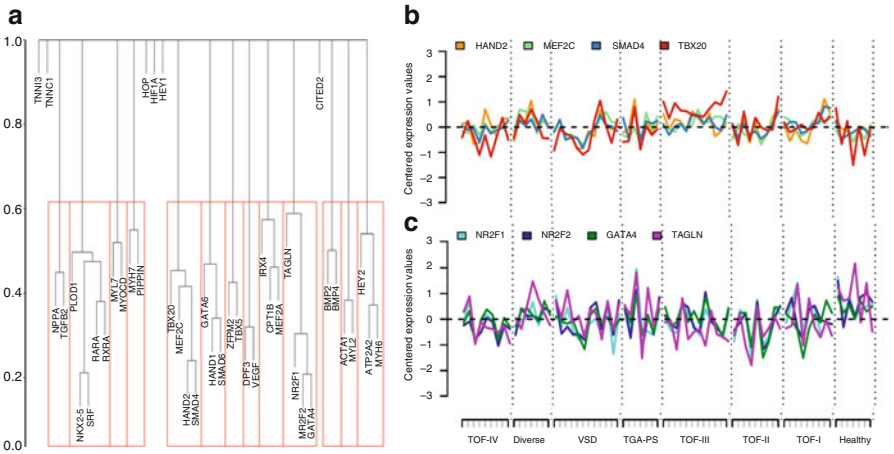

Fig. 12.1 Correlated gene groups. (**a**) Cluster dendrogram showing 13 correlated gene groups. Clustering was derived by cutting the cluster tree at the 0.001 quantile of a random distribution. The cluster distances are indicated on the y-axis. (**b**, **c**) Example of two correlated gene groups showing highly correlated patterns of expression in samples of patients and healthy individuals. Centered expression vectors were sorted by defined meta-phenotypes (Figure adapted from Toenjes et al. [5])

predict regulatory subnetworks in patients with diverse cardiac malformations. In applying linear models, specific disease-associated transcription profiles were identified in cardiac biopsies. Moreover, the combination with predicted transcription factor binding sites leads to the identification of regulatory subnetworks, which were further validated by chromatin immunoprecipitation followed by microarray analysis (ChIP-chip) and literature mining. For example, two correlated gene groups comprising HAND2, MEF2C, SMAD4 (SMAD family member 4), and TBX20 as well as GATA4, NR2F1/2 (nuclear receptor subfamily 2, group F, member 1 and 2) and TAGLN (transgelin), were shown to extensively regulate each other's expression and function, showing the complex molecular architecture underlying heart development (Fig. 12.1) [5].

In a study by Schlesinger et al., the global transcription network driven by Gata4, Mef2a, Nkx2-5, and Srf (serum response factor) was investigated in the mouse cardiomyocyte cell line HL-1. ChIP-chip data demonstrated the combinatorial regulation by these TFs and RNAi knockdown experiments showed that they partially compensate each other's function. In detail, genes bound by multiple TFs in their promoter and/or enhancer region are less likely differentially expressed in the knockdown of one respective factor, demonstrating the buffering capacity of the network [6]. Finally, the genome-wide combinatorial binding of Gata4, Nkx2-5, Tbx5, Srf, Mef2a, as well as the histone acetyltransferase p300 determined by ChIP followed by next-generation sequencing (ChIP-seq) in HL-1 cells has also been used to identify cardiac enhancer regions. Interestingly, the majority of loci bound by multiple cardiac TFs did not overlap with loci bound by p300 [7], a transcriptional coactivator commonly used to identify enhancers [8].

To dissect the spatiotemporal protein networks driving human heart development, a recent study constructed functional clusters from over 250 cardiac development genes based on morphological subgroups corresponding to the specific phenotype associated with their mutation [9]. In general, increased complexity of the heart during development is accompanied by a higher complexity at the molecular level. This is reflected by a higher number of functional modules in networks associated with "late phenotypes" compared to "early phenotypes." The TFs GATA4, TBX5, and NKX2-5 were found to be involved in the majority of networks in almost all stages of cardiogenesis, demonstrating their central role in the cardiac transcription network. Interestingly, the respective modules show a high degree of variation in their complexity and protein composition, leading to a flexible and combinatorial regulation by the core cardiac transcription factors. Taken together, these results underline the dynamics of cardiac transcription networks and are consistent with the striking phenotypic variability that can result from mutations of a single factor [10, 11].

12.2 Core Myogenic Transcription Factors

12.2.1 Nkx2-5

The transcription factor Nkx2-5 (NK2 homeobox 5) is essential for multiple aspects of heart development [12]. It is expressed in early heart progenitor cells and expression remains high in adult hearts [13]. Nkx2-5 physically interacts with other core cardiac TFs in a combinatorial manner. For example, Gata4, Tbx5, and Nkx2-5 synergistically activate the *ANF* promoter [14]. Moreover, Nkx2-5 interacts with Hand2 and Mef2c to determine ventricular identity [15, 16]. In combination with Srf and Gata4, it further activates the expression of sarcomeric genes [17]. Mice lacking *Nkx2-5* show abnormal morphogenesis of the heart tube as well as failure of left ventricular development and die early during embryonic development (lethality at E9.5) [18]. In addition, Nkx2-5 functions in the development and maintenance of the conduction system, demonstrated by progressive atrioventricular (AV) block observed in *Nkx2-5* heterozygous mice [19]. *NKX2-5* mutations in humans account for approximately 4 % of all CHD cases including AV block, septal defects, TOF, left ventricular noncompaction, double outlet right ventricle (DORV), AS, and Ebstein's anomaly [20, 21].

12.2.2 Mef2

Myocyte enhancer factor 2 (Mef2) proteins are a family of TFs that play a central role in cellular differentiation. They contain both a highly conserved MADS box and an immediately adjacent motif, the MEF2 domain, which together mediate dimerization, DNA binding, and cofactor interactions [22]. The *MEF2* genes *MEF2A*, *MEF2B*, *MEF2C*, and *MEF2D* are all expressed in every stage of the developing human heart [23] and are crucial for the expression of muscle-specific genes [24]. They interact with other key cardiac TFs, particularly to regulate the

expression of contractile proteins via interacting with members of the MyoD (myogenic differentiation) family, which play a key role in regulating muscle differentiation. For example, Mef2c regulates expression of Dpf3 (D4, zinc and double PHD fingers, family 3, also known as BAF45c) [25], a heart- and muscle-specific subunit of the BAF chromatin remodeling complex, and moreover interacts with Tbx5 to activate the expression of the *Myh6* (cardiac alpha-myosin heavy chain 6) gene [26]. Within cardiac muscle lineages, the expression of Mef2 itself is in turn regulated by NK2 proteins [27]. Moreover, the different Mef2 proteins can partly compensate each other's functions [28]. In addition to other TFs, Mef2 proteins can also interact with HATs (histone acetyltransferase, e.g., p300) and HDACs (histone deacetylase, e.g., HDAC4) to activate or repress the transcription of downstream targets [29]. HDAC4 expression is in turn repressed by the myogenic microRNA miR-1, which leads to an activation of Mef2 that together with MyoD initiates miR-1 expression in a feedback loop [30].

Mef2a is predominantly expressed in postnatal cardiac muscle cells, and mice lacking *Mef2a* die within the first week after birth due to myofibrillar fragmentation, mitochondrial disorganization, and impaired myocyte differentiation [31]. Mutations in Mef2a identified in patients implicate its signaling pathway in the pathogenesis of coronary artery disease and myocardial infarction [32]. In *Mef2c* null mice, failure to undergo cardiac looping and formation of the right ventricle have been reported [33], and a Mef2c enhancer has been defined that drives gene expression in the second heart field and has been widely used for conditional gene inactivation [34].

12.2.3 Tbx

Tbx proteins are characterized by a conserved 180 amino acid long region termed the T-box [35]. Members of this family including Tbx1, Tbx2, Tbx3, Tbx5, Tbx18, and Tbx20 are involved in early cardiac lineage determination, chamber specification, and the development of the conduction system [36]. Tbx2 and Tbx3 act redundantly during outflow tract (OFT) development and repress Tbx1 expression in ventral pharyngeal endoderm, while their own spatial expression is regulated by a feedback loop by Tbx1. Thus, the three factors form a crucial regulatory network for pharyngeal and OFT development, which is demonstrated by severe cardiac defects caused by knockout of *Tbx1* and either *Tbx2* or *Tbx3* [37]. Tbx5 is involved in multiple pathways and combinatorial interactions with other TFs [38]. For example, it physically interacts with Nkx2-5 to control gene expression in cells of the cardiac conduction system [39] or forms a transcriptional complex with Mef2c required for early heart development [26]. Finally, Tbx20 interacts with Gata4 to activate both *Mef2c* and *Nkx2-5* enhancers [40].

Heterozygous *Tbx5* knockout mice suffer from septation defects and AV block, similar to heterozygous *Nkx2-5* knockout mice [39]. Similar to the human *GATA4* mutant phenotype, double heterozygous *Tbx5* and *Gata4* knockout mice show atrioventricular septal defects (AVSD) and myocardial thinning [41]. In humans, *TBX5* missense mutations cause Holt-Oram syndrome [42], which is characterized by heart and limb malformations. Moreover, a homozygous variation in the *TBX5*

enhancer abrogating the cardiac expression of the gene was observed in a patient with isolated VSD [43]. Hemizygosity of *TBX1* is the major cause for the CHD phenotype in 22q11.2 deletion syndrome patients, which is characterized by conotruncal defects like TOF, DORV, and truncus arteriosus (TA) [44]. Mutations in *TBX3* [45] and *TBX20* [46] as well as in the *TBX18* promoter region [47] have also been identified in different CHD types.

12.2.4 Gata4

The zinc finger transcription factor Gata4 (GATA binding protein 4) is essential for cardiac development and plays a critical role for embryo survival [48, 49]. The Gata family of zinc finger TFs consists of six members, which all bind the DNA sequence "A/G GATA A/T." Gata4, Gata5, and Gata6 are expressed in the developing heart, and Gata4 and Gata6 remain expressed in adult cardiac myocytes. Both Gata4 and Gata6 regulate the expression of several cardiac-specific genes, and Gata4 is crucial for cardiac morphogenesis during mouse embryonic development [49]. Gata4 can physically interact with other cardiac TFs like Hand2 [50], Nkx2-5 [51], Mef2 [52], Srf [53], and Tbx5 [54]. In combination with Isl1 and Tbx20, Gata4 actives Mef2c and Nkx2-5 expression in the second heart field (SHF) [40]. Furthermore, Gata4 is known to regulate the transcription of several muscle-specific genes encoding contractile elements like the α- and β-myosin heavy chain (α- and β-MHC) proteins and α-actin as well as signaling molecules like atrial natriuretic factor (ANF) and brain natriuretic peptide (BNP) [55]. Gata4 DNA-binding affinity is enhanced by the HAT p300 [56], while it is negatively regulated by HDAC4, a transcriptional repressor of muscle gene expression [57]. In addition, Gata4 binds and participates in establishing active chromatin regions by stimulating histone 3 lysine 24 acetylation (H3K24ac) deposition [58].

Homozygous *Gata4* null mice die between embryonic day E7.0 and E9.5 because of severe morphogenic defects in heart tube formation [59]. Cardiogenesis is sensitive to Gata4 dosage, and graded reduction of the TF results in DORV, common atrioventricular canal (CAVC), and hypoplastic ventricular myocardium [60]. In human, *GATA4* mutations cause CHD including septal defects, tetralogy of Fallot (TOF), and pulmonary stenosis (PS) [12]. One *GATA4* mutation described in a family with septation defects specifically disrupted the interaction with TBX5, while the binding to NKX2-5 remained unaffected, suggesting a concerted role for GATA4 and TBX5 in cardiac septation [54].

12.2.5 Hand

Hand1 and Hand2 (heart and neural crest derivatives expressed 1 and 2) are basic helix-loop-helix transcription factors, which are expressed early in derivatives of the first and second heart field (FHF and SHF), respectively [1]. They are asymmetrically expressed in the developing ventricular chambers and play an important role in cardiac morphogenesis. *Hand2* knockout mice show abnormalities in the formation

of the right ventricle, most probably due to loss of the SHF [61, 62]. Embryonic stem cells (ESCs) lacking *Hand1* are unable to contribute to the outer curvature of the heart that gives rise to the left ventricle [63]. In developing mouse hearts, the deletion of *Hand1* has been associated with CHD including ventricular septal defect (VSD), atrioventricular valve malformation, hypoplastic ventricles, and outflow tract abnormalities 1 [64]. Nkx2-5 regulates the expression of Hand1 in the FHF, and double knockout of *Nkx2-5* and *Hand2* ablates both ventricular chambers, leaving only an atrial remnant [1]. In human, mutations in *HAND1* cause septation defects, while *HAND2* mutations have been identified in patients with TOF, DORV, and PS [12].

12.2.6 Isl1

The LIM homeodomain transcription factor Isl1 (Isl LIM homeobox 1) is required for the contribution of the SHF progenitor cells to cardiogenesis [2] and directly activates the expression of Mef2c [65, 66]. Isl1 represents a key factor of the transcriptional network regulating SHF development. The central role of Isl1 for cardiac development is demonstrated by knockout mice, which completely lack the derivatives of the SHF, namely, the outflow tract, right ventricle, and much of the atria [2]. Several *ISL1* SNPs have been associated with CHD in a large patient cohort [67]; however, their functional impact awaits further exploration.

The widely expressed serum response factor (Srf) is like Mef2, a MADS-box TF important for heart and muscle development. Srf is well-known to bind to a specific DNA motif, the CArG box, with the consensus sequence "$CC(A/T)_6GG$" in promoters of its target genes [68]. Several studies demonstrated the crucial role of Srf for heart and muscle development. For example, cardiac-specific deletion of *Srf* in mice results in embryonically lethal cardiac defects including abnormally thin myocardium, dilated cardiac chambers, poor trabeculation, and a disorganized interventricular septum [69]. Srf autoregulates its own expression [70] and is suggested to be a master regulator of the actin cytoskeleton and contractile apparatus [68]. Since Srf is ubiquitously expressed and has relatively low intrinsic transcriptional activity, its ability to regulate cardiac transcription is highly dependent on its interaction with both positive and negative co-regulators. For example, the combinatorial expression of Gata4, Nkx2-5, and Srf directs early cardiac gene activity [17]. Moreover, Srf enhances muscle gene expression in association with myocardin (Myocd), a smooth and cardiac muscle-specific transcriptional coactivator [71]. The Srf/Myocd complex recruits p300 to Srf-binding sites, which induces histone 3 acetylation (H3ac) and actives gene expression [72]. Furthermore, a strong correlation was found between the presence of H3ac, histone 2 lysine 4 dimethylation (H3K4me2), and Srf and p300 *in vivo* [73]. In addition, the homeodomain protein Hopx, which does not directly bind DNA, can inhibit SRF-dependent transcription by recruiting HDAC2 to Srf target genes, thereby modulating growth and proliferation of cardiomyocytes [74]. Similar to Mef2, Srf acts in feedback loop with a microRNA (miR-133) to modulate muscle proliferation and differentiation [30]. Furthermore, Srf regulates the transcription of other microRNAs like the smooth muscle-relevant miR-143 and miR-145, which in turn cooperatively targeted a network of

transcription factors including Myocd to promote differentiation and repress proliferation of smooth muscle cells [75]. In addition, Tbx1 promotes posttranscriptional regulation of Srf levels by proteasome-mediated degradation [76].

12.3 *Cis*-Regulatory Elements and Chromatin Structures

In humans, the number of protein-coding genes is only about 21,000, whereas the human body consists of about 10^{14} cells and approximately 200 different cell types, which originated from one pluripotent cell via numerous intermediate cellular stages. Thus, it seems reasonable that a large fraction of the sequence space is occupied by regulatory sequence elements defining spatiotemporal gene expression and protein functionality by "coding" for *cis*-regulatory elements and non-coding RNAs modulating transcription, translation, and posttranslational modification. In fact, 99 % of the human genome does not code for proteins and has been investigated by major international efforts led by the ENCODE consortium [77, 78]. Thus, a vertebrate gene is typically associated with a proximal promoter and multiple transcriptional regulatory elements (enhancers and silencers). These *cis*-regulatory elements are often distant from the promoter and sometimes act across intermediate genes. Forty-thousand human heart enhancers have been postulated [79], which are potential targets for DNA-binding transcription factors and consist of sequence motifs recognized by them. The specific sequence of a motif and interacting proteins, RNA cofactors, local physical properties of the DNA fiber, and surrounding chromatin determine the binding affinity of TFs and thereby modulate TF function. Chromatin structure is modulated, for example, by posttranslational modifications and variants of histones, presence of DNA methylation or chromatin related proteins, and RNAs (see Chaps. 13, 14, and 15). Thus, a complex molecular network of *trans*- and *cis-acting* elements directs spatiotemporal gene expression.

12.4 MicroRNAs as Downstream Targets

Downstream targets mediate a considerable multiplication effect of transcription factor function. Only a small proportion of differentially expressed genes in loss-of-function experiments are direct targets of the respective transcription factors [6]. The transcription factor Srf represents a well-studied example for regulating muscle-specific microRNAs like the cardiac-specific miR-1 and miR-133 as well as miR-143 and miR-145 [30]. RNAi-mediated knockdown of Srf supported the role of Srf as a microRNA activator such that the majority of the differentially expressed microRNAs were found to be downregulated. The differential expression of these microRNAs explained more than two thirds of the differentially expressed downstream target genes in Srf knockdown [6]. Figure 12.2 shows the Srf-based transcription network integrating Srf-binding events, H3ac, microRNAs, and differential expression in Srf knockdown. A further and more global study investigated the relationship between tissue-specific microRNAs and TFs across multiple tissues, including the heart and

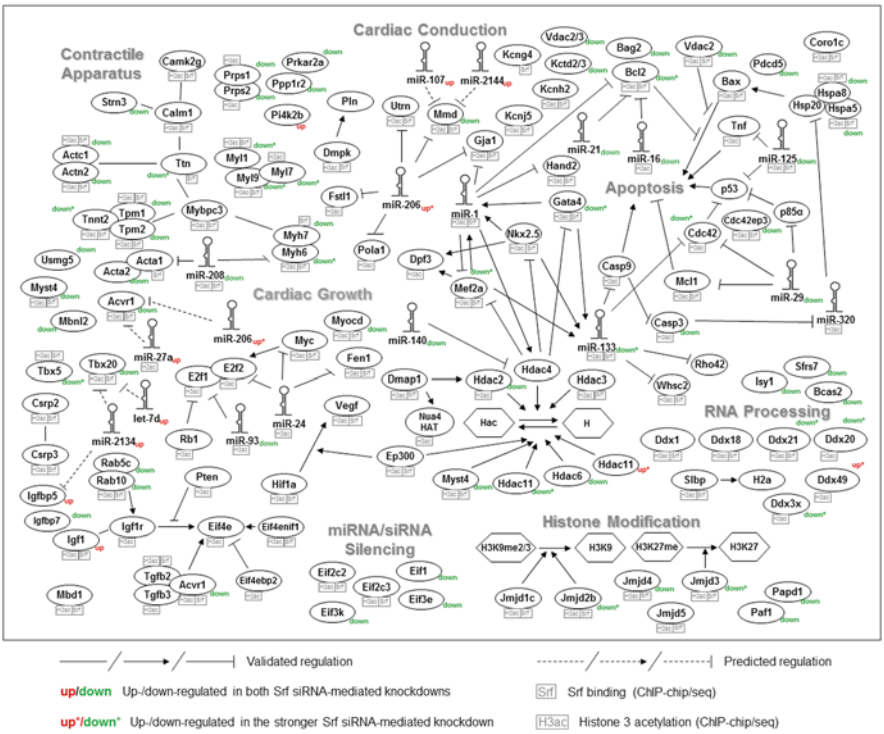

Fig. 12.2 Srf-based transcription network integrating Srf-binding events, histone 3 acetylation, microRNAs, and differential expression in Srf knockdown. The network is based on a literature search and findings from the Srf and histone 3 acetylation (*H3ac*), chromatin immunoprecipitation (*ChIP*), and Srf siRNA-mediated knockdown experiments in mouse cardiomyocytes. Srf binding and H3ac occurrence are depicted in small boxes and up- (*red*) or downregulation (*green*) in Srf knockdown is further indicated (Figure adapted from Schlesinger et al. [6])

skeletal muscle. Using ChIP-seq data for transcription factor binding sites (TFBS) from the ENCODE project, 2,347 interactions between TFs and tissue-specific microRNAs were identified, with the majority of the microRNAs being regulated by non-tissue-specific TFs. Moreover, TF-microRNA regulatory networks integrating verified or predicted interactions and expression data were constructed for each tissue. In the heart, the network comprises 33 TFs and 10 tissue-specific microRNAs, while in skeletal muscle 9 microRNAs are regulated by 52 TFs [80].

12.5 Disturbance of the Regulatory Network Underlying Congenital Heart Disease

More than 10 years ago, Kaynak et al. provided the first evidence that distinct types of congenital heart diseases are characterized by distinct gene expression profiles at a global scale [81]. With the development of the next-generation sequencing

technology, it became feasible to study the entire exome and even the entire genome in patients. It is now apparent that the majority of CHDs, which are sporadic and nonsyndromic, are based on multiple genomic alterations, which in concert alter the molecular network [82, 83]. These are not limited to cardiac-specific factors nor to the coding part of the genome. It is highly likely that network alterations driven by alterations in non-coding sequences will emerge and have a significant impact in driving the disease state. In general, human diseases including CHDs are often caused by heterozygous genetic variants that quantitatively affect dosage of the functional encoded gene product, as it is the case for copy number variations or haploinsufficiency [84, 85]. One example is Notch1 haploinsufficiency causing bicuspid aortic valve and severe aortic calcifications in related adults. Heterozygous nonsense mutations in Notch1 cause disruption of the epigenetic architecture, which results in derepression of latent pro-osteogenic and pro-inflammatory gene networks and triggers aortic valve calcification depending on hemodynamic shear stress [85].

Insights of the impact of non-coding variations have, for example, been gained by genome-wide association studies (GWAS) assessing the association between genetic variants and a phenotypic trait of interest in a large number of individuals. The TBX3-TBX5 locus and the NKX2-5 locus frequently are implicated regions in GWAS analysis of conduction anomalies. A meta-analysis of 14 GWAS on QRS duration in individuals of European descent implicated an intronic region of the *SCN10A* (sodium channel, voltage gated, type X, alpha subunit) gene as a major risk region for prolonged QRS duration [86]. Intriguingly, SCN10A is located next to SCN5A, which encodes the alpha subunit of the cardiac voltage gated Na+ channel and is known to cause several types of heritable arrhythmogenic disorders. In a close examination of this risk region in SCN10A-containing rs6801957, the sentinel SNP frequently found in ECG GWAS, reveals that it is occupied by TBX3, TBX5, NKX2-5, and several enhancer-associated coactivators, such as p300 and PolII, in the adult mouse heart. Analysis of the risk allele showed that the SNP alters the sequence of the *cis*-regulatory motif for TBX3/TBX5, which inhibits the response of the enhancer to these factors and decreases the activity of the enhancer in zebra fish *in vivo*, thus potentially reducing the expression level and dosage of SCN5A [87].

Conclusion

Cardiac development is tightly controlled by transcription factors that lead to correct temporal and spatial gene expression. The core cardiac transcription factors regulate each other's expression and can act in a combinatorial manner, resulting in a buffering capacity of the network. Moreover, co-regulators, epigenetic marks, and posttranscriptional regulators like microRNAs control their expression and functional activity. While transcription factors are clearly a main driving force for gene regulation, a number of studies now show the importance of multiple regulatory levels with a high degree of interdependency leading to a fine-tuned balance of gene expression. Disruption of cardiac transcription networks can lead to dramatic consequences for cardiogenesis and results in congenital heart disease. However, the realization of the complexity of the network

and most importantly its buffering capacity at different levels provide hope that we might be able to find ways to modulate it in order to arrive at a more favorable state without the need for genetic corrections. One focus could be the role of targetable epigenetic mechanisms driven by genetic alterations or environmental factors (see Chap. 16) to reduce the burden of CHDs and most importantly improve the long-term outcome.

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Post-transcriptional Regulation by Proteins and Non-coding RNAs

13

Amelia E. Aranega and Diego Franco

Contents

Abstract

Posttranscriptional regulation comprises those mechanisms occurring after the initial copy of the DNA sequence is transcribed into an intermediate RNA molecule (i.e., messenger RNA) until such a molecule is used as a template to generate a protein. A subset of these posttranscriptional regulatory mechanisms essentially are destined to process the immature mRNA toward its mature form, conferring the adequate mRNA stability, providing the means for pertinent introns excision, and controlling mRNA turnover rate and quality control check. An additional layer of complexity is added in certain cases, since discrete nucleotide modifications in the mature RNA molecule are added by RNA editing, a process that provides large mature mRNA diversity. Moreover, a number of

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posttranscriptional regulatory mechanisms occur in a cell- and tissue-specific manner, such as alternative splicing and non-coding RNA-mediated regulation. In this chapter we will briefly summarize current state-of-the-art knowledge of general posttranscriptional mechanisms, while major emphases will be devoted to those tissue-specific posttranscriptional modifications that impact on cardiac development and congenital heart disease.

13.1 Introduction

Posttranscriptional regulation comprises those mechanisms occurring after the initial copy of the DNA sequence is transcribed into an intermediate RNA molecule (i.e., messenger RNA) until such a molecule is used as a template to generate a protein. Posttranscriptional regulation is mainly mediated by distinct RNA-binding proteins (RBPs). RBPs are key components in RNA metabolism, regulating the temporal, spatial, and functional dynamics of RNAs. RBPs form dynamic interactions with coding, untranslated, and non-protein-coding RNAs in functional units called ribonucleoprotein (RNP) complexes [1, 2]. This enables the RBPs within RNP complexes to remain stably bound to the RNA throughout its journey from synthesis to degradation or to associate with the RNAs in a temporally and spatially specific manner. RNA molecules are constantly accompanied by RBPs, which are intimately involved in every step of RNA biology, including transcription, editing, splicing, transport and localization, stability, and translation. Altering the expression of RBPs has profound implications for cellular physiology, affecting RNA processes from pre-mRNA splicing to protein translation [1, 3]. RBPs therefore have opportunities to shape gene expression at multiple levels. This capacity is particularly important during development, when dynamic chemical and physical changes give rise to complex organs and tissues [2].

Modification of the nascent mRNA is a general mechanism that occurs in all cells within an organism. A subset of these posttranscriptional regulatory mechanisms essentially are destined to process the immature mRNA toward its mature form, conferring the adequate mRNA stability including modifications at the 5′ and 3′ ends (5′ capping and 3′ polyadenylation) as well as excision of pertinent introns by pre-mRNA splicing. mRNA turnover rate and quality control checking are performed by the nonsense-mediated decay (NMD) surveillance pathway. An additional layer of complexity is added in certain cases, since discrete nucleotide modifications in the mature RNA molecule are added by RNA editing, a process that provides large mature mRNA diversity. Given the fact that these posttranscriptional modifications would affect all RNA molecules, there are a very limited number of cases in which a discrete tissue layer or an organ, such as the heart, is affected since impairment impacts at the level of the organism. On the other hand, a number of posttranscriptional regulatory mechanisms occur in a cell- and tissue-specific manner, such as alternative splicing and non-coding RNA-mediated regulation. Alternative splicing is a major driver of mRNA diversity and consequently protein

diversity, affecting almost all genes within an organism. The use of alternative promoters or the generation of alternative mRNA species from a single gene locus has been reported widely in almost every biological context, providing extensive mRNA, and thus protein, diversity. In recent years a novel layer of regulation has been identified mediated by non-coding RNAs. Currently, short and long non-coding RNAs have been implicated modulating RNA expression at distinct biological levels, acting both as cis- and trans-acting factors. Importantly, tissue-specific expression of these short and long non-coding RNAs has been widely reported. In this chapter we will briefly summarize current state-of-the-art knowledge of general posttranscriptional mechanisms, while major emphases will be devoted to those tissue-specific posttranscriptional modifications that impact on cardiac development and congenital heart disease. While these processes are presented in the following subheadings as discrete events, it is important to highlight the intricate interrelationship between different posttranscriptional regulatory mechanisms.

13.2 mRNA Maturation: Generating Stability and Quality Control

The maturation of mRNA transcripts, from the time they are transcribed in the nucleus until they are exported into the cytoplasm, is accompanied by a series of general structural modifications (Fig. 13.1). A large number of RNA-binding proteins interact with the nascent transcript leading to the addition of modifications at the 5′ and 3′ ends as well along the coding sequence to basically stabilize the transcript and promote splicing whenever required [1, 2]. If impaired processing occurs, the NMD surveillance system is rapidly activated. In particular cases, editing of the nascent RNA transcript also occurs. Over the last years, we have gained much knowledge about the basic regulatory mechanisms orchestrating these events in eukaryotic cells, particularly in *Saccharomyces cerevisiae* and *Saccharomyces pombe*, while our understanding in metazoan cells has lagged behind.

13.2.1 5′ End Capping

Eukaryotic mRNAs are modified by the addition of a 7-methylguanosine "cap" to the first transcribed nucleotide in the nucleus (Fig. 13.1). This modification is necessary for efficient gene expression and cell viability from yeast to humans. The 7-methylguanosine cap is required for transcription elongation, splicing, translation, and general mRNA stability. On the other hand, the 5′ cap seems to be required for polyadenylation and nuclear export of mRNA in *S. cerevisiae* [4], but not in metazoan cells [5, 6]. Several factors have been reported to regulate mRNA cap methylation in yeast [7]. Triphosphatases such as Cet1p and Pct1 direct the hydrolyzation of RNA 5′ triphosphate to a diphosphate-RNA. Guanylyltransferases, such as Ceg1p and Pch1, catalyze the addition of CMP to the diphosphate-RNA to produce the guanosine cap [8], while the methylation of the guanosine cap is mediated

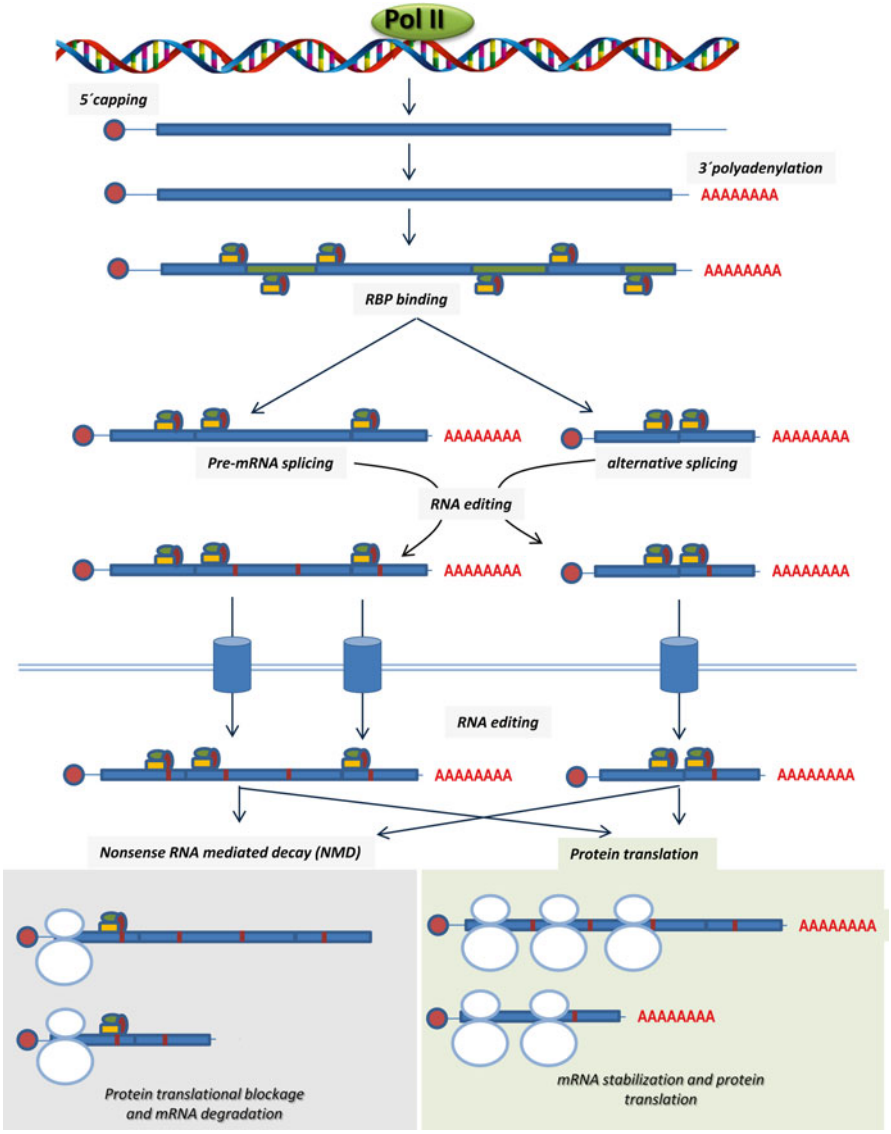

Fig. 13.1 Graphical representation of the distinct posttranscriptional regulatory mechanisms operating during the transcription, splicing, editing, quality control checking, and maturation of mRNA transcripts

by Abd1 and Pcm1. In mammals, the triphosphate and guanylyltransferase activities are found within the same peptide [9, 10] the capping enzyme of RNA guanylyltransferase and 5′ triphosphatase (RNGTT), while the RNA methyltransferase (RNMT) is encoded by a distinct protein [9–13]. Interestingly, guanylyltransferase

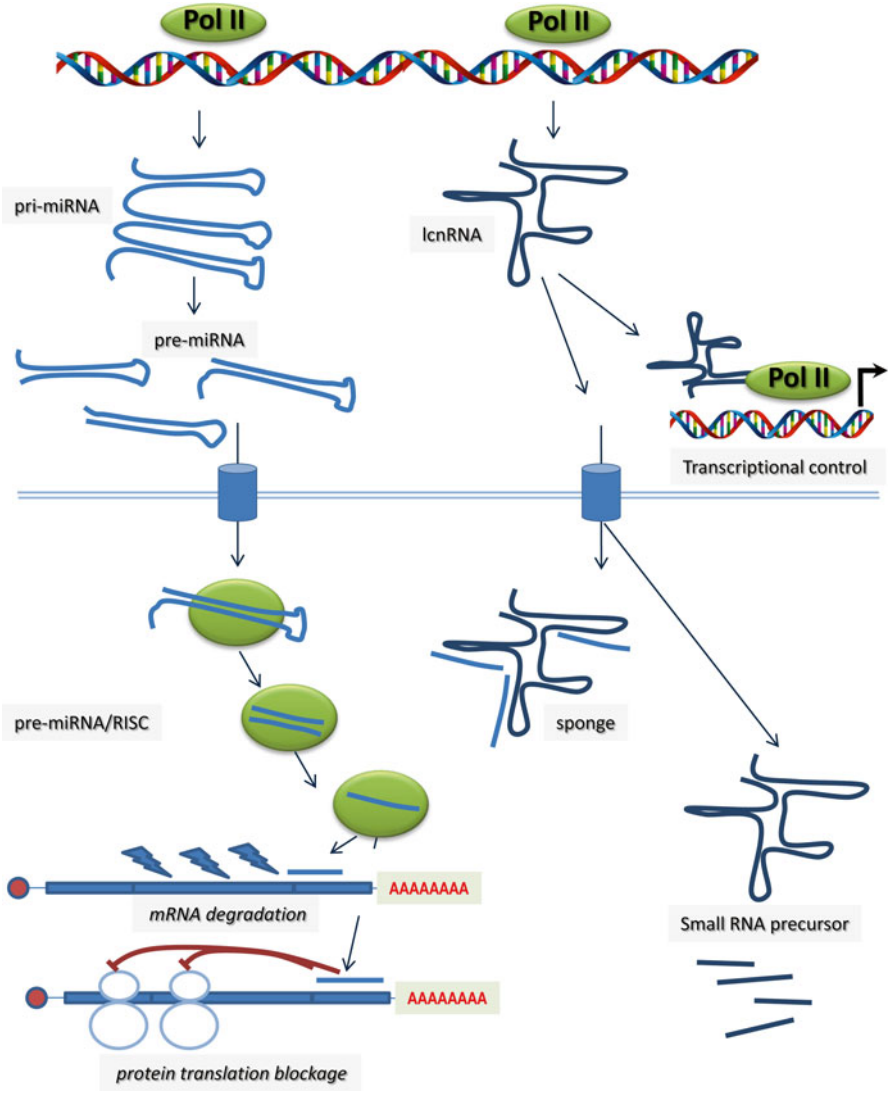

Fig. 13.2 Graphical representation of the microRNAs and long non-coding RNAs (lcnRNA) biosynthetic pathway and their functional roles during transcriptional and posttranscriptional regulation. microRNAs can elicit mRNA degradation and/or protein translation blockage. lncRNAs have been reported to actively contribute to transcriptional regulation and serve as sequestering small RNA system (sponge) or as template to generate smaller RNA molecules with, to date, poorly characterized functions

and methyltransferase are highly conserved in structure and function from yeast to humans, yet triphosphatases are widely divergent.

mRNA capping and cap methylation occur "co-transcriptionally," that is to say, the cap methyltransferase is recruited to RNA polymerase II as the RNA is

being transcribed, providing thus the means to promote transcription elongation [7]. Pre-RNA splicing is dependent on the 5′ cap since the splicing reaction has been demonstrated to be inhibited by the presence of free 7-methylguanosine [14]. The dependency of splicing on the 5′ cap is mediated by the cap-binding complex, which is a heteromeric complex formed by cap-binding protein (CBP) 80 and CBP20. From yeast to humans, the 5′ cap is necessary for the translation of almost all mRNAs, with the exception of mRNAs translated by an internal ribosome entry site [15]. The presence of a 5′ cap can also protect mRNA from degradation in *X. laevis* [16–18], while in *S. cerevisiae* inhibition of guanosine capping *in vivo* provoked rapid degradation in some but not all mRNAs, demonstrating the necessity for a guanosine cap to stabilize at a least a subset of mRNAs [4, 7, 19, 20]. Similarly, mRNA polyadenylation and nuclear export appear to largely be independent of the 5′ capping in *S. cerevisiae* [3] but dependent in other species such as *X. laevis* and humans [6, 21]. Thus, while the influence of 5′ capping is pivotal for subsequent mRNA biogenic steps such as transcriptional elongation, pre-mRNA splicing, and translation, species-specific differences seem to occur for degradation protection and mRNA polyadenylation. Given the essential role of 5′ capping in basal mRNA biogenesis, to date no specific defects affecting heart morphogenesis and/or muscle development have been reported.

13.2.2 3′ End Polyadenylation

Polyadenylation is a two-step nuclear process that involves an endonucleolytic cleavage of the pre-mRNA at the 3′-end and the polymerization of a poly-adenosine (polyA) tail (Fig. 13.1), which is fundamental for mRNA stability, nuclear export, and efficient translation during development [22]. The core molecular machinery responsible for the definition of a poly-A site includes several recognition, cleavage, and polyadenylation factors that identify and act on a given poly-A signal present in a pre-mRNA, usually an AAUAAA hexamer [22]. This mechanism is tightly regulated by both cis- and trans-acting factors, and its impairment can cause inefficient gene expression and thus disease. Previous studies have indicated that more than half of the human genes possess multiple polyadenylation sites [23], dubbed APA, which may produce mRNA isoforms with different protein-coding regions or 3′ UTRs of variable length. Interestingly, such a property is also documented in yeast [24]. The differential recognition of polyadenylation signals leads to long or short 3′ UTR of the transcripts. Usage of alternative poly(A) sites influences the fate of mRNAs by altering the availability of RNA-binding protein sites and miRNA binding sites. Abnormalities in the 3′-end processing mechanisms thus represent a common feature among many oncological, immunological, neurological, and hematological disorders [23, 25, 26], and the usage of APA and alterations in polyadenylation are beginning to be discovered and studied in human diseases [27, 28], yet to date no direct involvement in cardiovascular diseases has been reported.

13.2.3 Nonsense-Mediated Decay

Nonsense-mediated decay (NMD) is an evolutionary conserved surveillance pathway present in all eukaryotes studied to date. NMD plays an important role in the posttranscriptional control of gene expression. Approximately one-third of human genes generate pre-mRNAs that undergo alternative splicing, and similarly one-third of alternatively spliced transcripts are targeted for elimination by the NMD pathway [29]. Most alternatively spliced NMD targets appear to be generated in error [30], yet NMD also downregulates the level of other apparently normal transcripts [31–33]. NMD targets premature translation termination codons (PTC)-containing transcripts for rapid degradation (Fig. 13.1), thus protecting the organism from deleterious gain- or loss-of-function (dominant-negative effects) effects of the resulting truncated proteins [34–36]. As a rule, NMD degrades newly synthesized mRNAs during a pioneer round of translation [37–40] and occurs when a PTC is located more than 50–55 nucleotides upstream of the last exon-exon junction within the mRNA, and at least one intron and components of translation are present [41]. Importantly, there is a growing body of evidence supporting that mRNA decay in eukaryotes requires an exit from translation so that the mRNA is accessible to degradative activities [42–45].

The role of NMD in genetic diseases is emerging progressively. A pivotal role for NMD in cystic fibrosis as well as in Duchenne muscular dystrophy (DMD) has been documented (see for a review [46]), yet has only begun to be recognized in cardiac genetic diseases. Geiger et al. [47] recently reported that insufficient clearance of lamin A/C truncated mutations by NMD underlies the development of dilated cardiomyopathy in a human kindred. Similar findings have also been reported for nonsense mutations in hERG in the context of human long QT syndrome [48, 49]. Importantly an intricate relationship between NMD and the ubiquitin-proteasome system has been recently demonstrated in the context of hypertrophic cardiomyopathy [50], opening new ways to understand the complex RNA-protein interphase. In the context of congenital heart diseases, involvement of NMD has been proven for GATA binding protein 6 (*GATA6*) regulation in the setting of ventricular septal defect, patent ductus arteriosus, and congenital diaphragmatic hernia [51] and suspected in a kindred of syndromic patent ductus arteriosus as consequence the generation of aberrant transcription factor AP-2 beta (*TFAP2B*) splice variants [52].

13.3 mRNA Maturation: Generating Diversity (RNA Editing and Pre-mRNA Splicing)

13.3.1 RNA Editing

RNA editing relates to those molecular processes by which the RNA nucleotide sequence is conspicuously modified (Fig. 13.1). To date such changes have been observed in tRNA, rRNA, and mRNA molecules of eukaryotes, but not prokaryotes. RNA editing can modify an A-to-I (inosine) by the action of adenosine deaminase

that acts on RNA (ADAR), and similarly a C-to-U modification can be elicited by a protein complex composed by APOBEC-1 (apolipoprotein B mRNA editing enzyme, catalytic polypeptide 1), an RNA cytidine deaminase, and APOBEC-1 complementation factor (ACF). This C to U editing holoenzyme (APOBEC-1/ACF) is also in part regulated by CELF2 [53, 54].

Inosine is an essential modification introduced by specialized enzymes in a highly regulated manner generating thereafter transcriptome diversity. Adenosine to inosine (A-to-I) modification by the ADAR (i.e., ADAR1 and ADAR2) enzymes performs the most common type of RNA editing in metazoans [55], while C-to-U modifications seem to be confined to more discrete transcripts [53, 56–59]. A-to-I RNA editing most frequently targets repetitive RNA sequences located within introns and 5′ and 3′ untranslated regions (UTRs). ADARs use double-stranded RNA as substrates but allow structure interruptions such as bulges and loops. It is well known that these enzymes can use messenger RNA as targets for A-to-I editing and thereby recode the transcript. Both ADAR1 and ADAR2 have been proven to be able to also target short double-stranded RNA molecules, i.e., microRNAs and their precursors. Since the editing activity is found both in the nucleus and the cytoplasm, there are several steps during the microRNA maturation pathway that can be targeted for modification [60]. Although the biological significance of non-coding RNA editing remains largely unknown, several possibilities have been proposed, including its role in the control of endogenous short interfering RNAs [61].

RNA editing involving C-to-U modifications has been reported extensively to play a pivotal role in virus-associated human diseases, including human T lymphotropic virus (HTLV), hepatitis C virus (HCV), hepatitis B virus (HBV), and Epstein-Barr virus (EBV), among others [62, 63]. Furthermore, more recently a possible role in cancer development has also been proposed [63]. However, to date, no abnormalities in C-to-U RNA editing have been reported in cardiovascular diseases.

A-to-I RNA defective editing has been reported in various human diseases including viral infection susceptibility and cancer and neurological and psychiatric disorders [64–68]. Involvement of defective RNA editing in cardiovascular diseases is indirect and scarce [69, 70], yet an involvement in congenital heart diseases is likely to soon emerge.

13.3.2 Pre-mRNA Splicing and Alternative Splicing

RNA splicing is the molecular process by which introns are deleted from nascent immature mRNA providing the means to successfully liked exons back together and thus form a single mature mRNA molecule. RNA splicing is carried out by the assembly of over a hundred core proteins and five small nuclear RNAs into large ribonucleoprotein complexes, named spliceosomes [71]. Regulation of splicing is a complex process [72–74], and alterations of splicing potential have major consequences in distinct human diseases [75].

Alternative splicing is a major driver of protein diversity and allows the generation of distinct proteins from a single gene. It is estimated that almost 85 % of genes within the human genome undergo alternative splicing. Distinct mechanisms such as exon exclusion, intron retention, and the usage of alternative splice sites contribute to modify protein structure, localization, regulation, and function [76, 77]. Interestingly, genetic mutations in distinct spliceosome components have been reported in human families with distinct cardiac diseases such as myocardial infarction [78, 79] and dilated cardiomyopathy [80, 81], suggesting a functional link. Importantly, alternative splicing also plays a pivotal role during embryonic development. Differential expression of distinct spliceosome components has been reported during heart development [82]. Postnatal excitation-contraction coupling impairment has been reported in genetically engineered mice lacking ASF/SF2 spliceosome component [83], and mutant mice for SRp38, a spliceosome regulator, display early embryonic cardiac resulting in impaired calcium handling [84].

On the other hand, alternatively spliced variants have been documented widely in cardiovascular diseases such as cardiomyopathies, arrhythmias, and vascular defects leading to differential expression of sarcomeric proteins, ion channels, and cell signaling proteins [76, 77, 85–89]. An example of the impact of alternative splicing in adult heart physiology is illustrated by the diversity and functional consequences of alternative spliced variants of the troponin-tropomyosin complex (see for a review [90]). Multiple alternatively spliced variants are formed from each of the troponin isoforms, and deregulation of spliced variant expression is linked to dilated cardiomyopathy in different species [91–93]. Similarly, impaired ion channel splice variants also contribute to cardiac arrhythmogenesis, as reported for distinct components of the calcium handling and plasma membrane cardiac pumps [86, 88].

Multiple transcription factors, with critical roles in cardiac development, are alternatively spliced, such as T-box genes [94–96], myocardin [97], myocyte enhancer factor (Mef)-2 [98, 99], pituitary homeobox (Pitx)-2 [100–102], and GATA binding protein 4 (Gata4) [103]. In this context, Yehya et al. [104] identified an intronic retention variant of the *NFATC1* (nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 1) gene in patients with ventricular septal defects, suggesting that such a spliced variant might be a VSD-susceptibility gene. Bedard et al. [105] reported spliced variants of *ZIC3* (Zic family member 3) linked to patients with heterotaxy and congenital heart diseases. McCright et al. [106] reported that aberrant Notch2 alternative spliced variants leads to myocardial hypoplasia as well as eye and kidney defects. More recently, Ricci et al. [107] demonstrate that multiple genes were differentially spliced in hypoplastic left heart syndrome, suggesting a deregulation of cell metabolism and cytoskeleton and cell adherence. Interestingly, impaired alternative splicing in other genes also result in cardiac alterations. Impaired fibronectin splicing is associated with thoracic aortic aneurysm in patients with bicuspid aortic valve [108], while abnormal *SCN5A* (sodium channel, voltage gated, type V, alpha subunit) alternative spliced variants leads to fetal arrhythmias [109]. Furthermore, impaired expression of alternatively spliced *NXT2* (nuclear transport factor 2-like export factor 2) variants, a protein

involved in nuclear RNA export, also has been proven to affect cardiac development, particularly valve formation [110]. Ver Heyen et al. [111] reported that genetic engineered disruption of *SERCA2a/2b* (sarcoplasmic/endoplasmic reticulum calcium ATPase 2a/b) alternative splicing leads to 20 % increase in embryonic and neonatal mortality, as consequence of severe cardiac malformations. Buyon et al. [112] describes a spliced variant of congenital heart block-associated 52 kb autoantigen which is maximal at the time of fetal heart block, suggesting a putative role in its pathophysiology. These reports exemplify the potential causative role of impaired alternative spliced variants as key regulatory modulators of cardiac development. Increasing evidence of this is expected in the coming years as deep-sequencing technologies depict the magnitude of the alternative spliced transcriptome in congenital heart diseases.

13.4 Non-coding RNA-Mediated Posttranscriptional Control

Non-coding RNAs (ncRNAs) constitute a highly diverse group of RNA molecules in structure and function (see for a recent review [113]). Currently ncRNAs are broadly classified according to their size. Small ncRNAs are generally defined as those that are <200 nucleotides, whereas long non-coding RNAs (lncRNAs) can extend to tens or even hundreds of thousands of nucleotides in length. Small ncRNAs display a rather homogeneous structure, whereas lcnRNAs have more complex secondary structures. ncRNAs, such as ribosomal RNAs (rRNAs) and transfer RNAs (tRNAs), have been extensively studied given their prominent roles as components of the translational machinery. A similar situation occurs with small nuclear RNAs (snRNAs) and small nucleolar RNAs (snoRNAs) given their essential role in splicing. Over the last decade, great interest has arisen in a class of small regulatory ncRNAs that directly affect the expression and/or function of protein-coding genes, i.e., microRNAs (miRNAs). miRNAs were discovered in the early 1990s and since then represent the most extensively studied class of ncRNA. microRNAs display an average length of 22–24 nucleotides and are capable of interacting with the 3′ untranslated region of coding RNAs (mRNAs) eliciting blockage of protein translation and/or mRNA degradation [114]. Understanding of microRNA biogenesis has moved rapidly [115], whereas insights into the functional role of microRNAs are progressively emerging at a slower pace. Nonetheless, the functional relevance of distinct microRNAs in multiple aspects of cardiac development and diseases is now widely documented (see for recent reviews [116–118]).

Differential expression of microRNAs has been documented widely during embryonic [119–121], postnatal [122, 123], and the aging heart [124, 125] suggesting a pivotal role for microRNAs during different stages of heart development. Similarly, investigators have reported impaired microRNA expression in a large variety of cardiovascular physiopathological conditions, such as hypertrophic and/or dilated cardiomyopathy [126–131], heart failure [132–134], atrial fibrillation [135–139], and aortic aneurism [140]. The importance of microRNAs in congenital heart diseases is manifested by the embryonic defects observed in genetically engineered

mice. Conditional deletion of Dicer, an endonuclease required for the microRNA processing, with distinct Cre drivers, demonstrated the critical role of microRNA biogenesis in distinct temporal and tissue-specific contexts during cardiovascular development. Conditional ablation using an early cardiogenic deletor mouse strain (*Nkx2-5-Cre* mice) led to embryonic lethality due to cardiac hypoplasia [141], whereas ablation with myocardial-specific Cre driver line *(αMHC-Cre*) resulted in outflow tract defects and impaired chamber formation [142]. More recently Singh et al. [143] demonstrated that Dicer deletion in pro-epicardial cells compromised cardiac vascular development. In addition, germline deletion of discrete microRNAs such as miR-1-2 resulted in ventricular septal defects and early embryonic lethality [141], whereas miR-126 deletion leads to embryonic lethality due to vascular leakage [144]. These studies highlight the importance of microRNA biology for congenital heart diseases. In this context, an increasing number of studies are providing the impaired microRNA signature of distinct congenital heart diseases [145], such as ventricular septal defects [146], tetralogy of Fallot [147], corrected transposition of great arteries [148], univentricular left hearts [149], bicuspid aortic valves [150], and DiGeorge syndrome [151]. These studies provide novel insights for the prospective use of microRNA signature as biomarkers of prenatal diagnosis [152, 153]. However, in most cases, the impaired regulatory networks modulated by these microRNAs remain to be fully elucidated. In the coming years, we shall see an explosion on the understanding and functional consequences of microRNA regulation, with great hopes as to their therapeutic potential, including pediatric cardiology [154].

In addition to microRNAs, lncRNAs and circular RNAs are emerging also as post-transcriptional modulators. lcnRNAs might undergo alternative splicing and in some cases, but not in others, can be polyadenylated. lncRNAs can be located within the nucleus but also can be found within the cytoplasm thus potentially exerting a large number of biological functions. lncRNAs have been reported in a wide range of functions beyond posttranscriptional regulation such as cell cycle progression, differentiation, apoptosis, structural or cellular trafficking, as well as serving as precursors for smaller RNAs (see for a recent reviews [113, 154–156]). Differential expression of lncRNAs has been reported in the developing [157–159], adult [160] and aging [161] heart as well as in ventricular cardiac hypertrophy [161], heart failure [134], myocardial infarction [162], and cardiac ischemia [163]. Interestingly, a pivotal role of *myheart* lcnRNA has been reported in the context of cardiac hypertrophy [164, 165]. Importantly, differentially expression of lcnRNAs also has been reported in hearts with congenital heart defects, such as ventricular septal defect [166] and tetralogy of Fallot [167]. Overall these data suggest a plausible role for lncRNAs in congenital heart diseases, and the first evidences for this have recently been reported. Seminal works demonstrated that genetic deletion of *fendrr* and *braveheart*, two cardiac enriched lncRNAs, respectively, leads to impaired cardiogenesis [168, 169]. On the other hand, understanding of the functional role of circular RNAs is very incipient, with yet some evidence that they can act as microRNA sponges [170, 171]. In the coming years, it is expected that unraveling the functional roles of lcnRNAs and circular RNAs will guide toward the understanding of the etiology of distinct cardiovascular diseases, including there in congenital heart diseases.

Conclusion

Posttranscriptional regulation is a complex process. This chapter has highlighted distinct processes that sequentially modify the nascent mRNA molecule into a mature form with, in many cases multiple distinct variants. It is important to emphasize that complex regulatory networks between these processes are well documented such as for the multiple roles of 5′ capping and 3′ polyadenylation in mRNA stabilization, elongation, and translation among others, but importantly emerging evidence demonstrates that microRNAs and lncRNAs also participate in these intricately interlinked regulatory mechanisms [172], i.e., modulating alternative splicing [173]. Thus, we could foresee that in coming years, impaired posttranscriptional regulatory networks would be linked to distinct congenital heart diseases, as recently reported by Xu et al. [174].

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Post-translational Modification

14

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Contents

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Abstract

Posttranslational modifications, including chemical and covalent conjugation of small proteins, play dynamic regulatory roles underlying the functional modulation of proteins. Posttranslational modifications mediate virtually all cellular processes including cell proliferation, differentiation, apoptosis, and epigenetics in both physiological and pathophysiological conditions. In this chapter, we will summarize the major progresses in this field and expect to help to understand the importance of acetylation, methylation, ubiquitination, and SUMO conjugation in cardiac function and diseases. Reviews on phosphorylation posttranslational modifications were excluded because of space.

14.1 Protein Methylation and Demethylation

Protein methylation is the process of transferring a methyl group to a target substrate catalyzed by methylation enzymes. Protein methylation occurs on lysine, arginine, histidine, proline amino acids on substrates such as histones, transcription factors such as STAT3 and p53 [1], coactivators/corepressors such as p300 and RIP140 [2–4], and other signaling molecules such as cytochrome c [5], calmodulin [6]. Histones are important for epigenetics and gene regulation. Histone methylation on the lysine and arginine of histone tails is involved in regulating chromatin structure, gene transcription. For instance, methylation of lysine 4 and arginine 17 of histone 3 (H3) is associated with transcriptional activation [7, 8], while methylation of lysine 9 of H3 is related to gene silencing [9]. The major methylation enzymes include the Su(var)3-9, SET domain-containing proteins such as the Enhancer of zeste homologs Ezh1 and Ezh2, SETD1A and SETD1B, and SMYD proteins, and the non-SET domain-containing protein DOT1L (DOT1-like histone H3 methyltransferase). It is noteworthy that methylation of histones is a reversible process by several groups of demethylases. Removal of methylated lysine and arginine is achieved via amine oxidation, hydroxylation, or deimination. The demethylation enzymes include PADI4 (peptidylarginine deiminase, type4), the amine oxidase LSD1 (lysine-specific demethylase 1) (KDM1), and the Jumonji (JmjC) domain-containing protein family [10]. Histone methylation is implicated in heart failure in both human patients and rodent disease models. Altered trimethylation of H3K4 and H3K9 was observed in a genome-wide methylation analysis of human failing hearts and in a rat cardiomyopathic model [11]. However, the status of any specific enzymes involved in cardiac methylation modifications remains to be revealed.

14.1.1 Methylation Enzymes and Heart Development/Function

A well-studied methyltransferase, Ezh2, is a SET domain-containing enzyme and an epigenetic modifier. Ezh2 enzymatic activity requires the presence of other components in the polycomb repressive complex 2 (PRC2) [12, 13]. Ezh2 mainly catalyzes

bi- and trimethylation at lysine 27 on histone 3 (H3K27), subsequently conferring transcriptional repressive activity. In addition to its methyltransferase function, Ezh2 also exhibits coactivator activity via physical association with other factors such as estrogen receptor and β-catenin, likely independent of its enzymatic activity [14]. Ezh2 plays essential roles in normal cardiac development and in the maintenance of cardiac homeostasis. Cardiac conditional ablation of Ezh2 achieved by Nkx2-5-cre reduced H3K27me3 levels in hearts and resulted in embryonic lethality of the mutant mice with a wide spectrum of cardiovascular structural defects including atrial septal defects (ASDs), ventricular septal defects (VSDs), double-outlet right ventricle (DORV), and persistent truncus arteriosus (PTA) [15, 16]. Surprisingly, ablation of Ezh2 at a later developmental stage achieved by cTnT-cre failed to cause any discernible heart defects [16], suggesting that Ezh2 is required for the early cardiac morphogenesis but is dispensable for heart development after ~ E9.5. Mechanistically, increased apoptosis, decreased cell proliferation, and deficiency in the endothelial-to-mesenchymal transition contributed to the defective development of endocardial cushions (ECs), which are critical for normal cardiac structural formation during mouse embryogenesis [17]. These findings demonstrate that Ezh2 is essential for cardiomyocyte survival and death. Recently, GATA4, a core transcription factor that directs normal cardiac development and served as an Ezh2 substrate by methylation on lysine 299 [18], interfered with its physical interaction with P300 and subsequently suppressed GATA4's transcriptional activity [18]. Hey2, a bHLH transcription factor critically involved in the formation of ECs [19], was identified as a direct downstream target of Ezh2. However, whether Hey2 is directly implicated in the cardiac phenotypes induced by Ezh2 knockout in murine heart remains to be elucidated.

Conditional inactivation of Ezh2 using Mef2c-cre at E7.5 in the anterior heart field did not cause embryonic death [20]. However, mutant mice exhibited postnatal cardiac hypertrophy, associated with the elevated expression of a number of hypertrophic genes including Six1, which is otherwise silent in the mature cardiomyocytes. Overexpression of Six1 in cultured cardiomyocytes caused hypertrophy, while reduced Six1 levels in Ezh2-deficient hearts ameliorated the cardiac hypertrophic phenotype [20]. Hence, Ezh2 plays an important role in postnatal cardiac homeostasis in part via suppressing Six1 expression in cardiomyocytes.

DOT1L, a methyltransferase that catalyzes bimethylation on histone3 lysine 79 (H3K79me2), does not contain a SET domain. DOT1L is highly expressed in embryonic hearts. Knockout of DOT1L resulted in death of mutant mice around embryonic day 10.5 (E10.5) [21]. Mutant mice exhibited a variety of developmental defects including those in the heart [21], further confirmed by a conditional knockout murine model. Cardiomyocyte-specific deletion of DOT1L by α-MHC-cre led to dilated cardiomyopathy and premature postnatal death accompanied by abnormalities in conduction system, such as AV block [22]. Dystrophin, which is involved in maintaining normal cardiac function [23], was also significantly downregulated in DOT1L null hearts. Correspondingly, AAV9-mediated expression of a shorter form of dystrophin in DOT1L null hearts rescued cardiac phenotypes including cardiac dysfunction and conduction abnormalities, subsequently diminishing the lethality of mutant mice [22]. In addition, DOT1L levels were shown to be downregulated in human dilated

cardiomyopathic muscles [22]. These studies demonstrate the importance of DOT1L in both early heart formation and postnatal cardiac function and suggest the potential involvement of DOT1L in human cardiac muscle disorders.

14.1.2 Demethylation Enzymes and Heart Development/ Function

The Jumonji protein family represents a major class of demethylation enzymes. Although over 20 Jumonji proteins have been identified, studies on their roles in cardiovascular development, function, and diseases have been limited. Jumonji proteins contain a highly conserved JmiC domain that harbors histone demethylase activity. However, Jarid2 (Jumonji, JMI), the founding member of Jumonji family, contains substitutions in the catalytic domain and believed to silent enzymatic activity [24]. Jarid2 was shown to occupy the same genomic regions as PRC2 and indirectly promoted demethylation by suppressing PRC2's methyltransferase activity. The phenotypes associated with the Jarid2 knockout vary largely upon the mouse genetic background [25]. Jarid2 null mice obtained by gene trap led to congenital heart defects including ventricular septal defect and double-outlet right ventricle [26]. Interestingly, only specific ablation of Jarid2 in the endothelium, but not in other cardiac regions, recapitulated the cardiac structural phenotypes observed in the streamline knockout mice [27]. Furthermore, a significant upregulation of Notch 1 expression was observed in the Jarid2 mutant hearts [27]. During heart development, Notch signaling plays a well-established role in endothelial differentiation and function [28]. Therefore, Jarid2 may mediate endothelial development and function at least partially via regulating Notch signaling.

JMJD2A (lysine-specific demethylase 4A, KDM4A), another member of Jumonji family, is a lysine-specific histone demethylase that demethylates trimethylated lysine 9 and 36 on histone3 (H3K9me3 or H3K36me3) [29], thus initiating transcriptional repression which requires its demethylase activity [30]. Cardiomyocyte-specific knockout of JMJD2A with α-MHC-cre diminished cardiac hypertrophy induced by transverse aortic constriction (TAC)-generated pressure overload [31]. Accordingly, cardiac-specific overexpression of JMJD2A, also controlled by α-MHC promoter, exacerbated cardiac hypertrophy in response to TAC. Mechanistically, the expression of FHL1 (four and a Half LIM domains protein 1), mutations of which were causally linked to human cardiac hypertrophy [32], further increased in JMJD2A null hearts. Also, FHL1 gene activity responded to pressure overload-induced cardiac hypertrophy [33]. Furthermore, JMJD2A expression levels were increased in human hypertrophic cardiac muscles [31], further demonstrating the involvement of JMJD2A in cardiac hypertrophy found in human patients and murine models. Interestingly, a recent study revealed an isoform of JMJD2A, named ΔN-JMJD2A, which lacks the N-terminal enzymatic domain and exhibits no demethylase activity but was positively involved in skeletal muscle differentiation via promoting H3K9 demethylation [34]. It is speculated that ΔN-JMJD2A must recruit other demethylases such as JMJD2C and/or LSD1 to fulfill its demethylation duty

[34]. It will be interesting to determine whether this isoform and the full-length JMJD2A have similar or overlapping roles in the context of cardiovascular development and function. In addition, JMJD6, initially described as a phosphatidyl serine receptor [35], was confirmed to be a histone arginine demethylase for H3R2me2 and H4R3me2 [36]. Global deletion of JMJD6 in mice caused embryonic lethality with a wide range of organ defects including the heart, although no details of cardiac defects and the underlying mechanisms were revealed [35].

14.2 Protein Acetylation and Deacetylation

Acetylation is the process in which an acetyl group from acetyl-coenzyme A is transferred to the ε amino groups of lysine residues in histones and non-histone proteins, while deacetylation removes acetyl groups. Acetylation and deacetylation of histone epigenetic marks are controlled by histone acetyltransferases (HATs) and histone deacetylases (HDACs), central to epigenetic chromatin regulatory programs. The imbalance between acetylation and deacetylation contributes to development of a number of diseases including fibrosis [37], neurodegeneration [38], and some cancers [39, 40]. This balance is also important in maintaining cardiac homeostasis and is linked to cardiac muscle disorders such as cardiac hypertrophy and heart failure. In this subsection we will discuss the roles of major acetylases and deacetylases in cardiac function and pathogenesis and will also mention the potential application of HDACs inhibitors in cardiac remodeling and heart failure in the clinic.

14.2.1 Acetyltransferases and Cardiac Function/Diseases

Based on the subcellular localization, HATs may be divided into two categories: nuclear HATs and cytoplasmic HATs [41]. The former includes GNATs (Gcn5-related N-acetyltransferases), p300/CBP family, and other acetyltransferases such as MYST proteins, which harbor bromodomain that binds acetylated substrates. Cytoplasmic HATs mainly serve to recognize newly synthesized proteins that have not been unacetylated and will not be discussed here.

p300 (E1A binding protein p300) and its related CBP (CREB-binding protein) are HATs well studied in muscle and heart. Both p300 and CBP harbor a highly conserved HAT domain and a bromodomain that can physically interact with acetylated lysines; they also function as HATs and/or coactivators for many transcription factors. Although p300 and CBP both are expressed in heart during early cardiogenesis, cardiac-specific knockout CBP did not result in any discernible cardiac phenotypes at both baseline and under isoproterenol stimulation nor did it cause any significant changes in the expression levels of cardiac regulatory proteins [42]. Thus, CBP is not required for normal cardiac development and function, although some *in vitro* studies showed that repressing CBP activity in cardiomyocytes attenuated phenylephrine-induced cell size increase [43], indicative of the possible implication of CBP in cardiac cell hypertrophy *in vitro*.

In contrast to CBP, p300 appears to be relevant for early cardiovascular development and for the maintenance of postnatal cardiac homeostasis. Mice carrying genetic deletions of p300, as a heterozygous mutant and homozygous mutants, died in early embryos [44]. The mortality rate of p300 heterozygotes is also associated with mouse genetic background [44]. Null p300 mice died between embryonic days E9 and E11.5 with pericardial edema, with reduced trabeculation [44], indicating embryonic heart failure with reduced cellularity. Structural contractile protein genes encoding MHC and α-actinin were poorly expressed in mutant hearts than in wild-type hearts and accompanied by a decrease in cell proliferation [44]. Furthermore, using a gene, knock-in approach revealed that the acetyltransferase activity of p300 was essential for normal cardiac morphogenesis [45]. In the adult heart, p300 also plays an important role in cardiac growth and function. The levels of p300 were substantially increased in the hearts under pathological conditions, such as pressure overload in animal models and in human failing hearts [46]. Moreover, p300 haploinsufficient mice inhibited pressure overload-induced cardiac hypertrophy by ~50 % [46], whereas overexpression of p300 in cardiomyocytes promoted cell proliferation and cardiac hypertrophy [46, 47]. Consistent with the above findings, p300 promoted left ventricular remodeling after myocardial infarction, which is associated with its acetyltransferase activity [48].

14.2.2 Cardiac Enriched Proteins as Acetylation Substrates of p300

Given p300's critical transcriptional role in cardiovascular function and diseases, identification of its cardiac enriched protein targets other than histones may also lead toward fully appreciating the significant roles of p300 in cardiovascular system.

GATA4, a zinc finger-containing transcription factor, is subjected to a variety of posttranslational modifications. Acetylation of GATA4 by p300 is one of them and mainly occurs on the lysine residues K311/318/320/322 [49], which promotes the transcriptional activity by increasing GATA4 DNA binding [49, 50]. Increased levels of GATA4 acetylation also coincide with increased p300 levels in heart, suggestive of a p300-dependent activated GATA4-transcriptional network [46, 48].

Nkx2-5, a NK2 homeodomain transcription factor critical factor for normal cardiogenesis, is a p300 target. Acetylation site of Nkx2-5 by p300 remains to be identified. Interestingly, Nkx2-5 was found to complex with either p300 or HDAC5 on the sodium calcium exchanger (Ncx1) gene promoter, depending on its acetylation status [51].

Myocardin belongs to the SAP superfamily (SAF-A/B, Acinus, PIAS), and its expression is limited to cardiac and smooth muscle cells during embryonic development [52]. Myocardin is a serum response factor (SRF)-dependent coactivator and is essential for normal development of cardiac sarcomeric structure and function [53]. Mutations in myocardin are likely to be associated with human cardiovascular disorders [54]. Myocardin can be acetylated by p300, which increased its physical association with SRF, and the physical interaction between p300 and myocardin was essential for the acetylation of myocardin [55].

Myocyte enhancer factor-2 (Mef2) proteins are MADS (MCM1, agamous, deficiens, serum response factor)-box-containing transcription factors and have four family members named Mef2A, Mef2B, Mef2C, and Mef2D. Mef2 proteins are involved in development of both heart and skeletal muscle. For instance, knockout of Mef2C resulted in the absence of the right ventricular [56], while overexpression of Mef2C specifically in murine hearts governed by α-MHC promoter promoted development of a dilated cardiomyopathy and heart failure [57]. Acetylation on lysine 4 in the MADS box of Mef2C by p300 promoted its DNA binding and transcriptional activity [58].

KLF5 (Krüppel-like factor 5, also named as BTEB2 and IKLF) is a zinc finger-containing transcription factor involved in cardiovascular pathophysiology, as evidenced by the findings that angiotensin II induced the expression of KLF5 in heart and that reduced levels of KLF5 in mice attenuated wall thickness, cardiac fibrosis, and hypertrophy in response to external pro-hypertrophic stimuli such as angiotensin II [59]. KLF5 was acetylated on K369R, localized to its DNA binding domain (DBD), by p300 [60]. Although acetylation did not have any significant effects on the DNA binding of KLF5, the acetylation-deficient mutant KLF5 lost its capacity to activate target gene promoter as well as to promote cell proliferation and growth [60]. However, how acetylation affects the transcriptional activity of KLF5 in the context of cardiovascular system remains to be determined.

NFAT (nuclear factor of activated T cells) c1, that belongs to NFAT transcription factor family that consists of five members, is involved in diverse cellular activities and normal development of several vertebrate organs [61, 62]. NFATc1, a downstream effector of calcineurin, plays a well-defined role in cardiac valve development and maturation, as evidenced by the findings that NFATc1$^{-/-}$ mice lacked outflow valves and died in uterus from heart failure [63, 64]. During osteoclast differentiation, NFATc1 can be acetylated by p300 and PCAF, which is induced by RANKL. This acetylation stabilizes NFATc1 and increases its transcriptional activity [65]. Also, acetylation of NFATc1 is mediated by Erk1/2 signaling via phosphorylation of p300 in skeletal muscles. Lysines 351 and 549 are the major acetylation sites by p300, and acetylation-deficient NFATc1 exhibited impaired DNA binding and transcriptional activity [66].

In addition, some transcription factors that are ubiquitously expressed but also important for cardiac gene regulation and cardiac function are also acetylation substrates, including SP1 [67], NF-kappaB [68], and Smad3 [69].

14.2.3 Acetylation and Mitochondrial Function in Heart Diseases

Mitochondria are the major energy-producing organelles in the heart and are also subjects of cardiac muscle disorders and heart failure. Numerous mitochondrial proteins were observed as acetylation targets in heart during heart failure [70]. Analysis of pressure overload-induced cardiac hypertrophy and heart failure by MALDI-TOF/TOF mass spectrometry analysis of 2D protein Western blot revealed that mitochondrial energy generation-related enzymes such as ATP synthase beta subunit, acyl-CoA dehydrogenase (LCAD), aspartate

aminotransferase, and mitochondrial malate dehydrogenase (MMDH) were hyperacetylated, while Sirt3, a deacetylase (see below), was significantly downregulated [70]. LCAD was acetylated on eight lysine residues, K42, 156, 189, 240, 254, 318, 322, and 358 [71], which repressed its enzymatic activity [71, 72]. In line with these findings, the failing heart induced by pressure overload exhibited a significant reduction in fatty acid oxidation without changes in LCAD [73], suggesting that hyperacetylation may repress the activity of LCAD. More recently, the murine cardiac-specific knockout of Ndufs4 gene, a component of mitochondrial complex I, heightened heart failure in response to pressure overload [74]. In general agreement, the murine pressure overload mouse model elicited increased global protein acetylation in failing hearts [74] revealing that the vast majority of mitochondrial proteins are acetylation substrates. It will be important to determine how acetylation affects specific mitochondrial protein activity in the setting of cardiac pathophysiology

14.2.4 Deacetylases and Cardiac Function/Diseases

HDACs, which deacetylate acetyl-L-lysine side chains in histone and non-histone proteins to produce L-lysine and acetate, consist of four classes based on their structures [75, 76]: Class I HDACs include 1, 2, 3, and 8; Class II HDACs, IIa 4, 5, 7, and 9 and IIb 6 and 10; Class III sirtuins 1–7, related to the yeast Sir2 (Silent Information Regulator 2); and Class IV HDAC11. Class I and II HDACs are zinc-dependent hydrolases, whereas Class III HDACs are not zinc independent but nicotinamide adenine dinucleotide (NAD+) dependent for their enzymatic activities [77]. Also, Class IIa HDACs render minimal enzymatic activity compared with other HDACs. HDACs are involved in mediating a vast diversity of physiological/pathophysiological events in cells and tissues. The consequence of some HDACs in cardiac diseases has been extensively studied. Generally, Class I HDACs are believed to be detrimental to cardiac function and promote cardiac remodeling by suppressing antihypertrophic gene expression once activated, whereas Class2 HDACs are found to antagonize hypertrophic response induced by external stimuli. For instance, HDAC2 promoted cardiac hypertrophy via mediating the PI3K-Akt-Gsk3b signaling pathway [78], but HDAC5 or HDAC9 attenuated hypertrophic response to multiple external stimuli [79, 80]. Here, we list the cardiac phenotypes of knockout and/or transgenic murine models of HDAC Class I, II, and III members based on the updated publications in the field (Table 14.1). The reader is also referred to some previously published excellent reviews on this topic [89, 90].

14.2.5 HDAC Inhibitors in Cardiac Hypertrophy and Heart Failure

The development and application of HDACs inhibitors in the treatment of human cardiomyopathy and heart failure may be one of many therapeutic strategies. Indeed, TSA, a chemical HDAC inhibitor, suppressed myocardial fibrosis in murine models

Table 14.1 Histone deacetylases (HDAC) knockout (KO)/overexpression models and cardiac phenotypes

Name of enzyme	Major cardiac phenotypes	Related mechanisms	References
HDAC1 & 2	*Double KO*: Dilated cardiomyopathy, arrhythmias, and premature death	HDAC1 and 2 are functionally redundant. Double KO dysregulates the expression of genes encoding ion channels and skeletal muscle contractile proteins	[81]
HDAC3	*KO*: Variety of cardiac structural defects including double-outlet right ventricle, ventricular septal defects, interrupted aortic arch type B *Overexpression*: Increased postnatal cardiomyocyte proliferation without hypertrophy	Defective smooth muscle differentiation, decreased expression of the notch ligand Jaged1	[82, 83]
HDAC2	*KO*: Attenuated cardiac hypertrophy in response to hypertrophic stimuli *Transgenic*: augmented hypertrophy	Modulating PI3K-Ak5-Gsk3b signaling pathway	[78]
HDAC4	*KO*: no obvious cardiac phenotypes		[84]
HDAC5 & 9	*Single KO*: exaggerating hypertrophic response to pressure overload but not to chronic b-adrenergic stimulation *Compound KO*: embryonic/early postnatal death with ventricular septal defects and thin ventricular wall	HDAC5 and 9 are functionally redundant. HDAC9 mediates the transcriptional activity of MEF2	[79, 80]
HDAC6	*KO*: attenuating the cardiac dysfunction in response to AngII stimulation.	Unclear	[85]
HDAC7	*KO*: embryonic lethality due to blood vessel dilation and rupture	Suppressing the expression of matrix metalloproteinase 10 via physical interaction with MEF2	[86]
HDAC8	*KO*: Global KO mice exhibited no discernible cardiac phenotypes but had skull defects	Dysregulation of homeobox genes in cranial neural crest cells	[87]
HDAC11	*KO*: No discernible gross phenotypes; had some effects on immune system	Unclear	[88]

of cardiac hypertrophy induced by either aortic banding or Hopx gene overexpression [91, 92]. Sodium valproate, another chemical HDAC inhibitor, ameliorated right ventricular hypertrophy induced by pulmonary artery banding in a rat model [93]. Some excellent reviews are recommended for those interested [94, 95]. In all, these findings may hold promise for the clinical application of HDAC inhibitors in cardiac diseases.

14.3 Ubiquitination and Cardiac Function/Diseases

Ubiquitin, a highly conserved 76 amino acid protein (~16kd), which was discovered in the 1970s [96, 97], is the founding member of the super family of ubiquitin-like proteins or modifiers. Ubiquitin can be covalently conjugated to its targets via a series of chemical cascades that requires activating enzyme E1, conjugating enzyme E2 and ligases E3 (Fig. 14.1), consequently either causing targets' degradation or altering targets' activity, depending on the position of lysine that is used for covalent ubiquitin-ubiquitin linkage [98]. For instance, the substrates with polyubiquitination formed on lysine 48 of ubiquitin are usually delivered to proteasome complex for proteolysis; however, monoubiquitination or polyubiquitination linked on lysine 63 of ubiquitin usually alters the activity of substrates (Fig. 14.1). Ubiquitination is a reversible process that can be de-ubiquitinated by a group of enzymes [99]. Selectivity of ubiquitination substrates is mainly governed by several hundred E3 ligases that have been identified up to date; however, only a few are muscle-specific E3 ligases and will be discussed in this chapter regarding their association in heart diseases.

14.3.1 Ubiquitin Proteasome System and Heart Diseases

The proteasome is composed of a large multienzymatic protease complex that degrades the vast majority of intracellular proteins, including nonfunctional (misfolded or damaged) or functional proteins in an ATP-dependent manner. The major components of ubiquitin proteasome system (UPS) are the 26S proteasome and ubiquitin. Polyubiquitinated proteins formed on lysine 48 on the ubiquitin molecule are usually targeted for proteasome-mediated degradation. Recent studies indicate an important role for UPS in cardiac diseases.

Familial Dilated Cardiomyopathy The familial dilated cardiomyopathy associated with UPS is the desmin-related myopathy. Desmin is a filament protein essential for normal cardiomyocyte structure, and its stability is mediated by a chaperon protein named αB-crystallin, a heat shock protein that recruits desmin for proteasome-associated degradation. Mutations of desmin and/or αB-crystallin, like p.Arg120Gly, substantially impair the function of αB-crystallin [100] and cause dilated cardiomyopathy and heart failure [101, 102]. This human dilated cardiomyopathy was recapitulated in the mouse model in which αB-crystallin-Arg120Gly mutant was specifically expressed in cardiomyocytes. The transgenic mice developed mitochondrial disorganization and dysfunction and cardiomyopathy and eventually were succumbed to heart failure [103]. Further studies revealed the impairment of UPS and increased aberrant protein aggregates underlying the development of cardiomyopathy [104], indicative of the implication of UPS in desmin-related cardiomyopathy.

Familial Hypertrophic Cardiomyopathy The most common familial hypertrophic cardiomyopathy is caused by the mutations on MYBP3 gene, which encodes cardiac myosin-binding protein C (cMBP-C) [105] (see Chap. 60). Most mutations

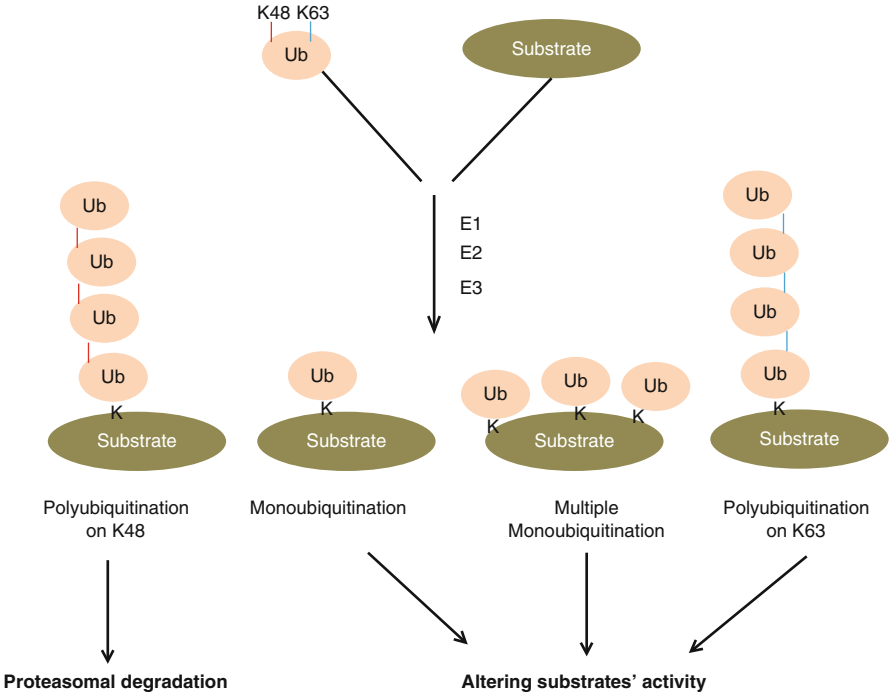

Fig. 14.1 The conjugation of ubiquitin. Ubiquitination involves activating enzyme E1, conjugating enzyme E2 and ligase E3. Up to date single E1, a few E2, and couple hundred E3 have been identified, and E3 mainly determines the substrate specificity of ubiquitination. Ubiquitination may have several different forms: polyubiquitination on lysine 48, mainly resulting in proteasomal degradation of substrates, and polyubiquitination on lysine 63 of ubiquitin or monoubiquitination (one ubiquitin attached to one lysine on the substrate), or multiple monoubiquitination (multiple ubiquitins attached to lysines on different locations of substrate protein), consequently resulting in the change in the function of substrates

on MYBP3 generate truncated proteins [106], likely degraded by UPS [107], thus increasing the UPS workload. Indeed, impaired UPS was observed in the cultured cardiomyocytes with adenoviral-mediated expression of truncated cMBP-C [107], and transgenic mice overexpressing truncated cMBP-C in heart developed cardiac hypertrophy as expected [108].

Ischemic Heart Disease The suppression of UPS activity by coronary ischemia/reperfusion was first reported in 2001 [109] and later confirmed [110]. Decreased UPS activity led to increased accumulation of abnormal protein aggregates, which contributes to cardiac dysfunction after ischemic injury. Correspondingly, overexpression reduced the aggregation of aberrant proteins, infarct size, and improved cardiac function after ischemia/perfusion [111]. In contrast, inhibition of the proteasome activity in cardiomyocytes heightened the myocardial ischemia/reperfusion injury in mice [112]. Mechanistically, increased reactive oxidative species and decreased ATP content may underlie dysfunctional UPS activity observed in the ischemia/reperfusion heart [110, 113].

Cardiac Remodeling and Heart Failure Cardiac remodeling occurs in response to elevated workload such as hypertension or pathological stimuli, such as ischemia, and may progress to heart failure, characteristic of reduced cardiac pump function. Although the implication of UPS in cardiac remodeling and heart failure has been previously studied, the findings are confounding. The UPS activity was reported to be deficient in some studies but to be elevated in others on the human failing hearts and/or in the animal models with cardiac remodeling and heart failure [114–116]. Recently, a selective instead of a general suppression of the proteasomal function caused by oxidative injury [117] further complicates the issue. More extensive studies will be needed to clarify these issues.

14.3.2 Ubiquitination E3 Ligases and Cardiac Diseases

As mentioned above, the specificity and selectivity of substrates for UPS-mediated degradation are governed by ubiquitination E3 ligases, which are divided into three classes: the RING finger-containing proteins, the U-box-containing proteins, and the HECT-domain-containing proteins. The major cardiac ubiquitin E3 ligases that have been shown to play a role in cardiac physiology and pathophysiology include atrogin-1, the muscle RING finger (MuRF) family (MuRF1, 2, and 3), carboxyl-terminus of heart shock protein 70-interacting protein (CHIP), and murine double minute 2 (MDM2).

Atrogin-1 Atrogin-1 (atrophy gene 1, or muscle atrophy F-box, MAFbx) harbors an F-box motif, characteristic of functional domain identified in the components of the SCF (skp1, cullin, F-box protein) ubiquitin ligase complex. Atrogin-1 is specifically expressed in the cardiac and skeletal muscles in humans, mice, and rats [118, 119], thus defined as a muscle and cardiac-specific E3 ligase. Atrogin-1 interacts with the SCF ubiquitin ligase complex and brings its substrates for degradation. One of atrogin-1's substrates is calcineurin. Calcineurin plays an important role in cardiac pathological hypertrophy in response to pressure overload and pro-hypertrophic stimuli [120]. Overexpression of atrogin-1 promoted degradation of calcineurin and repressed NFATc4 nuclear translocation, inhibiting cardiac hypertrophy induced by pressure overload [121]. However, another study showed that expression of atrogin-1 was substantially elevated in heart by pressure overload and that knockout of atrogin-1 was found to repress the cardiac hypertrophy induced by both pressure overload and β-adrenergic activation [122]. Mechanistically, stabilization of IkB-a and repression of NF-kB underlies this protective effect rendered by atrogin-1 ablation [122]. Currently, additional work will be needed to reconcile the abovementioned conflicting studies.

MuRF1 MuRF1, a RING domain-containing E3 ligase, was found to play a critical role in mediating cardiac troponin 1 stability [123, 124]. An earlier study showed that MuRF1 null mice had hearts with comparable mass relative to wild-type mice measured at baseline but were more resistant to hypertrophy regression [125]. However, MuRF1 knockout mice developed physiological cardiac hypertrophy, and MuRF1 was not required for cardiac atrophy [126]. These discrepancies could be attributable to different experimental settings used in these two studies, such as age

and sex of animals used, the duration of stimulation, etc. Also, overexpression of MuRF1 facilitated heart failure in response to aortic banding [127]. More importantly, MuRF1 expression levels were increased in the cardiac muscles obtained from the patients with therapeutic cardiac atrophy after left ventricular assist device placement [125], indicative of a potential role for MuRF1 in human heart diseases.

MDM2 MDM2 also harbors a RING domain like MuRF1. MDM2 expression levels were elevated in murine hearts challenged with oxidative stress and pressure overload [128]. Overexpression of MDM2 in cultured cardiomyocytes protected them from hypoxia-reoxygenation-induced injury, while suppression of MDM2 activity blocked its protective activity [129]. Several major ubiquitination targets whose degradation is mediated by MDM2 are p53 [130, 131], ARC (apoptosis repressor with caspase recruitment domain) [128], β2-adrenergic receptor and β-arrestin [132], insulin-like growth factor 1 receptor [133], and FoxO1 [134, 135].

CHIP CHIP is a U-box-containing E3 ligase, and also a chaperone of the heat shock proteins (HSP) 70 and 90. CHIP is highly enriched in heart [136], and $CHIP^{-/-}$ mice showed high mortality and severe cardiac hypertrophy compared with wild-type mice [137]. Overexpression of CHIP in murine hearts was protected from ischemia-induced cell death [138], thus demonstrating the protective role of CHIP in the maintenance of cardiac homeostasis. As an E3 ligase, CHIP has some common targets for proteasome-induced degradation as MDM2, including p53 and FoxO1 [139, 140].

14.3.3 Deubiquitinase and Heart Disease

Deubiquitinases play an important role in balancing ubiquitination-deubiquitination and thus maintains stable protein homeostasis. Currently about 79 deubiquitinases have been predicted in the human genome [141]. The involvement of deubiquitinases in immune system and cancer has been previously studied [142, 143], in which suppressing deubiquitinase activity may be a potential therapeutics for cancer [144]. More recently, mutations on USP8, a deubiquitinase gene, were causally linked to human Cushing's disease [145]. However, the information regarding the implication of deubiquitinases in heart diseases is quite limited. Analysis from heart samples obtained from human dilated cardiomyopathy indicated dysregulated expression of deubiquitination enzyme isopeptidase-T and the ubiquitin-fusion degradation system-1 [146], two important deubiquitinases. However, how these changes really contribute to the pathogenesis of cardiomyopathy remains obscure.

14.4 SUMO Conjugation and Cardiac Function/Diseases

SUMO (small ubiquitin-like modifier) belongs to the ubiquitin-like superfamily. So far four SUMO family members, named SUMO-1, SUMO-2, SUMO-3, and SUMO-4, have been identified; however, SUMO-4 appears not to be conjugatable in its natural form [147]. SUMO-1 shares ~50 % homology with SUMO-2 and SUMO-3, but the

active SUMO-2 and SUMO-3 proteins exhibit ~97 % identify. SUMO-1 and SUMO-2/3 may have distinct roles in regulating cellular activities, because (a) they exhibit substrate specificity to some degree [148]; (b) SUMO-2/3 appears to be more responsive than SUMO-1 to external stimuli, at least in cultured cell lines [149]; and (c) SUMO-1 exhibits different subcellular localizations and dynamics than SUMO-2/3 [148].

Like ubiquitination, sumoylation is also an enzymatic process that requires E1 (heterodimeric), E2 (Ubc9), and E3 and is ATP dependent (Fig. 14.1). Sumoylation is also a reversible process. Sentrin-specific proteases (SENPs) are responsible for deconjugating SUMO conjugation and also responsible for maturation of initially synthesized inactive SUMO precursors [150]. SUMO E3 ligase may enhance the specificity and efficiency of SUMO conjugation (see below). It is noteworthy that sumoylation may occur on a single or multiple lysine residues of a given substrate, named mono- or multisumoylation, respectively (Fig. 14.2). SUMO moieties can also be conjugated to the lysine residue within a SUMO protein that is attached to a substrate, forming a polymeric (poly-)SUMO chain (i.e., polysumoylation). Given the transient and dynamic nature of SUMO conjugation and the fact that in most cases only a small fraction of a given substrate is modified in any given time frame, it has been a challenge to detect

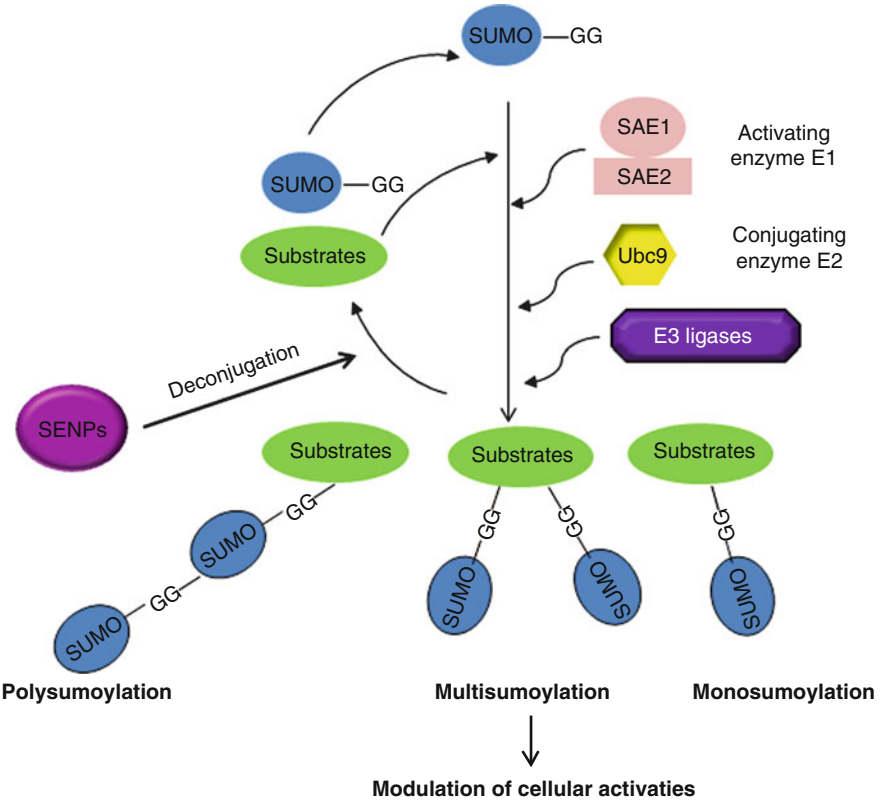

Fig. 14.2 SUMO conjugation machinery. Sumoylation requires heterodimeric activating enzyme (*E1*), conjugating enzyme (*E2*), and E3 ligase. SENPs deconjugate SUMO substrates. SUMO conjugation may occur in three forms: mono-, multi-, and polysumoylation

endogenous sumoylated targets. Although the majority of SUMO substrates identified so far is nuclear, a number of SUMO targets are also localized in other subcellular compartments such as cytoplasm, plasma membrane, and mitochondria [151–155].

SUMO E3 Ligases SUMO E3 ligases (Table 14.2) may catalyze SUMO conjugation by activating non-consensus SUMO acceptor site(s) and/or promoting multisumoylation/polysumoylation. SUMO E3 ligases are also believed to play a role in substrate discrimination. Among these known SUMO E3 ligases, PIAS family that consists of four isoforms (PIAS1, PIAS3, PIASx, and PIASy) is the most potent SUMO E3 ligase. However, the ligase activity of PIAS proteins might not always be attributable to the RING domain [166]. Of note, PIAS proteins themselves are SUMO substrates, although the SUMO acceptor sites remain to be identified [178, 179]. PIAS family members appear to have overlapping/redundant function, because knockout of PIAS1, PIAS2, or PIAS4 in mice did not lead to any severe and lethal phenotype(s) [180–183], while double knockout of PIAS1 and PIAS4 caused mouse embryonic lethality before E11.5 [184].

Desumoylation Enzyme SENPs The family of SUMO-specific proteases (SENPs) consists of six members (SENP1, 2, 3, 5, 6, 7) in human that are specifically involved in the SUMO conjugation pathway [185]. SENPs cleave the C-terminal extensions of the SUMO proteins for maturation (endopeptidase activity) and free SUMO proteins that are attached to substrates (isopeptidase activity) (Fig. 14.1). SENPs differ in the capacity of their endopeptidase/isopeptidase activity as well as in their discrimination of substrates/SUMO isoforms [186, 187]. For instance, SENP1 and SENP2 exhibit both endopeptidase/isopeptidase activities for all SUMO precursors, while SENP3, 5, 6, and 7 preferentially target SUMO-2/3 [185]. Data for SENP3, 5, 6, or 7 knockouts are not available, but knockout of either SENP1 or SENP2 caused embryonic lethality [188–190], suggesting the non-redundant role of SENP proteins in murine embryonic development.

SUMO Targeting Sequence SUMOs target the lysine (K) residue mainly localized in the known consensus sequence ψKXE/D (ψ is a bulky hydrophobic amino acid, X is any residue, D is aspartic acid, and E is glutamic acid) [191, 192]. However, sumoylation can also occur on noncanonical lysine residue(s) within some substrates [193, 194]. SUMO E3 ligases can induce the conjugation of SUMO to the noncanonical lysine residue(s) [195]. It is also worth noting that not all proteins that harbor this four-amino acid sequence motif are SUMO targeted. Interestingly, SUMO-2/3 also has a consensus sequence ValLysThrGlu that contains the lysine 11 [196], which is the critical site for poly-SUMO chain formation.

14.4.1 Involvement of SUMO Conjugation in Cardiovascular Function in Animal Models

The implication of SUMO conjugation in oncogenesis has been studied widely. Recent findings suggest that SUMO may be important for cardiac function and is involved in cardiovascular disorders. Indeed, SUMOs directly modify many

Table 14.2 Overview of SUMO E3 ligases

SUMO E3 ligases	References
HDAC4	[156–158]
Krox20	[159]
MAPL	[160]
MMS21/NSE1	[161]
Pc2	[162–165]
PIAS	[166, 167]
RanBP2	[168–170]
Rhes	[171]
SF2/ASF	[172]
TLS/FUS	[173]
TOPOR	[174, 175]
TRAF7	[176]
TRIM28	[177]

substrates that are important for cardiovascular development and function (Table 14.3). For instance, Nkx2-5, Tbx5, and Zic3 are all important regulators for mouse cardiovascular development/function. While SUMO conjugation enhanced the activity of Tbx5 and Nkx2-5 [203, 219, 222], it repressed Zic3 function, at least partially, via modulating the nuclear localization of Zic3 [221], raising the possibility of a direct implication of SUMO conjugation in congenital heart diseases (CHDs). In support of this hypothesis, a subset of SUMO-1 null mice exhibited premature death with cardiac structural defects [223, 224], although this phenotype was likely affected by genetic background [225, 226]. The importance of SUMO-1 conjugation in early heart development was further highlighted by the observation that overexpression of SENP2, a pan-SUMO isopeptidase in mouse hearts, decreased SUMO conjugation and caused cardiac structural abnormalities as well as early demise, and simultaneously cardiac overexpression of SUMO-1 rescued heart defects and decreased the mortality rate of SENP2 transgenic mice [227].

In the SENP2 gain-of-function model, the conjugation of SUMO-1, SUMO-2, and SUMO-3 was diminished due to upregulated desumoylation activity [227]. Although a subset of SENP2-Tg mice died from CHDs, the surviving SENP2-Tg mice developed cardiac hypertrophy with age, accompanied by impaired cardiac function and elevated fibrosis [227], implicating the SUMO conjugation pathway in the development of cardiomyopathy. It will be interesting to dissect the role of SUMO-1 from that of SUMO-2/3 in development of cardiomyopathy, if any.

14.4.2 Potential Implication of SUMO Conjugation in Cardiovascular Diseases in Humans

14.4.2.1 SUMO and CHDs

Accumulating evidence points to a role of the SUMO conjugation pathway in cardiovascular disorders in humans. The abovementioned SUMO substrates such as

Table 14.3 SUMO substrates important for cardiovascular development/function

SUMO targets	Major sumoylation site(s)	References
Connexin 43	Lysine 144/237	[151]
Drp1	Multiple lysine residues	[153, 194]
Erk5	Lysine 6/22	[197]
ERRα	Lysine 14/403	[198]
Ezh2	N/A	[199]
GATA4	Lysine 366	[167]
Lamin A	Lysine 201	[200]
Mef2c	Lysine 391	[157]
Msx1	Lysine 9/127	[201]
Myocardin	Lysine 445	[195]
Nfatc1/C	Lysine 349/702/914	[202]
Nkx2-5	Lysine 51	[203]
PARP1	Lysine 203/486	[204]
PGC-1α	Lysine 183	[205]
PPARγ	Lysine 107	[206–208]
PPARα	Lysine 385 (murine) Lysine 185 (human)	[209, 210]
Prox1	Lysine 556	[211, 212]
Retinoid x receptor α	Lysine 108	[213]
SERCA2a	Lysine 480/585	[214]
Smad4	Lysine 113/159	[215–217]
SRF	Lysine 147	[203, 218]
Tbx5	N/A	[219]
TRPM4∂	Non-identified	[220]
Zic3	Lysine 248	[221]

Nkx2-5 and Zic3 all are linked etiologically to human congenital heart defects. A number of disease-linked naturally occurring Nkx2-5 and Zic3 mutants showed significantly decreased SUMO conjugation [221, 228]. Cardiac-specific expression of the Nkx2-5 SUMO site mutant (lysine 51 to arginine, Lys51Arg) in wild-type mice did not cause severe phenotype(s); however, it did induce CHDs in Nkx2-5 haploinsufficient mice (Nkx2-5$^{+/-}$) [228]. Given the comparable expression level of the transgene K51R and wild-type Nkx2-5 in compound transgenic Lys51Arg:Nkx2-5$^{+/-}$ mouse hearts and the decreased sumoylation level of a number of human Nkx2-5 mutants, there is a likelihood that the altered sumoylation of Nkx2-5 contributes to the development of CHDs associated with these Nkx2-5 mutants. Another interesting finding is that sequencing analysis of the SUMO-1 gene of control and newborn patients with both cleft lip/palate and atrial septal defects (ASDs) reveals mutations in a *cis*-regulatory element of the SUMO-1 gene, mutations that repressed the reporter activity by ~50 % in the *in vitro* assays [224]. However, to further link SUMO-1 gene with human genetic diseases, more human samples will be needed for analysis, and family pedigree studies will also be required.

14.4.2.2 SUMO and Cardiomyopathy

Mutation of lamin A, a nuclear structural protein, is implicated etiologically in inherited dilated cardiomyopathy [229] (see Chap. 59). SUMO modifies the lysine residue 201 in a consensus sequence MetLysGluGlu of lamin A and the mutation of glutamic acid 203 to either glycine (Glu203Gly) [230] or lysine (Glu203Lys) [231] (which are associated casually with familial dilated cardiomyopathy and conduction disease), negatively affects lamin A sumoylation leading to altered nuclear localization [200]. These two mutations displayed similar molecular phenotypes exhibited by the sumoylation-deficient Glu201Arg mutation [200]. Thus, deficient sumoylation of lamin A is implicated directly in familial dilated cardiomyopathy.

SERCA2a, an ATPase and a critical factor in handling Ca^{2+} homeostasis during excitation-contraction coupling, was identified as a SUMO substrate on two sites: K480 and K585 [214]. The levels of both free and SUMO-1 conjugated SERCA2a were significantly decreased in human failing hearts [214]. Recovery of SUMO-1 conjugation globally or conjugated to SERCA2a improved cardiac function in large animal models [232]. These findings illustrate the participation of SERCA2a sumoylation in human heart failure.

More recently, SENP5 was shown to be implicated in cardiomyopathy and heart failure. SENP5 was upregulated in the human cardiomyopathic heart muscles, and transgenic mice overexpressing human SENP5 specific in hearts recapitulated human cardiomyopathy [233]. The transgenic hearts exhibited increased apoptosis and decreased cell proliferation with enlarged mitochondria. Moreover, overexpression of Bcl2 in the SENP5-Tg hearts rescued cardiac dysfunction [233]. Mechanistically, SUMO conjugation to Drp1, an important factor for mitochondrial fission, was decreased in the SENP5-overexpressing hearts, and knockdown of Drp1 in cultured cardiomyocytes repressed the activation of apoptotic pathway by SENP5 [233]. Thus, SENP5 induces cardiomyopathy mainly via targeting mitochondria.

The abovementioned studies serve as examples demonstrating that deficiency in SUMO conjugation to a particular substrate may contribute to abnormal cardiac muscle disorders. However, the cardiac phenotypes presented in globally sumoylation-deficient murine models should be a consequence of the total or net effect on the activity of multiple SUMO substrates, rather than an effect on the function of a single SUMO target. How to associate the functional consequence originating from a global sumoylation change with a specific group of targets that work in the same or similar signaling pathways is an interesting topic for future investigation.

14.4.2.3 SUMO and Conduction Diseases

Cardiac conduction disorders pose a significant health threat (see Chaps. 62 and 63). The aforementioned sumoylation-deficient lamin A mutants also are associated with familial conduction disease. Another cardiac conduction disease is progressive familial cardiac conduction block I, which exhibits autosomal dominant inheritance [234]. A point mutation from glutamic acid 7 to lysine (E7K) in TRPM4 gene, which encodes transient receptor potential cation channel, subfamily M, member 4

(a Ca^{2+}-activated nonselective cation channel), was responsible for the development of familial heart conduction block [220]. TRPM4 is a SUMO substrate, and the mutant E7K displays a resistance to the desumoylation by SENP1, subsequently protecting it from proteasomal degradation and leading to increased channel activity [235]. SUMO also targets other cardiac ion channel proteins such as Kv2.1 [236] and Kv1.5 [237] and regulates their functions; however, whether sumoylation of these proteins are involved in cardiac arrhythmia needs to be investigated.

14.4.2.4 Potential Implication of SUMO Conjugation in the Ischemic Heart

Several lines of evidence showed that there was an increase in conjugation of SUMO-1 and SUMO-2/3 in cerebral ischemia, which was protective against tissue damage [238–241]. However, no systemic study has yet suggested the involvement of a global change in SUMO conjugation during ischemia/reperfusion. However, there is one study in which a globally increased SUMO-1 conjugation was observed in the hypoxic heart associated with elevated SUMO-1-attached HIF1α [242]. Erk5 (extracellular signal-regulated kinase 5), an important mediator of ischemic/reperfused injury and inhibitor of apoptosis, was suppressed by sumoylation [243]. Sumoylation of Erk5 was increased in myocardial infarction, in H_2O_2-induced inflammation, and in the aortas of diabetic mice [197, 243]. Although data regarding how sumoylation affects HIF1α stability and function are controversial [188, 244–246], an increase in the level of SUMO-1 conjugated Erk5 appears to promote inflammation, therefore worsening the injury. Given that many SUMO substrates are implicated in ischemic/reperfused heart injury, the exact role the SUMO conjugation pathway plays in this particular pathophysiological setting remains to be determined.

Conclusion

Since the discovery of the first SUMO protein, the number of novel SUMO substrates has increased rapidly, and the understanding of how the SUMO conjugation pathway is involved in a variety of cellular activities as well as the underlying molecular mechanisms has improved significantly. However, to fully decipher the role played by SUMO conjugation in cardiovascular disease, several issues remain to be addressed. For example, is there any functional difference between SUMO-1 and SUMO-2/3 in terms of regulating cardiovascular function? Although a global change in SUMO-1 conjugation was observed in the human failing hearts [214], given the complexity of pathophysiological conditions associated with end-stage heart failure, is this change associated with cardiomyopathy or a consequence of long-term treatment? Also, how SUMO-2/3 functions in the human diseased hearts requires systemic study. Another interesting topic is whether drugs with cardiovascular side effects induce any significant changes in the levels of SUMO conjugation. In yeast, the sumoylation pathway was implicated in doxorubicin-induced cytotoxicity [247]. Whether or not doxorubicin can mediate sumoylation in the heart, which at least partially contributes to doxorubicin-linked cardiomyopathy, is an interesting area to explore.

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Epigenetics

15

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Contents

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Abstract

Epigenetics is the study of heritable changes to the genome and gene expression patterns that are not caused by direct changes to the DNA sequence. Examples of these changes include posttranslational modifications to DNA-bound histone proteins, DNA methylation, and remodeling of chromatin architecture. Collectively, epigenetic changes provide a layer of regulation that affects transcriptional activity of genes while leaving DNA sequences unaltered. Sequence variants or mutations affecting enzymes responsible for modifying or sensing epigenetic marks have been identified in patients with congenital heart disease (CHD), and small molecule inhibitors of epigenetic complexes have shown promise as therapies for adult heart diseases. Additionally, transgenic mice harboring mutations or deletions of genes encoding epigenetic enzymes recapitulate aspects of human cardiac disease. Taken together, these findings suggest that the evolving field of epigenetics will inform our understanding of congenital and adult cardiac disease and offer new therapeutic opportunities.

15.1 Introduction

Coordinated expression of genes coded by the DNA sequence of the genome requires precise temporal and spatial interactions between protein factors in the nucleus and DNA. Work over the last half century has revealed that these interactions are highly influenced by the manner in which DNA is packaged and organized as chromatin. The basic unit of chromatin is the nucleosome, a complex of 147 bases of DNA wound around an octameric core of histone proteins. The spacing and number of nucleosomes determines chromatin density, with the DNA within highly dense heterochromatin being less accessible to nuclear factors than sequences within relaxed, less dense euchromatin. As a result, genes within heterochromatic regions tend to be silenced, while euchromatic loci typically are active. Clusters of nucleosomes are further condensed so that the genome can be packaged efficiently into the nucleus.

The first epigenetic modification to be identified was methylation of DNA itself. Methylation of cytosine residues typically is associated with transcriptional silencing, and a number of enzymes are responsible for adding and removing these methyl groups. Another type of epigenetic modification involves posttranslational modifications of histone proteins, including methylation and acetylation of lysine residues. These modifications modulate the recruitment of additional protein complexes that regulate chromatin density and conformation. A host of "writer" and "eraser" enzymes exists to add and remove specific modifications from histone tails,

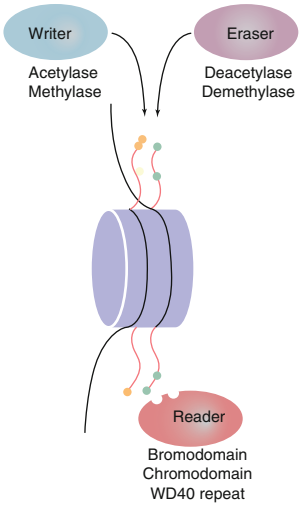

Fig. 15.1 Epigenetic regulators modify histone tails of a nucleosome. Epigenetic factors can be categorized into "writers," "erasers," and "readers." Writers add specific modifications to the histone tails of a nucleosome, while erasers remove these modifications. Readers function to recognize epigenetic modifications and can recruit other regulatory factors. Examples of each class of epigenetic regulator are provided

and the description of these growing families of enzymes is evolving rapidly (Fig. 15.1). Histone methyltransferases and deacetylases are among the most studied of these "writer" and "eraser" enzymes, respectively, in the context of cardiac development and disease. Covalent modifications of histone proteins are recognized by "reader" proteins that affect downstream changes in chromatin in response to changes in epigenetic marks (Fig. 15.1). Included in this class of proteins are chromodomain-containing proteins such as CHD7, a member of a larger family of ATP-dependent chromatin-remodeling complexes. Mutations in the gene-encoding CHD7 recently have been identified in patients with congenital heart disease [1]. This chapter will provide an overview of epigenetic changes during cardiovascular development and highlight the various families of epigenetic proteins and their roles in congenital heart disease. Additionally, we will highlight how changes in nuclear architecture may influence cardiac development.

15.2 Epigenetic Changes During Cardiac Development

The explosion of next-generation sequencing technologies coupled with the improved efficiency of directed differentiation of embryonic stem cells into cardiac lineages has enabled mapping of the landscape of histone modifications over the course of cardiac differentiation. Most prominently, work by Paige et al. and Wamstad et al. have shed significant light on the dynamics of gene activation and repression during cardiac specification [2, 3]. By taking snapshots at a series of

well-defined time points in mouse and human ESC differentiation, these groups defined the presence of various histone modifications across the genome during cardiogenesis using chromatin immunoprecipitation coupled with massively paralleled sequencing (ChIP-Seq). Specifically, they observed unique histone modifications marking transcription start sites (trimethylation of histone H3 at lysine 4; H3K4me3), enhancers (monomethylation of histone H3 at lysine 4; H3K4me1, and acetylation of histone H3 at lysine 27; H3K27ac), and inactive chromatin (trimethylation of histone H3 at lysine 27; H3K27me3), thus providing an initial "map" of the dynamic epigenetic landscape during cardiac development. A theme that emerges from these surveys is that genes specific to noncardiac lineages such as endoderm and ectoderm derivatives are rapidly marked by repressive histone modifications, resulting in repression of these lineages. These findings support the hypothesis that cell differentiation requires active silencing of alternative lineages.

These studies also suggest that cardiac-specific enhancers are marked by H3K4me1 [3]. Integration of these epigenetic maps with publicly available databases will guide further studies of epigenetic changes during cardiac development.

15.3 Histone Modifications

Epigenetic studies have focused on understanding how histone proteins undergo posttranslational modifications, which amino acid residues are subject to these modifications, and how various combinations of modifications affect gene expression. Methylation and acetylation are two of the most common and well-studied histone modifications. Histones can be methylated on a variety of residues and Jumonji domain-containing proteins act as demethylases. Histone acetyltransferases (HATs) acetylate lysine residues primarily on histone tails, and histone deacetylase enzymes (Hdacs) reverse this process. Additional forms of histone modification have been described and continue to be discovered, though application to cardiac development and disease awaits further study [4].

15.3.1 Histone Deacetylase Enzymes

Histone deacetylases (Hdacs) remove acetyl groups from lysine residues, typically resulting in compaction of chromatin and repression of gene expression. Histones represent only one type of the many possible substrates for Hdacs, which can also remove acetyl groups from lysine residues of many other proteins, including tumor protein 53 (p53) and GATA binding protein 4 (Gata4) [5, 6]. Hence, these enzymes are more accurately known as lysine deacetylases (Ldacs) and nonhistone targets likely account for many important developmental functions that remain to be fully elucidated. There are five classes of Hdac proteins: I, IIa, IIb, III, and IV. Class I includes Hdacs 1, 2, 3, and 8, and these enzymes are expressed in most tissues [7–9]. Hdacs 1 and 2 are members of several repressive complexes including the Sin3 deacetylase, repressor element-1 silencing transcription factor corepressor 1/deacetylase (CoREST),

nucleosome-remodeling deacetylase (NuRD), and polycomb-repressive complex 2 (PRC2) complexes [10]. Hdacs 4, 5, 7, and 9 comprise the class IIa subfamily of Hdacs. Class IIa Hdacs exhibit minimal deacetylase activity *in vivo* and *in vitro* due to evolutionary divergence of a critical tyrosine residue within the catalytic pocket. These Hdacs are characterized by a highly conserved myocyte enhancer factor 2-binding domain (Mef2). In addition, class IIa Hdacs act as signal integrators through interactions with 14-3-3 chaperone proteins to facilitate shuttling in and out of the nucleus [9]. Class III sirtuins require NAD^+ for deacetylation activity and have been studied in the context of cardiac hypertrophy [11, 12]. Hdac11 is the sole member of class IV and is expressed in the heart; however, its function remains poorly described [13].

15.3.1.1 Hdac1 and Hdac2

Hdac1 and Hdac2 share a large degree of amino acid homology, are thought to have mostly redundant functions, and are both expressed in the myocardium [14, 15]. Global *Hdac1*-null mice die in utero at approximately E10.5, but myocardial specific deletion of *Hdac1* does not result in gross cardiac abnormality [14]. Global *Hdac2* deletion by a gene trap allele results in partial lethality, but surviving mutants exhibit only slightly thickened myocardial walls under homeostatic conditions [15]. However, these mutant mice are resistant to the cardiac hypertrophy that results fom *β*-adrenergic stimulation or transaortic constriction. Montgomery et al. generated a floxed allele of Hdac2, and global deletion of *Hdac2* (by crossing the floxed allele to a cytomegalovirus-Cre recombinase mouse) results in lethality of all mice within 24 h of birth [14]. The differences between the phenotypes of the gene trap and floxed alleles are likely due to differences in genetic background and/or efficiencies of *Hdac2* inactivation. Combined deletion of *Hdac1* and *Hdac2* in cardiomyocytes results in death within 2 weeks of birth due to cardiac arrhythmias and cardiomyopathy, along with ectopic expression of fetal ion channels and contractile proteins [14]. It should be noted that compound deletion of *Hdac1* and *Hdac2* just a few days earlier in cardiac development leads to intrauterine demise, highlighting the changing role of Hdacs during cardiac development, especially during critical times of progenitor cell commitment, proliferation, and differentiation.

15.3.1.2 Hdac3

Like other class I Hdacs, Hdac3 is expressed in the myocardium throughout development and into adulthood. Germline deletion leads to lethality during gastrulation, before the heart is formed, and any cardiac phenotype can be appreciated [16]. Specifically, genetic deletion of *Hdac3* in early cardiac progenitors results in embryonic lethality late in gestation and ventricular hypoplasia [17]. Hdac3 acts as a repressor of T-box transcription factor 5 (Tbx5), a known driver of cardiogenesis, which also plays critical roles in development of the cardiac conduction system and atrial septation [5, 18, 19], though it is unclear if deacetylation of Tbx5 protein by Hdac3 is required for cardiogenesis. Myocyte specific deletion of *Hdac3* during mid-late gestation with alpha–myosin heavy chain-Cre recombinase (αMHC-Cre) permits survival until 3–4 months of age when mice succumb to severe cardiac hypertrophy and fibrosis [20]. Interestingly, deleting *Hdac3* in postnatal myocardium and skeletal

muscle with myosin creatine kinase-Cre (MCK-Cre) results in a very mild phenotype and no lethality unless the mice are stressed by a high-fat diet [21]. Switching the mice from normal chow to a high-fat diet results in severe hypertrophic cardiomyopathy and fibrosis followed by death of all animals within several weeks. Myocyte specific deletion of *Hdac3* is also characterized by extensive metabolic dysregulation of both lipid and glucose processing. Consistent with these findings, Hdac3 is unique among Hdacs for its interaction with the nuclear receptor corepressor-silencing mediator for retinoid and thyroid receptor (NCoR/SMRT) complex that has been previously implicated in metabolic regulation [22–24].

Hdac3 is also a potent regulator of cell proliferation and directly regulates p21 [25, 26]. Previous work in transformed cells and hematopoiesis implicated Hdac3 in regulating cell cycle progression and DNA replication [26, 27]. Transgenic overexpression of *Hdac3* in cardiomyocytes causes increased proliferation and ventricular hyperplasia [25]. Expression analysis indicated that Hdac3 represses several cyclin-dependent kinase inhibitors, reducing levels of these cell cycle checkpoints and promoting rapid proliferation of cardiomyocytes.

15.3.1.3 Hdac5 and Hdac9

These two class II Hdacs are expressed in the myocardium and skeletal muscle, and individual genetic loss yields viable adult mice without an overt cardiac phenotype under homeostatic conditions [28, 29]. However, combined deletion of *Hdac5* and *Hdac9* results in sensitivity to hypertrophic signals, phenotypically opposite to that of *Hdac2*-null animals. Loss of *Hdac5/9* leads to overactive Mef2 signaling in response to calcineurin-dependent signaling, which normally leads to export of class IIa Hdacs from the nucleus and inhibition of Mef2 activity. The absence of *Hdac5/9* leaves Mef2 within the nucleus where it can activate hypertrophic gene programs [29]. Class IIa Hdacs also modulate other transcription factor complexes, including serum response factor (SRF), myocardin, and calmodulin-binding transcription activator 2 [30], and it is likely that deletion of both *Hdac5* and *Hdac9* has effects on these regulators and Mef2 throughout development.

15.3.1.4 Hdac7

Global deletion of *Hdac7* leads to midgestation death at E11.5 due to vascular defects [31]. Mutant mice have vascular leak and lack smooth muscle cells around the dorsal aorta. Hdac7 complexes with Mef2 to repress matrix metalloproteinase 10 (Mmp10) in endothelium, which has been shown to influence the integrity of surrounding smooth muscle [31]. Consistent with a primary role of Hdac7 in endothelial cells, knockdown studies of *Hdac7* in endothelial cell cultures results in abnormal cellular morphology and tube formation [32].

15.3.1.5 Sirtuins

Sirtuins are class III Hdacs and require nicotinamide adenine dinucleotide (NAD) for their catalytic activity. There are seven sirtuins in mammals with various expression patterns, cellular localizations, and functions [11, 12]. Sirt1 is the best-studied sirtuin and is located in the nucleus and cytoplasm [33, 34], while Sirt2 is restricted

to the cytoplasm [35]. Sirt3, Sirt4, and Sirt5 are localized to the mitochondria primarily [36]. Sirt6 and Sirt7 are localized to the nucleus [37, 38]. Sirt6 is involved in deacetylation of H3K9 and H3K56 [38], while Sirt7 modulates gene transcription dependent on RNA polymerase-I [37]. Multiple sirtuin proteins have been implicated in the pathogenesis of cardiac hypertrophy, but to our knowledge, there is no clear role for sirtuins in the most common forms of congenital heart disease.

15.3.2 HAT Proteins: Histone Acetyltransferases

Histone acetyltransferase enzymes catalyze the addition of acetyl groups to lysine residues of histone tails. This results in relaxation of chromatin, greater accessibility of the transcriptional machinery to DNA, and an increase in gene expression.

The most well-studied HAT is p300, with genetic deletion resulting in a generalized lack of cell proliferation, myocardial thinning, and death between E9.5 and E11.5 [39]. A single amino acid mutation abrogating the acetyltransferase activity results in myocardial thinning as well, but these mutants live to E12.5–E15.5 [40]. p300 also acetylates nonhistone proteins, including Gata4, which plays multiple roles in cardiac myocyte specification, differentiation, and proliferation [5, 41]. Acetylation of Gata4 enhances its transcriptional activity [42]. Interestingly, Hdac2, functioning with the atypical homeodomain protein, Hopx, can deacetylate Gata4 and represses its transcriptional activity [5]. This pathway is important in cell cycle exit and reducing the proliferative capacity of myocytes in late gestation. These are examples of traditional "epigenetic" factors acting on nonhistone proteins to indirectly affect cardiac gene expression.

15.4 Histone Methylation

Multiple arginine and lysine residues on histone proteins are methylated. Gene activation or gene repression is dependent on which residue of the histone tail is methylated. Gene activation is associated with methylation at lysine (K) 4, K36, and K79 of histone H3, while gene repression is associated with methylation of K9 and K27 [4]. To date, histone methyltransferases and demethylases have not been studied as comprehensively in the context of cardiac development as Hdac and HAT proteins.

15.4.1 Jumonji Proteins

Methyl groups are removed from histone tails by a family of proteins containing JumonjiC-domains. Two members of this family, jumonji AT-rich interactive domain 2 (Jarid2) and jumonji domain-containing 6 (Jmjd6), have important roles in cardiac outflow tract septation. Jarid2 is expressed in the developing myocardium of mice, as early as E8.0. The developing outflow tract, ventricular septum, and ventricular walls express relatively high levels of Jarid2 as compared to the atria. Jarid2 remains

expressed in the myocardium in the adult mouse but at lower levels than during development. Global deletion of *Jarid2* results in hypertrabeculation of myocardium and outflow tract defects, including double outlet right ventricle (DORV) [43, 44]. Jarid2 controls expression of Notch1, and its downstream target, Neureglin1, both implicated in the control of trabecular growth [44]. Similarly, *Jmjd6* knockout mice demonstrate DORV [45]. The nature of the defects suggests that these proteins affect neural crest function, though conditional deletion in the neural crest will be necessary to establish the tissue-specific roles of these proteins.

15.4.2 *WHSC1* and Wolf-Hirschhorn Syndrome

Wolf-Hirschhorn syndrome is characterized by mental retardation, epilepsy, craniofacial abnormalities, and myocardial septal defects. Genetically, the syndrome is correlated with mutations and/or translocations of *WHSC1* (Wolf-Hirschhorn syndrome candidate 1), which encodes a histone methyltransferase that specifically methylates H3K36 and H4K20 [46]. Knockout *Whsc1* mice are characterized by myocardial septal defects, bone defects, and growth delay. However, haploinsufficiency of *Whsc1* in murine models does not result in the overt features associated with the clinical syndrome [47]. However, Whsc1 is known to associate with NK2 homeobox 5 (Nkx2-5), and compound haploinsufficiency of *Whsc1* and *Nkx2-5* in murine models recapitulates the atrial and ventricular septal defects associated with the clinical syndrome [47]. Some human mutations involve deletions of large pieces of chromosome 4, including the nearby Msh Homeobox 1 (*MSX1*) gene, which is highly expressed in the mesenchyme that contributes to craniofacial structures including teeth. Some of the typical craniofacial features of Wolf-Hirschhorn syndrome, such as dental abnormalities and cleft lip and/or palate, are more prominent in patients when *MSX1* is lost as well [48].

15.4.3 Smyd Proteins

The SET and MYND (Smyd) proteins are a family of five proteins characterized by a SET domain, which catalyzes methyltransferase activity, and a protein–protein interaction MYND domain [49]. The MYND domain allows Smyd proteins to interact with Hdacs, and Smyd1 represses gene transcription in an Hdac-dependent manner [50]. Interestingly, multiple Smyd family members have been detected in the cytoplasm and can be shuttled into the nucleus. This suggests that Smyd proteins likely have nonnuclear functions such as methylation of nonhistone proteins [49]. Smyd1 is the founding member of this family of proteins, and knockout studies in mice demonstrate a critical role in cardiac myocyte maturation and morphogenesis of the right ventricle [50]. This defect may be due to, at least in part, Smyd1 regulation of *Heart and Neural Crest Derivatives Expressed 2* (*Hand2*) and *Iroquois Homeobox 4* (*Irx4*) expression [50]. Zebrafish studies confirm a critical role for *smyd1b*, one of two zebrafish orthologues; knockdown of *smyd1b* results in mispatterning of

myofibers and lack of myocardial contraction [51, 52]. Smyd2, known to associate with the Sin3 complex [53], is strongly expressed in the embryonic myocardium and highly related to Smyd1 [54]. *Smyd2* knockdown in zebrafish results in cardiac dysfunction and myocyte disorganization [55]. However, knockout studies in mice demonstrate Smyd2 is dispensable for normal cardiogenesis [54]. *Smyd3* knockdown in zebrafish results in abnormal cardiac looping and defective expression of essential myocyte genes [56].

15.4.4 MLL2 and Kabuki Syndrome

Kabuki syndrome is an autosomal dominant syndrome characterized by mental retardation, distinctive craniofacial abnormalities, skeletal anomalies, and short stature [57]. The incidence of congenital heart disease in patients with Kabuki syndrome is 30–55 %. There is a wide spectrum of reported defects including aortic coarctation, septal defects (atrial and ventricular), and hypoplastic left ventricle [58, 59]. Type I Kabuki syndrome is associated with mutations in the mixed-lineage leukemia protein 2 (*MLL2*), a histone methyltransferase belonging to the Trithorax family of proteins [60]. MLL2 is required for methylation of H3K4 [61, 62]. Mutations in *MLL2* have been detected in 48–75 % of Kabuki patients, and nonsense or frameshift mutations are the most common types of mutations detected. Type II Kabuki syndrome, a more rare form of the disorder, is associated with mutations in *KDM6A*, a histone demethylase that specifically acts on H3K27 [63].

15.5 Epigenetic Gene Variants and Sporadic Congenital Heart Disease

The study by Zaidi et al., as part of the Pediatric Cardiac Genomics Consortium, provided a fundamental advance in our understanding of epigenetics and congenital heart disease by sequencing the exomes of 362 trios (affected patients and their parents) with a variety of congenital heart disorders, along with a cohort of 264 unaffected trios [1]. Interestingly, there was an overrepresentation of mutations or variants in genes encoding epigenetic enzymes in affected patients when compared to unaffected controls. Cohorts were largely composed of patients of European ancestry. Probands were affected by a variety of forms of congenital heart disease, but mostly composed of conotruncal defects ($n = 153$ patients, transposition of the great arteries, DORV, TOF, partial truncus arteriosus, and aortic arch abnormalities), LV obstructive lesions ($n = 132$, hypoplastic left heart syndrome, aortic coarctation, bicuspid aortic valve), and heterotaxy ($n = 70$). Extracardiac abnormalities including craniofacial defects were present in 22 % of probands. The authors identified mutations predicted to be pathologic in 28 genes. These predictions depended on whether the mutations resulted in premature termination, splicing variants and/or frameshift of the coding sequence. The analyses also demonstrated that these mutated genes

were amongst the highest expressed genes in the developing heart (25 % percentile or higher). Mutations in five genes were specifically associated with histone methylation of H3K4: *MLL2*, *WDR5*, *CHD7*, *KDM5A*, and *KDM5B*. H3K4me is known to mark active promoters and genes "poised" to be expressed. MLL2, as previously discussed, deposits the methylation of H3K4 [62]. JARID1A and JARID1B are responsible for removing methyl groups on H3K4, and CHD7 recognizes this histone modification [64]. Additionally, WD repeat domain 5 (WDR5) has been identified as part of the MLL complex [65]. Interestingly, the proband identified with a *CHD7* mutation did not manifest typical features of CHARGE syndrome, though a patient with *MLL2* mutation did display characteristic craniofacial abnormalities typical of Kabuki syndrome. The group also identified mutations in *ring finger protein 20* (*RNF20*) and *ubiquitin-conjugating enzyme E2B* (*UBE2B*), factors involved in the ubiquitination of H2BK120, which is required for H3K4 methylation.

15.6 DNA Methylation

In addition to histone proteins, the C5 position of cytosine nucleotides can be directly methylated (5-mC). The DNMT family of proteins (DNA methyltransferases) is composed of three proteins that catalyze transfer of methyl groups onto DNA in CpG islands. DNMT1 recognizes previously methylated DNA to reinforce existing patterns after DNA replication. DNMT3a and DNMT3b are *de novo* DNMTs and catalyze methylation of previously unmethylated cytosines [66]. More recently, it was discovered that the methylcytosine dioxygenase (TET) family of proteins catalyzes DNA demethylation through the conversion of 5-mC to 5-hmC as well as 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) [66]. Methyl-CpG-binding domain proteins, including MeCP2, recognize methylated DNA and recruit histone-modifying complexes. Mutations in *MECP2* have been implicated in the pathogenesis of Rett syndrome, a condition characterized by regression of skills, significant neurological difficulties, and stereotypical movements [67]. However, it was recently determined that approximately 20 % of patients with Rett syndrome have a prolonged QT interval on their electrocardiogram, which can lead to sudden cardiac death in some patients [68]. Further work is needed to determine if mutations in TET proteins or DNMTs in humans are associated with congenital heart disease.

15.7 Chromatin Architecture

The organization and three-dimensional structure of chromatin has emerged as a significant regulator of gene transcription. Dense, heterochromatic regions are associated with repressed gene activity and euchromatic regions with a more open conformation permit increased access by transcription factors to the DNA and are associated with increased gene transcription. Regulating higher order chromatin structure in this way allows for simultaneous control of large genomic regions and coordination of gene expression – a requirement for large-scale processes such as cell differentiation and stress response. For example, epigenetic mapping has

demonstrated that as cells exit pluripotency during development and become more lineage restricted, levels of heterochromatin increase and the genome becomes more compact while cell fates stabilize [69, 70]. Chromatin-remodeling complexes such as Brg1-associated factor (BAF), CHD, and inositol requiring 80 (INO80) contain ATPase domains that catalyze nucleosome restructuring–detaching histone proteins from the DNA helix and reassembly of the complex elsewhere. In addition, these complexes may contain domains for sensing histone modifications or other epigenetic marks and reorganize the chromatin accordingly. Finally, emerging evidence has also identified three-dimensional positioning of chromatin within the nucleus as an additional layer of regulation of chromatin structure and transcriptional activity.

15.7.1 ATP-Dependent Chromatin Remodelers

Chromatin remodeling complexes are a class of "writers" that are characterized by SWI-like ATP-dependent catalytic activity. There are several classes of chromatin remodeling complexes including the switching defective/sucrose nonfermenting (SWI/SNF), chromodomain, helicase, DNA binding (CHD), and INO80 complexes. These complexes all contain a core ATPase domain that serves to disassemble the DNA-histone contacts to restructure and reposition nucleosomes. The complexes also serve as bridges to other regulatory factors and readers of histone modifications, making them important regulators of chromatin architecture and organization.

15.7.2 SWI/SNF Complex

The SWI/SNF complex was identified and purified in yeast with a vertebrate homolog BAF composed of 12 subunits. The core ATPase subunit is encoded by one of two highly homologous genes, SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily a, member 4 (also known as brahma-related gene 1; *Brg1*) and SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily a, member 2 (*Brm*); however, most studies suggest that Brg1 is the dominant ATPase in cardiovascular development [71].

Brg1 is expressed from the onset of embryogenesis, and *Brg1*-null embryos do not advance past implantation [72]. Endothelial-specific deletion of *Brg1* results in embryonic lethality at E10.5 and deficient yolk sac vasculogenesis [73]. Loss of *Brg1* in the endocardium results in hypotrabeculation. Further analysis revealed that Brg1 acts to repress a metalloproteinase, *Adamts1*, responsible for breakdown of the cardiac jelly present in the early embryonic heart. Loss of *Brg1* allows premature expression of Adamts1, early degradation of the cardiac jelly, and lack of normal trabeculation. Deletion of *Brg1* in cardiac myocytes results in reduced cell proliferation and thin compact myocardium, ventricular septal defects, and lethality at E11.5 [71]. In developing cardiomyocytes, Brg1 complexes with Hdacs and poly (ADP-ribose) polymerases (PARP) to repress the adult isoform of myosin heavy chain and activate the fetal form, permitting normal embryogenesis. Loss of *Brg1* induces

premature expression of the adult myosin isoform and leads myocytes to exit the fetal state too rapidly. Conditional deletion of *Brg1* results in hypoplastic right ventricle, shortened outflow tract, and embryonic lethality at E11.5 [71]. Smooth muscle depletion of *Brg1* leads to reduction of contractile proteins and dilated cardiomyopathy secondary to patent ductus arteriosus [74–77]. Taken together, these tissue-specific deletions suggest that Brg1 is an essential regulator of cardiac development as a core ATPase of the BAF complex, and its abrogation can lead to a variety of congenital heart defects in animal models.

Polybromo 1 (Baf180) is another subunit of the BAF complex critical for normal cardiac development. Baf180 contains six bromodomains and serves as a "reader" of acetylated histone tails. Global deletion of *Baf180* is embryonic lethal by E15.5 with ventricular hypoplasia and ventricular septal defects [78]. Epicardium-specific deletion disrupts normal vasculogenesis, resulting in coronary artery abnormalities [79]. Further analysis reveals that Baf180 is required for normal expression of elements of the retinoic acid pathway, a core regulatory mechanism of myocardial patterning. D4, zinc and double PHD fingers, family 3 (Baf45c or Dpf3) is a BAF subunit that also serves as a "reader," in this case of histone tails modified by the addition of acetyl and methyl groups. Knockdown in zebrafish results in abnormal cardiac looping and poor cardiac function [80]. Notably, human patients with tetralogy of Fallot (TOF) exhibit increased expression of Baf45c in the right ventricle [81]. Cui et al. showed that Baf45c is also upregulated in the hypertrophied left ventricle of patients with aortic stenosis and hypertrophic cardiomyopathy [82]. Mechanistically, phosphorylation of Baf45c (Dpf3a) by casein kinase 2 induces hypertrophy by releasing HEY repressors from chromatin [82].

SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily d, member 3 (Baf60c) has been shown to interact with Nkx2-5, Tbx5, and Gata4 to activate multiple genes critical for cardiogenesis [83]. Loss of function studies in mice and zebrafish have confirmed Baf60c regulates multiple steps of cardiogenesis including cardiac looping and morphogenesis [84–86]. Recent lineage tracing studies suggest that Baf60c-expressing cells give rise to the majority of the heart, in addition to extracardiac structures [87].

Williams syndrome is an autosomal dominant disorder notable for growth deficiency, dysmorphic facies, and cardiovascular abnormalities including pulmonary artery stenosis, supravalvular aortic stenosis, bicuspid aortic valve, aortic coarctation, and ASD [88]. Williams syndrome is marked by loss of 7q11.23 encompassing 23 genes including Williams syndrome transcription factor (*WSTF*), which is recruited into the BAF complex [89].

15.7.3 CHD Chromodomain Proteins

The CHD chromatin remodelers are complexes made up of at least a chromodomain, helicase, and DNA-binding domain. *CHD7* has been implicated in human disease as its haploinsufficiency results in CHARGE syndrome [64]. The syndrome features several cardiac defects including septal defects, endocardial cushion abnormalities, and conotruncal malformations. Furthermore, the aforementioned study

by Zaidi et al. implicated *CHD7* as a genetic cause of congenital heart disease. Studies in mice reveal that loss of one copy of *Chd7* results in congenital heart disease including truncus arteriosus and interrupted aortic arch [90]. Recent studies suggest that this phenotype is mediated by a genetic interaction with Tbx1.

15.7.4 INO80 Complexes

Inositol requiring 80 (INO80) complexes contain DNA helicases to unwind double-stranded DNA. In vertebrates, pontin and reptin are two closely related helicases that commonly complex with one another and have been extensively studied in zebrafish. Disruption of either of these enzymes disrupts INO80 chromatin-remodeling activity and affects cardiac morphogenesis in fish [91]. Pontin represses cardiomyocyte proliferation, and Reptin plays an opposite role to promote proliferation through counteracting effects on the β-catenin pathway. Currently, little is known of their functions in mammalian heart development and disease.

15.7.5 BET Proteins: Chromatin "Readers"

The bromodomain and extra terminal (BET) family of proteins consist of three ubiquitously expressed members (Brd2-4) and a testis-specific isoform, BrdT. BET proteins are characterized by tandem bromodomains that allow for recognition of acetylated lysine residues on histone tails and other nuclear proteins. Pharmacologic inhibition of BET family members with the small molecule inhibitor JQ1 is sufficient to inhibit development of pathologic cardiac hypertrophy [92, 93]. JQ1 can competitively bind the bromodomains of BET family proteins, thereby inhibiting the "reader" ability [94]. Inhibition of cardiac hypertrophy was postulated to be secondary to inhibition of transcription at cohorts of genes relevant to a pro-hypertrophic phenotype. However, the role of BET proteins during cardiogenesis remains unknown. *Brd2* deficiency in mice leads to defective neural tube closure and death at approximately E13.5 [95, 96]. *Brd4* deficiency in mice leads to death shortly after implantation [97].

15.8 Gene Positioning Within the Nucleus

An emerging concept within the field of chromatin organization is that the localization of genes within the nucleus can affect gene expression. Several recent studies have revealed, for example, that the conformation of chromatin at the nuclear periphery versus in the nucleoplasm is very different [98]. More specifically, peripheral chromatin bound at the nuclear pore complex tends to be more transcriptionally active than chromatin at the nuclear membrane between the pores, where gene expression is repressed [99–101]. The inner surface of the nuclear membrane is coated by a thin layer of intermediate filament proteins called lamins. Chromatin found at the nuclear lamina tends to be transcriptionally silent, and forced tethering of reporter genes to the nuclear periphery can result in repression [102, 103]. Genome-wide mapping studies

have revealed that the organization of chromatin at the nuclear lamina versus the interior of the nucleus/nucleoplasm is dynamic and varies over the course of cell differentiation [104]. The significance of these changes during development is still a matter of intense study; however, it is clear that several diseases with cardiac manifestations are caused by mutations in nuclear lamina-associated proteins. These laminopathies include Hutchinson-Gilford progeria syndrome and Emery–Dreifuss muscular dystrophy and are due to mutations in genes encoding lamin A and its interacting protein emerin, respectively [105, 106]. While these diseases are caused by disruptions of the nuclear lamina, further study is required to establish whether changes in chromatin organization are responsible for the observed phenotypes.

In addition to lamina filaments, the nuclear membrane is also decorated with thousands of nuclear pore complexes (NPC). These large protein complexes of at least 30 different nucleoporin proteins are typically thought of as regulators of selective transport in and out of the nucleus [107]. In addition to this transport role, nuclear pore proteins interact with chromatin, and genome-wide surveys have demonstrated that nuclear pore complexes tend to associate with transcriptionally active chromatin [101, 108, 109]. Much work remains to clarify the role of the nuclear pore complex in development and transcriptional regulation, although interesting connections have begun to emerge. For example, screens have identified several chromatin-remodeling complexes including chromatin structure remodeling (RSC) as interacting partners with the NPC [110]. Mutations in *nucleoporin 155 kDa* (*Nup155*) in mice results in atrial arrhythmias [111]. Hdac4 can physically interact with Nup155, and this interaction is necessary for Hdac4-mediated gene regulation at least in some settings [112].

Conclusion

The rapidly growing appreciation of the complex array of enzymes and associated proteins that mediate epigenetic regulation of gene expression has transformed our understanding of the etiology of congenital heart disease. The specific functions of various subclasses of epigenetic modifiers during heart development remain poorly elucidated, and the range of interacting proteins, RNA molecules, and other moieties have not been fully defined. Nevertheless, gain and loss of function studies in animal models, and human genetic studies, strongly suggest that the developing cardiovascular system is acutely sensitive to epigenetic perturbations, and it seems likely that these sensitivities are further modulated by environmental exposures. Epigenetic regulation of cardiovascular development is a rapidly evolving field that will gain increasing relevance for the understanding and treatment of cardiac disorders in the future.

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Environmental Signals

16

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Contents

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Abstract

Environmental factors have long been known to play a role in the pathogenesis of congenital heart disease (CHD), but this has not been a major focus of research in the modern era. Studies of human exposures and animal models demonstrate that demographics (age, race, socioeconomic status), diseases (e.g., diabetes, hypertension, obesity, stress, infection, high altitude), recreational and therapeutic drug use, and chemical exposures are associated with an increased risk for CHD. Unfortunately, although studies suggest that exposures to these factors may cause CHD, in most cases, the data is not strong, is inconclusive, or is contradictory. Although most studies concentrate on the effects of maternal exposure, paternal exposure to some agents can also modify this risk. From a mechanistic standpoint, recent delineation of signaling and genetic controls of cardiac development has revealed molecular pathways that may explain the effects of environmental signals on cardiac morphogenesis and may provide further tools to study the effects of environmental stimuli on cardiac development. For example, environmental factors likely regulate cellular signaling pathways, transcriptional and epigenetic regulation, proliferation, and physiologic processes that can control the development of the heart and other organs. However, understanding of the epidemiology and risk of these exposures and the mechanistic basis for any effects on cardiac development remains incomplete. Further studies defining the relationship between environmental exposures and human CHD and the mechanisms involved should reveal strategies to prevent, diagnose, and treat CHD induced by environmental signals.

16.1 Introduction

Since the beginning of the modern, molecular era of developmental biology, it has become commonplace to investigate the role of signaling pathways and genetics in human disease and in animal models. However, genetic influences on developmental processes cannot explain all CHDs [1]. Single-gene mutations in humans and animal models can cause heterogeneous phenotypes, suggesting that other factors regulate normal and abnormal morphogenesis. Genetic background explains some of this variation, but much of it may be due to environmental signals. Indeed, it has been estimated that nongenetic factors may play a role in up to 30 % of CHD [1], and recent data are revealing the mechanisms by which environmental factors can regulate signaling pathways and gene transcription to cause various forms of CHD.

Cardiac morphogenesis is a complex process that extends from early gestation until well after birth, although the embryonic period (day 18–60 of human gestation) is generally the critical window for cardiac teratogenesis. Over the last 30 years, the integration of many signaling pathways has been shown to regulate an array of transcriptional regulatory factors to control cardiac morphogenesis, and mutations in many of these pathways cause CHD in animals and humans. More recent research has defined a role for epigenetic regulation of cardiac morphogenesis. Environmental

factors potentially may modulate all of these processes to influence cardiac development. This chapter will summarize the evidence for the role of environmental factors in cardiac morphogenesis and the mechanisms underlying these processes.

16.2 Role of Environmental Signals During Cardiac Development

16.2.1 General Evidence and Clinical Studies

Cardiac teratogens are environmental or physiologic factors that cause cardiac dysmorphogenesis and CHD. Generally, teratogens are thought to be environmental toxins, to which the mother and fetus are exposed, but human conditions, such as different demographic categories and maternal diseases, can be considered teratogenic.

Prior to the 1980s, anecdotal evidence suggested that environmental factors played a role in the development of CHD. For example, the association of congenital rubella infection and CHD was reported in 1941 [2], and thalidomide use was associated with CHD in the 1950s and 1960s. These and other findings prompted large-scale epidemiologic studies to investigate the association of environmental factors and CHD. Two of the most important were case control studies in Maryland and Virginia (the Baltimore-Washington Infant Study (BWIS)) and in Finland [3, 4]. Comparison of these studies to more recent ones is summarized below and presented in more detail elsewhere [1, 5, 6].

The lack of clear data supporting the cardiac teratogenic potential of environmental factors is striking. In comparing data from various studies, the significance of individual environmental factors often is quite variable. For example, five medium/large studies show relative risk or odds ratio of 0.9–1.3 for having a child with CHD after using selective serotonin reuptake inhibitors (SSRIs) during pregnancy, but in only one was there statistically significant risk [6, 7]. Adding to this confusion are smaller case reports and series that lack adequate controls. Finally, these studies are complicated by the presence of overlapping risk factors in the population, which make choosing appropriate controls and comparing individual studies difficult.

16.2.2 FDA Drug Risk Categories

Animal models are an excellent method to examine cardiac teratogenesis, but these studies can be difficult to interpret when the concentrations of reagents used in these studies are not consistent with human exposures or when data from human studies are lacking or inconsistent with the model. To assess the risk of various drugs during pregnancy, the Food and Drug Administration (FDA) has developed five risk categories (Table 16.1) that reflect results of animal and human studies and the potential risks and benefits to the fetus and mother. Although not specific for cardiac teratogenesis, the risk category of drugs discussed below (Table 16.2) should be considered when contemplating the use of these agents during pregnancy. Note that the

Table 16.1 Definition of FDA risk categories for birth defects

Risk category	Description
A	Adequate and well-controlled studies have failed to demonstrate a risk to the fetus in the first trimester of pregnancy (and there is no evidence of risk in later trimesters)
B	Animal reproduction studies have failed to demonstrate a risk to the fetus and there are no adequate and well-controlled studies in pregnant women
C	Animal reproduction studies have shown an adverse effect on the fetus and there are no adequate and well-controlled studies in humans, but potential benefits may warrant use of the drug in pregnant women despite potential risks
D	There is positive evidence of human fetal risk based on adverse reaction data from investigational or marketing experience or studies in humans, but potential benefits may warrant use of the drug in pregnant women despite potential risks
X	Studies in animals or humans have demonstrated fetal abnormalities and/or there is positive evidence of human fetal risk based on adverse reaction data from investigational or marketing experience, and the risks involved in use of the drug in pregnant women clearly outweigh potential benefits
N	Not classified

Source: http://chemm.nlm.nih.gov/pregnancycategories.htm

Table 16.2 FDA risk categories of various potentially cardio-teratogenic drugs

Risk category	Drugs
A	Folate
B	Amoxicillin (±clavulanic acid), ampicillin, cefotaxime, metronidazole
C	Acetaminophen, beta-carotene, bupropion, caffeine, cocaine (topical), codeine, fluconazole, fluoxetine, ibuprofen, Marinol, naproxen, rifampicin, sertraline, sulfamethoxazole/trimethoprim, theophylline, tretinoin topical, zidovudine
D	Aspirin (in the third trimester), lithium, paroxetine, phenytoin, valproic acid
X	Retinoic acid agents (alitretinoin, etretinate, isotretinoin), thalidomide
N	Acetaminophen, aspirin, diazepam, marijuana

FDA is revising its labeling of drugs for risk during pregnancy and that these categories are not applied to non-pharmacologic environmental agents.

16.2.3 Risks Assessment

The risk of developing CHD after different exposures can be defined in a number of ways, depending on the methods. Generally, odds ratios (ORs) are derived from case-control studies, while relative risks (RR) are derived from randomized control trials or cohort studies. As the following sections will summarize data from various studies, OR and RR will be combined. The range of OR/RR without confidence intervals will be noted in some cases, and the reader is referred to excellent reviews for more details [1, 5, 6].

The relative importance of any particular environmental factor is a combination of the prevalence of exposure, the relative risk of associated CHD, and the clinical

severity of the CHD. For example, the extremely low prevalence of uncontrolled phenylketonuria during pregnancy mitigates the high risk of CHD in these pregnancies. In contrast, although the risk for CHD after *in utero* exposure to diabetes, fever, tobacco smoking, ethanol ingestion, seizures, or obesity may (or may not) be low, the relative importance to society may be high due to the high prevalence of these exposures.

16.3 Environmental Signals During Cardiac Development

The following sections review data from human studies of potential cardiac teratogens. Please note that studies of the risk and etiology of congenital defects in conditions such as those listed below are complicated by the association of some of these conditions with each other, making accurate assignment of any single risk factor difficult.

16.3.1 Human Demographics

16.3.1.1 Age

Younger and older age in both parents is associated with increased risk for all CHD or specific types of CHD, although these associations are inconsistent between studies. Men older than 25 years and women older than 30 years and both men and women under 20 years of age may have increased risk.

16.3.1.2 Race

Race may alter risk for CHD. Some studies suggest that Caucasian infants are more likely to have left-sided obstructive lesions, septal defects, Ebstein's anomaly, and some conotruncal defects and are less likely to have atrial septal defects (ASD) and valvular pulmonary stenosis (PS) than African American infants. One study showed equal risk for Hispanic women compared to Caucasian women. Eastern Asian infants are also more likely to have a supracristal/outlet ventricular septal defect (VSD).

16.3.1.3 Socioeconomic Status

Lower socioeconomic status may be associated with increased risk for CHD, with significant OR/RR of 1.6 and 3.4 in two studies.

16.3.2 Human Conditions and Diseases

16.3.2.1 Diabetes

Pregestational and gestational maternal diabetes can cause many cardiac and noncardiac defects, particularly when it is uncontrolled in the early stages of pregnancy. Lesions include laterality defects, conotruncal defects, atrioventricular septal defects (AVSD), VSDs, and left-sided obstructive lesions. The OR/RR of any form of CHD in infants of diabetic mothers ranges from 1.8 to 18. This risk is decreased with good glycemic control. In addition, obstructive and nonobstructive hypertrophic cardiomyopathy can occur in infants of diabetic mothers. At any risk above

baseline, the increasing rate of diabetes in the general population has worrisome implications for the global incidence of CHD.

16.3.2.2 Fever/Maternal Infections

Maternal fever is teratogenic in animal models. Human gestational fever in the first trimester increases risk for all CHDs, although data are inconsistent among studies. A variety of CHD types may be associated with fever, with the most common being conotruncal defects. However, interpreting data in humans is complicated by the effects of the infectious sources of and the therapies for fever. For example, the relationship between maternal rubella infection and a patent ductus arteriosus (PDA), PS, peripheral pulmonic stenosis (PPS), and VSDs is well documented. Influenza exposure during pregnancy may also increase risk for CHD, and gestational HIV exposure increases risk for dilated cardiomyopathy.

16.3.2.3 Folate Deficiency

Data supporting a risk for CHD in pregnancies complicated by folate deficiency are largely derived from studies showing that folate supplementation during early stages of pregnancy decreases risk for CHD. Although results among these studies are inconsistent, the potential benefit of folate supplementation during pregnancy on the risk of CHD strengthens the recommendation for this therapy. Additional studies suggest that hyperhomocysteinemia, which can result from folate and vitamin B12 deficiency, may also increase risk for CHD. Finally, multivitamin/folate use may ameliorate the effects of fever, alcohol, lithium, and hyperhomocysteinemia on the risk for CHD [8].

16.3.2.4 High Altitude/Hypoxia

Being born at high altitude causes maternal hypoxia and increases risk for ASDs and PDAs, but this has not been extensively studied.

16.3.2.5 Hypertension

Hypertension may increase the risk of CHD up to twice that of controls, but no specific lesion stands out.

16.3.2.6 Maternal Stress

Increased maternal stress has been associated with an increased risk (OR/RR = 1.4–2.7) for CHD, particularly conotruncal defects, in some studies.

16.3.2.7 Obesity

The association of obesity with CHD has been inconsistent among various studies, and OR/RR ranges between <1 and 3. However, increased risk is observed with increasing levels of obesity.

16.3.2.8 Phenylketonuria

There is a strong association of phenylketonuria with CHD, with an RR of ≥6 and a rate of up to 14 % of pregnancies with high levels of phenylketonuria having newborns with CHD. However, good dietary control can reduce or eliminate this risk.

The most likely CHD lesions to be seen are left-sided obstructive lesions, tetralogy of Fallot (TOF), VSDs, and PDAs.

16.3.2.9 Reproductive History

Multiparous women are more likely to have children with CHD, while nulliparous women may have increased risk for children with ASDs, TOF, and VSDs. A history of reproductive problems carries increased risk for ASDs, AVSDs, Ebstein's anomaly, and TOF. Assisted reproductive technology is associated with increased risk for congenital lesions, including CHD such as aortic valve stenosis, ASDs, coarctation of the aorta (CoA), TOF, and VSDs.

16.3.3 Dietary and Recreational Drug Exposures

16.3.3.1 Alcohol

The original description of fetal alcohol syndrome included CHD. However, heavy maternal alcohol consumption during pregnancy is inconsistently associated with CHD, particularly ASDs, conotruncal defects, and VSDs. It is difficult to gauge the exact level of exposure required to increase risk in these studies.

16.3.3.2 Caffeine

Caffeine has not been shown to cause CHD in humans, although animal models of *in utero* exposure to caffeine may alter cardiac function in adults [9].

16.3.3.3 Tobacco Smoke

Active, and, possibly, passive, exposure to tobacco smoking appears to increase risk for CHD, particularly conotruncal defects, although the risk is relatively low and inconsistent in a number of studies.

16.3.3.4 Other Recreational Drugs

Maternal cocaine use may increase risk for CHD, such as ASDs, AVSDs, looping defects, PPS, and VSDs. Marijuana use by the mother may increase risk for Ebstein's anomaly, tricuspid atresia, and VSDs. Paternal cocaine use increases risk for ASDs, looping defects, VSDs, and/or tricuspid atresia, and paternal marijuana use increased risk for VSDs in the BWIS [3, 10].

16.3.4 Medications

16.3.4.1 Antibiotics

In general, antibiotic use during pregnancy is not thought to increase risk for CHD. However, sulfonamide-containing antibiotics inhibit folate metabolism and may increase risk for CHD (OR/RR = no risk or 1.8–3.4), such as CoA and hypoplastic left heart syndrome, and this risk may be decreased by folate supplementation. In addition, the antifungal agent metronidazole was associated with CHD in

the BWIS, but this finding has not been validated by other studies. In contrast, fluconazole appears to be safe during pregnancy. The risk of antiretroviral therapy (e.g., zidovudine) during pregnancy appears to be low for causing CHD. Antibiotics are generally listed under FDA Risk Category B or C, and a few common antibiotics are listed in Table 16.2.

16.3.4.2 Anticonvulsant Medications

Epilepsy may increase risk for CHD, due to the disease itself, comorbid conditions, or increased detection of CHD from increased surveillance. As a class, anticonvulsants do not increase risk for CHD, but some individual agents, such as phenytoin and valproic acid (ASDs), may increase this risk.

16.3.4.3 Antidepressant Medications

The BWIS suggested that antidepressant medications may increase the risk of CHD if used during pregnancy (OR/RR = 3.0, range 1.2–7.6), but other studies do not necessarily confirm this. For example, studies of the risk of SSRI and serotonin-norepinephrine reuptake inhibitor use during pregnancy are very mixed. Fluoxetine (VSDs), paroxetine (ASDs, VSDs, right ventricular outflow tract obstruction (RVOTO)), and sertraline (ASDs, VSDs) have been found to cause CHD in some studies, but many other studies have not demonstrated significant risk. Furthermore, a recent cohort study using a nationwide Medicaid database containing almost one million pregnant women found no significant risk of CHD in women taking antidepressants during the first trimester of pregnancy compared to other women with depression who did not take these drugs [7]. This lack of risk was seen in the group in aggregate and for individual drugs and drug classes. This included a lack of association of paroxetine or sertraline use with RVOTO or VSDs, respectively [7].

The association of tricyclic antidepressant use during pregnancy with CHD is also inconsistent. In addition, lithium use during pregnancy is thought to increase risk for CHD, particularly Ebstein's anomaly, despite questions about the magnitude of this risk. Finally, maternal depression itself may increase the risk for delivering a child with CHD, for reasons discussed in the last section.

16.3.4.4 Antihypertensive Medications

Studies of the association between various antihypertensive medications, including angiotensin-converting enzyme inhibitors and beta-blockers, during pregnancy and CHD give inconsistent results, and any CHD associated with these agents could be due to the underlying hypertension.

16.3.4.5 Anti-inflammatory Agents

Reports on the risk of CHD when mothers have taken aspirin, ibuprofen, and other nonsteroidal anti-inflammatory drugs (NSAIDs) have been inconsistent, although associations with AVSDs, bicuspid aortic valves, conotruncal defects, interrupted aortic arch, and VSDs have been reported. In addition, the use of a number of NSAIDs in late pregnancy has been associated with premature closure of the ductus arteriosus and conversely with PDAs. Neither maternal acetaminophen nor corticosteroid use appears to increase risk for CHD.

16.3.4.6 Retinoic Acid

Animal and human studies demonstrate that vitamin A analogs affect cardiac development. Retinoic acid agents, even most topical formulations that may be used to treat acne, are listed in FDA Risk Category X. In contrast, beta-carotene use seems to be safe during pregnancy.

16.3.4.7 Sedatives/Narcotics

The small amount of data available on the effects of sedatives and narcotics during pregnancy on CHD risk is inconsistent.

16.3.4.8 Thalidomide

The use of this agent as an antiemetic in the 1960s led to congenital heart defects, particularly ASDs, conotruncal defects, and VSDs. Its use is contraindicated during pregnancy.

16.3.5 Chemical Exposures

16.3.5.1 Air Pollution

The risk for CHD in the children of women exposed to air pollution, particularly carbon monoxide, is unclear, although animal and human studies suggest that such a risk may exist. This risk may be due to the molecules in the polluted air or the particulate matter, to which the molecules can bind.

16.3.5.2 Organic Solvents and Other Exposures

Exposure to other environmental agents that may be cardiac teratogens, such as chlorinated molecules, heavy metals, hazardous waste, organic solvents, and radiation, is inconsistently associated with the development of CHD. For example, organic compounds may increase risk for conotruncal defects and VSDs, but this risk has not been seen in all studies. It is not clear yet if xenoestrogens such as phthalates and bisphenol molecules increase risk for CHD, and some studies show more risk after paternal exposure compared to maternal exposure.

16.3.5.3 Pesticides

Gestational exposure to pesticides may increase risk of CHD, but results are inconsistent. Associated lesions include conotruncal defects, total anomalous pulmonary venous return (TAPVR), and VSDs.

16.4 Potential Signaling Pathways Affected by Environmental Agents

Understanding how the environmental agents discussed above can affect cardiac morphogenesis to cause CHD is currently problematic due to a lack of both basic knowledge about the effects of these factors on the processes that control cardiac development and clinical confirmation of these effects. Over the last 30 years,

extensive research has defined many molecular mechanisms that control heart morphogenesis, and each year new mechanisms are discovered, but linking these vital pathways to environment signals has not been a major focus of research. In the following sections, a summary of how these cardiac regulatory pathways may be regulated by environmental factors is presented.

16.4.1 Extracellular and Intracellular Signaling Pathways and Transcription Factors

Even before the heart fields form in the early gastrulating embryo, extracellular and intracellular signaling pathways feed into transcription factor networks to regulate the specification, proliferation, differentiation, migration, and function of cells in the heart (Chaps. 11 and 12). Although effects of environmental stimuli on these pathways during cardiac development have received little attention, some published data in animal models suggest that these cascades can be regulated by environmental factors to cause CHD.

16.4.1.1 Hypoxia Signaling Pathways

Altered oxygen content (hypoxia, hyperoxia) has been shown to control cardiac morphogenesis, proliferation, and differentiation from early development to the postnatal period via a number of signaling pathways, the most well known being hypoxia-inducible factor (HIF) signaling [11, 12]. Hypoxia and hyperoxia can also increase oxidative stress, which also feels back to control the HIF pathway (see below). Pregnancy at high altitude may directly control hypoxia signaling, while secondary effects on these pathways may occur due to environmental factors that alter oxidative stress or mitochondrial function. However, no specific link between environmental factors and hypoxia signaling pathways has been demonstrated in human at this time.

16.4.1.2 Oxidative Stress Pathways

There is little specific human data on environmental control of reactive oxygen species (ROS) signaling in the developing heart, but treatment with antioxidants during diabetic pregnancies can prevent CHD [1]. Many environmental factors, including alcohol, diabetes, therapeutic drugs (e.g., phenytoin, thalidomide, valproic acid), heavy metals, hyperhomocysteinemia, pesticides, pollution, tobacco smoke, and xenoestrogens, may cause oxidative stress *in utero* by increasing ROS production or decreasing antioxidant defenses. These changes could affect the activity of cardiac transcription factors such as HIFs, GATA binding protein 4 (GATA4), myocyte enhancer factor 2C (MEF2C), and NK2 homeobox 5 (NKX2-5) and alter proliferation and differentiation [12, 13].

16.4.1.3 Retinoic Acid Receptor Signaling

Retinoic acid/vitamin A deficiency or abundance can affect retinoic acid receptor nuclear transcription factors that control laterality signaling pathways involving left-right determination factor (LEFTY), nodal growth differentiation factor

(NODAL), and paired-like homeodomain 2 (PITX2) as well as pathways that control myocyte patterning, proliferation, and differentiation [14].

16.4.1.4 Wnt Signaling

Alcohol, folate, lithium, and homocysteine may work via Wnt signaling to regulate/disrupt the expression of genes involved in early cardiac development [8].

16.4.1.5 Miscellaneous Pathways

Caffeine may affect adenosine and phosphodiesterase signaling to alter cardiac development [9]. Xenoestrogens and other pollutants may regulate hormone signaling pathways in early heart development [13]. Furthermore, stress or exogenous steroid treatment during pregnancy may alter maternal glucocorticoid levels, while maternal diabetes could affect embryonic/fetal insulin signaling to affect the developing heart. SSRIs could affect serotonin signaling pathways in developing myocardium and cardiac neural crest cells. NSAIDs inhibit cyclooxygenases to close the PDA and could affect other aspects of cardiac development via this pathway.

16.4.2 Physiologic Factors

16.4.2.1 Ion Homeostasis

Lithium inhibits various channels and exchangers, and other environmental agents may regulate ion homeostasis to control cellular physiology. For example, diabetes and caffeine can modify calcium release from the sarcoplasmic reticulum to alter myocyte contractility and calcium signaling pathways [9, 15].

16.4.2.2 Mitochondria

Mitochondria regulate myocyte physiology, differentiation, and embryonic survival. Hypoxia may regulate mitochondrial function as low oxygen concentrations may limit oxidative phosphorylation. In addition, hypoxia may paradoxically increase oxidative stress, which can affect mitochondrial function. Disruption of canonical Wnt signaling, via glycogen synthase kinase-3β, or other pathways could also affect mitochondrial activity. These changes in mitochondrial function can regulate ROS signaling pathways that control cardiac myocyte differentiation [16]. Finally, environmental signals converging on mitochondria may affect apoptotic and necrotic pathways.

16.4.3 Epigenetics: DNA and Histone Modifications and Non-coding RNAs

Epigenetics is the study of heritable mechanisms that regulate gene transcription without modifying the sequence of the DNA. The three epigenetic processes (DNA methylation, histone modification, and non-coding RNAs) control chromatin remodeling and mRNA processing and can regulate cardiac morphogenesis, metabolism,

and myocyte differentiation (see Chap. 15). For example, in humans, mutations in histone methylation pathways are associated with CHD [17]. Published data from other systems or work in the mature heart suggest that many of the factors listed in Sect. 14.3 control epigenetic mechanisms, but these phenomena have not been studied in detail in the developing heart.

16.4.3.1 DNA Methylation

Folate deficiency and hyperhomocysteinemia may affect DNA methylation as these molecules are essential to this pathway. *In utero* exposure to alcohol, caffeine, and hypoxia alters DNA methylation [8, 9, 18]. DNA methylation in the developing heart may also be altered by air pollution-, diabetes-, and heavy metal-induced oxidative stress and isotretinoin and thalidomide [15, 19, 20].

16.4.3.2 Histone Modification

Valproic acid inhibition of a histone deacetylase may explain some of its teratogenicity [20]. Diabetes and antidepressants (SSRI and tricyclic) also affect chromatin remodeling via effects on histone-modifying enzymes [15, 20].

16.4.3.3 Non-coding RNAs

Data suggests that air pollution, alcohol, diabetes, heavy metals, and xenoestrogens affect the expression of a number of microRNAs [15, 19]. Long non-coding RNAs have only recently been studied, so little is known about their environmental regulation.

16.4.4 Multiple Pathways May Be Involved

Finally, as should be apparent in the previous sections, a single environmental agent may affect multiple pathways that can modulate developmental processes in the heart. In fact, the signaling pathways and physiologic processes affected by environmental agents likely contribute to their epigenetic effects to create an integrated cascade.

Conclusion

Every cellular pathway that regulates metabolism, proliferation, differentiation, and cell death can be affected by many environmental factors. However, although studies in humans and animals support a role for these factors in CHD, data are insufficient. Further studies of the causative role of human environmental exposures in the pathogenesis of CHD and of the cellular mechanisms by which these factors exert these effects should be undertaken to design strategies to prevent, diagnose, and treat CHD induced by environmental signals.

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The Contractile Apparatus of the Heart

17

Ingo Morano

Contents

Abstract

In the present chapter, I will describe basic structural and functional features of the contractile apparatus of muscle cells of the heart, namely, cardiomyocytes and smooth muscle cells. Cardiomyocytes form the contractile myocardium of the heart, while smooth muscle cells form the contractile coronary vessels. Both muscle types have distinct properties and will be considered with respect to their cellular appearance (brick-like cross-striated versus spindle-like smooth), arrangement of contractile proteins (organized in sarcomeres versus non-sarcomeric organization), calcium activation mechanisms (thin-filament versus thick-filament regulation), contractile features (fast and phasic versus slow and

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tonic), energy metabolism (high oxygen versus low oxygen demand), chemomechanical energy conversion (high adenosine triphosphate (ATP) consumption and short duty ratio versus low ATP consumption and high duty ratio), excitation-contraction coupling (calcium-induced calcium release versus pharmacomechanical coupling), and molecular motors (type II myosin isoenzymes with high adenosine diphosphate (ADP)-release rate versus myosin isoenzymes with low ADP-release rates).

17.1 Introduction

Part of the work has been published in Chap. 22 [1] in "Neuroscience – From Molecules to Behavior" (Galizia and Lledo eds 2013), Springer-Verlag (with kind permission from Springer Science+Business Media). The heart is a permanently contractile organ. Each day, the human heart contracts approximately 86,000 times, pumping about 3,500–4,500 l of blood through the body and performing about 100 kJ of work. The tone of coronary vessels regulates the perfusion of the heart with blood [2].

17.2 The Myocardium

The heart consists of the epicardium, which covers the outer surface, and the endocardium, which covers the inner surfaces, with the myocardium in between. The myocardium consists of sheets of contractile cells, the cardiomyocytes, which are interconnected by electron-dense specialized cell-cell junctions, the intercalated disks, providing mechanical stability and electrical communication between neighboring cardiomyocytes.

A heart beat, or cardiac cycle, consists of a contraction period (systole) and a relaxation period (diastole) that generates ejection of blood from the heart and filling of the heart, respectively. Cardiomyocytes are cross-striated, longitudinally arranged cells (around 150 µm long and 20 µm wide). They contain a well-developed sarcoplasmic reticulum (SR), forming longitudinal vesicles and terminal cisternae that store large amounts (mM concentrations) of Ca^{2+} buffered by specific Ca^{2+}-binding proteins, in particular calsequestrin [3]. Invaginations of the sarcolemma, the T-tubules, communicate with terminal cisternae of the SR to form dyads (a single T-tubule associated with one terminal cisternae; cf. triads in skeletal muscle fibers). Calcium release channels (ryanodine receptors, RYR) are inserted into the terminal cisternae of the SR. Three RyR isoforms are expressed: RyR1 in the skeletal muscle, RyR2 in the cardiac muscle, and all three, RyR1, RyR2, and RyR3, in the smooth muscle. The RyR is a homotetramer and forms a macromolecular complex with multiple interacting partners, including anchoring proteins, protein kinases, phosphatases, and accessory regulatory proteins (triadin, junction, calsequestrin). Specific voltage gated L-type calcium channels (dihydropyridine

receptors, DHPR) are located mainly in the T-tubular membrane of the sarcolemma. DHPR comprise a voltage-sensing and pore-forming α1s subunit and four accessory subunits called α2/δ, ß, and γ subunit. In contrast to skeletal muscle fibers, DHPR do not directly bind to RYR2 in the terminal cisternae of the SR. There is a small space of 0.05–0.2 µm between DHPR and RYR2 called the "dyadic cleft."

Cardiomyocytes contain myofibrils, i.e., tubular cross-striated contractile structures about 1 µm in diameter that extend along the entire length of the cardiomyocyte (around 150 µm). The myofibrils have regions with high birefringence (anisotropic "A-bands") and regions with lower birefringence, i.e., isotropic "I-bands" around the Z-lines and "H-bands" in the middle of the A-bands around the M-lines. The distance between Z-lines is called a sarcomere with a length of approximately 2 µm in the resting cardiomyocyte (Fig. 17.1).

Sarcomeres consist mainly of longitudinally arranged thin (actin) filaments (polymerized G-actin molecules, around 1 µm in length, 50 nm thick), thick (myosin) filaments (polymerized myosin molecules, around 1.6 µm in length, 100 nm

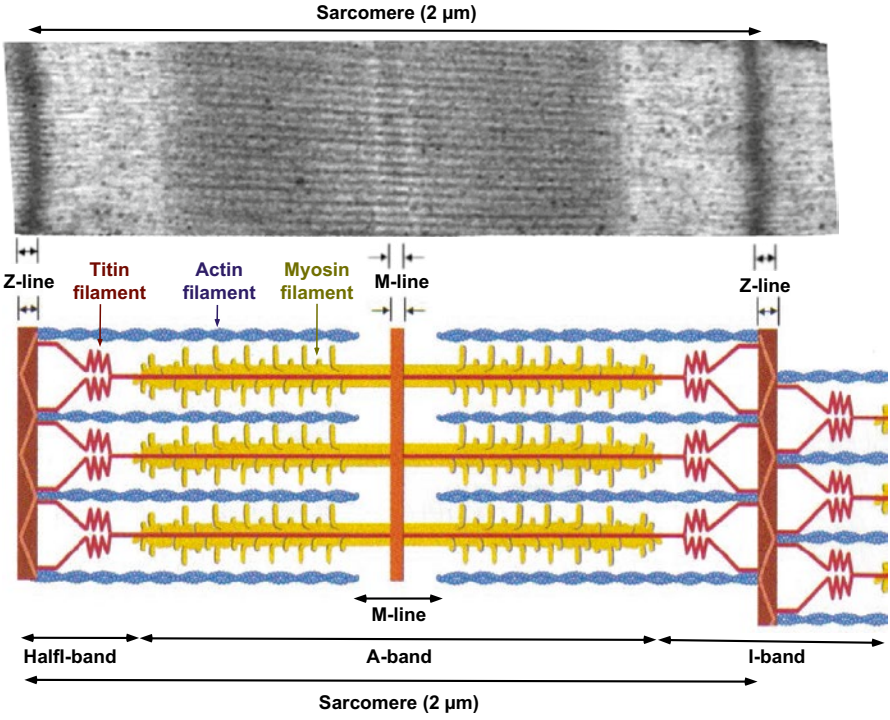

Fig. 17.1 Structure of a cardiac sarcomere. *Top*: electron microscopy of a cardiomyocyte sarcomere (Photograph Sh. Mahmoodzadeh 2014, with permission). *Bottom*: schematic interpretation (from: Chapter 6 "Kontraktionsmechanismen", Linke and Pfitzer; in: "Physiologie des Menschen" 2011; Schmidt, Robert F., Lang, Florian, Heckmann, Manfred (eds); Springer-Verlag, with kind permission from Springer Science+Business Media). *Abbreviations*: *A-band* anisotropic band, *I-band* isotropic band, *H-zone* "helle Zone" (bright zone), *M-band* middle band

thick), and filaments made of titin (Fig. 17.1). Actin filaments are anchored at the Z-lines, and myosin filaments are centered within the sarcomeres, vertically interconnected by the central M-line. Titin (also called connectin) is a giant elastic protein with spring-like elements in the I-band region, roughly 3900 kDa and 1 μm in length, which directly connects Z- with M-lines of the sarcomere. The actin filament mainly is associated with filamentous tropomyosin as well as troponin complexes. The Z-line with its characteristic zigzag structure (Fig. 17.1) is predominantly composed of globular α-actinin but contains many additional structural and regulatory proteins (e.g., CapZ, desmin, myotilin, telethonin). Costameres are protein assemblies that align the Z-lines of subsarcolemmal myofibrils with the dystrophin-glycoprotein complex (DGC) at the sarcolemma via cytoskeletal actin filaments. Thus, costameres couple force generation of myofibrils with the sarcolemma in striated muscle. The DGC consists of a cytoplasmic syntrophin complex, a transmembrane sarcoglycan complex, and a transmembrane/extracellular dystroglycan complex, which binds to the extracellular matrix protein laminin. The cytoplasmic rod-shaped huge protein dystrophin anchors cytoskeletal actin filaments to the DGC complex.

The structure maintaining the thick filament and titin arrays is the M-line located in the middle of the A-band. The main proteins forming the M-line are muscle-specific creatine kinase, myomesin 2 (M-protein), and myomesin 1.

17.3 The Myosin Filament

According to the sequence of their motor domain, myosins recently were classified into 35 classes [4]. Those myosins which form dimers and assemble into thick filaments are called conventional or type II myosins and were first roughly prepared and named by W. Kühne in 1863 [5].

Type II myosins (constituting around 35 % of the total skeletal muscle protein) are the motor proteins of all muscle types. They are composed of two heavy chains (MYH). Each MYH associates with two different light chains (MLC), the essential (ELC) and regulatory (RLC) light chains.

The MYH (e.g., chicken skeletal MYH = 1938aa, ≈200 kDa) can be proteolytically cleaved into a C-terminal 150 nm alpha-helical rod domain and a pear-shaped ≈ 20 nm N-terminal head domain with associated MLC, called subfragment 1 or S-1. Proteolytic cleavage of the myosin rod domain yields subfragment 2 (S-2) and light meromyosin (LMM). They contain multiple heptad-repeat sequences, allowing dimerization to form a coiled-coil superstructure. S-1 and S-2 together are termed heavy meromyosin (HMM). Limited proteolysis of S-1 produces (from N- to C-terminus) a 25 K, a 50 K (with a large cleft and the actin-binding sites), and a 20 K domain, the converter domain, and the α-helical lever arm. The ATPase binding site forms a cleft at the 25 K/50 K interface. The lever arm contains two IQ motifs in tandem, namely, IQ1 for ELC binding and IQ2 for RLC binding [6]. Full-length ELC is designated as alkali 1 (A1), while alternatively spliced ELC forms with the N-terminal 46aa deleted are termed alkali 2 (A2). The C-terminus of A1 with its four EF-hand domains binds to the myosin lever arm while its antenna-like N-terminus interacts with the thin filament.

The coiled-coil rod domains of myosin associate to form end-polar (striated muscles) or side-polar (smooth muscle) myosin filaments. Each end-polar myosin filament (≈1.6 μm in length, 100 nm in diameter; thick filament) contains about 300 myosin molecules. The myosin heads of end-polar thick filament project in a helical array with a periodicity of 42.9 nm and an axial interval of 14.3 nm – except for a 0.15 μm bare zone, where there are only overlapping myosin rods. The myosin heads bind as "cross-bridges" (XB) to specific sites on the actin filament for contraction generation. The myosin filament is associated with myosin-binding protein C (C-protein) at regular intervals.

17.4 The Actin Filament

Actin (which comprises about 19 % of total skeletal muscle protein) is a globular protein (G-actin) with a 5.5 nm diameter and molecular weight of 42 kDa. G-actin can be divided into four subdomains with a central ATP bound. G-actin molecules polymerize under physiological conditions to form a helical filamentous structure (F-actin) with a length of 1 μm and diameter of 50 nm (thin filament), each actin with a central ADP bound. In particular, subdomain 1 which contains both the N- and C-termini of actin is located at the periphery of the thin filament and available for myosin interactions. F-actin appears by electron microscopy as two twisting strands of globular subunits. The axial separation of the crossover points is about 35 nm; thus every 7th actin has the same orientation.

F-actin is anchored to the Z-line by its plus end (i.e., the filament site of preferential growth) and binds to the capping protein tropomodulin at its minus end. Myosin-S1 forms arrowhead complexes with actin filaments which point to the minus end (i.e., toward the center of the sarcomere). Hence, type II myosin is termed a minus-end-directed motor. The final thin filament contains about 200 actin monomers associated with regulatory troponin-tropomyosin complexes (ca. 30 tropomyosin molecules and ca. 30 troponin complexes) regularly spaced along the thin filament.

Tropomyosin (Tm) consists of two α-helical polypeptide chains, each of ca. 35 kDa, forming a ≈42-nm-long coiled coil. Tm molecules aggregate end to end to form continuous strands running along the actin filament. Interactions between tropomyosin and actin are electrostatic.

The troponin (Tn) complex consists of three components, TnI, TnT, and TnC, with a total molecular weight of ≈80 kDa. Tn complexes are located every 35 nm on F-actin (i.e., every seven actins along the long pitch helix), probably at the sites of overlap between neighboring tropomyosin molecules. TnT (≈36 kDa) is a rod-like protein (18.5 nm long) which binds with its C-terminal half to both TnI and TnC and with its N-terminal half to both Tm and actin. TnI (≈24 kDa) holds the Tn complex together by binding to both TnC and actin. Cardiac TnI has a ≈30aa N-terminal extension with several phosphorylation sites. TnC (≈18 kDa) is the Ca^{2+} sensor of the contractile apparatus. It has a dumbbell-like shape with an N- and a C-terminal lobe. Binding of Ca^{2+} to TnC at the low-affinity sites at the N-lobe (two sites in skeletal, one site in cardiac TnC) represents the physiological trigger

regulating contraction. The C-lobe of TnC (containing two high-affinity Ca^{2+}/Mg^{2+} binding sites) interacts with TnI and TnT.

In addition to the regulatory Tn-Tm complexes, F-actin associates with nebulin filaments (skeletal muscle ~700 kDa) or its smaller cardiac homologue nebulette (~100 kDa). Nebulin binds to actin and Tn-Tm complexes and is anchored to the C-terminus of the Z-disk, where it binds via its N-terminus to tropomodulin. Nebulin and nebulette act as molecular rulers that determine thin filament length and may also modulate actomyosin interactions.

17.5 Cardiomyocyte Contraction

Cardiomyocytes have characteristic passive as well as active properties. Passive elasticity, i.e., tension generated upon stretch during rest, mainly depends on the elastic titin filaments. Furthermore, compression of titin spring elements during sarcomere shortening may provide the restoring force that sets the sarcomere length to resting levels when activation ceases.

An activated cardiomyocyte contracts, i.e., generates force and shortens with a load. Force generation without shortening is termed isometric and shortening at constant load/force generation, isotonic. The energy for muscle contraction comes from the hydrolysis of ATP. Hydrolyzed ATP can rapidly be resynthesized by different metabolic processes. ATP could effectively be resynthesized by transfer of P_i from phosphocreatine to ADP through the activity of creatine kinase (Lohmann reaction). Adenylate kinase (or myokinase) produces ATP by conversion of two ADP molecules to one ATP and one AMP molecule, which is removed by AMP deaminase to form inosine monophosphate and NH_4. In the heart, ATP is mainly synthesized through aerobic metabolism, i.e., conversion of glucose, fatty acids, and lactate to acetyl CoA and subsequent oxidation to CO_2 and H_2O in the mitochondria (Krebs cycle, electron transport chain). Hence oxygen consumption of the heart (around 0.1 ml/g/min) is high, accounting for approximately 10 % of the total oxygen consumption of the body.

Mechanochemical energy transformation is accomplished by cyclic interaction of the cross-bridges (XBs) with the thin filament. During this process, the XBs bind independently, generate force through a conformational change (the power stroke), and detach from the thick filaments (the cross-bridge theory; [7]). Recent X-ray crystallographic 3-D analysis of myosin-S1 fixed at different states of the XB cycle revealed the domain movements. According to a recently modified model [8] (Fig. 17.2), ATP binds to the ATP-binding cleft of the myosin XB (M), which causes the rapid detachment of the XB from the actin (A) filament (A + M-ATP). ATP is hydrolyzed, forming the M-ADP-Pi state (pre-power-stroke state, lever arm up, 50 K-cleft open). The M-ADP-Pi state weakly attaches to actin through ionic interactions (A-M-ADP-P_i). Releasing Pi then forms a strong stereospecific actin-binding state required for the power stroke of the XB (A-M-ADP). The power stroke is executed by movement of the myosin lever arm by ≈10 nm (lever arm down, 50 K-cleft closed). Detachment of the force-generating XB from the actin filament is achieved by the release of ADP and binding of ATP and restoration of the lever

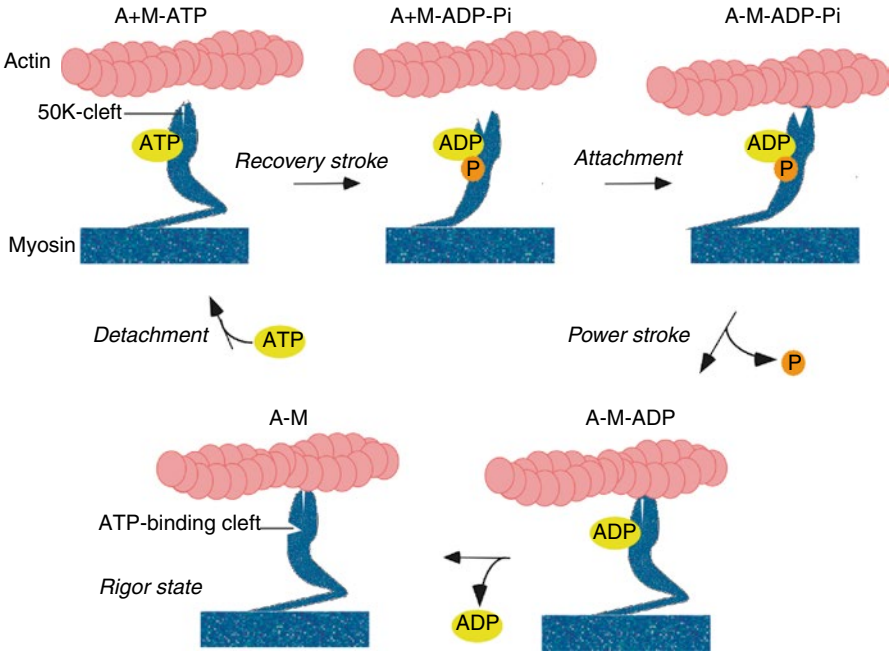

Fig. 17.2 Simplified scheme of chemomechanical coupling. A–M: the myosin cross-bridge is bound in a nucleotide-free force-generating state to actin (rigor position; lever arm "down," 50 K-cleft closed). A+M-ATP: ATP binds to myosin which detaches from actin. A+M–ADP–Pi: ATP is quickly hydrolyzed moving the lever arm "up," 50 K-cleft open (recovery stroke). A–M–ADP–P_i: myosin attaches to actin in a non-force-generating pre-power-stroke state (lever arm up, 50 K-cleft open); A–M–ADP: upon release of *Pi*, myosin is shifting into its force-generating state (power stroke), with the lever arm "down," 50 K-cleft closed. A–M: Release of ADP forms the transient rigor state; rapid binding of ATP to the active site of the myosin cross-bridge then causes the rapid detachment of the A–M into the A+M–ATP state. *Abbreviations*: *A* actin, *M* myosin, *Pi* inorganic phosphate, *ATP* adenosine triphosphate, *ADP* adenosine diphosphate

arm of the XB to its non-force-generating position (recovery stroke). In the absence of ATP, the nucleotide-free XBs remain strongly bound to the actin filament producing a rigor mortis state (A-M; lever arm down, 50 K-cleft closed; the swinging lever arm model [9]). Crystal structures have also shown that a small conformational change in the ATP-binding domain of the XB (movement of 0.5 nm) is amplified into the ≈10 nm movement of the lever arm.

17.5.1 Isometric Contraction

Isometric force generation of a muscle cell (F_0) during steady-state contraction depends on the number of XBs in the force-generating state and the force a XB can exert (F', ≈6 pN). Since XBs independently interact with the thin filament during muscle activation, only a fraction "n" of the total amount of actively cycling XBs

(n_{tot}) generates force at any one time. "n" therefore depends on the XB-kinetics, i.e., the rates of transitions of XBs from force- into the non-force-generating states (detachment rate; g_{app}) and the rate of transitions of XBs from non-force- into force-generating states (attachment rate; f_{app}), namely:

$$n = f_{app} / \left(f_{app} + g_{app} \right) \text{ [7, 10] and therefore } F_0 = F' \cdot n_{tot} \cdot \left[f_{app} / \left(f_{app} + g_{app} \right) \right].$$

There is a bell-shaped dependency of force generation on the muscle/sarcomere length. The optimal force is elicited at resting sarcomere length, i.e., around 2 μm, while smaller or larger sarcomere lengths gradually decrease force generation. The level of thick and thin filaments overlaps, and the resulting different amounts of force-generating XBs provide the structural basis for this observation [11]. The heart is working on the ascending limb of the length-tension curve. An increased filling volume of the heart, which sets the sarcomeres into a more optimal filament overlap zone, could therefore be acutely compensated by an increased force generation and stroke volume (Frank-Starling mechanism). In addition, Ca^{2+} sensitivity of the myofilaments increases upon stretching the sarcomeres, supporting the increased force and ejection volume after increased diastolic filling.

17.5.2 Isotonic Contraction

Cardiomyocytes shorten by the relative sliding of thick and thin filaments, and the distance between Z-lines (the sarcomere length) decrease (the sliding filament model; [12]). The velocity of shortening depends on the load in a characteristic hyperbolic mode [13]. Power and efficiency curves can be calculated from the force-velocity relationship, showing a characteristic bell-shaped function with maximal efficiency at around one third of maximal shortening velocity and zero efficiency at maximal isometric force generation or maximal shortening velocity.

If an isometrically contracting muscle is allowed to shorten quickly (1 ms; "quick release"), there is an instantaneous decline of tension due to the discharge of the elastic element of the force-generating cross-bridges. A quick release of roughly 1 % of muscle length at optimal overlap (which equals a step size of around 10 nm per half-sarcomere) causes a complete drop to zero force. Larger shortening distances then compress elastic spring components of the XBs, generating force in the reverse direction. The fraction of compressed ("negative") XBs increases that of pulling ("positive"), i.e., force-generating XBs decreases with increasing shortening velocities. To allow fast shortening, the detachment rate of negative XBs ("g2") – and consequently ADP release rate and ATP consumption – should be higher during shortening than during isometric contraction. As a consequence, the maximal shortening velocity (V_{max}) of a muscle depends on "g2." In fact, the different V_{max} values of fast and slow muscles types result from distinct gene expression of myosin isoenzymes with higher and lower ADP release rates, respectively. Attenuated force-bearing capacity and increased ATP consumption during muscle shortening could explain both the hyperbolic force-velocity relationship and the increased heat

released during shortening (Fenn effect). Activated muscles produce heat and work. The work performed by an XB is around 22 kJ/mol. Since the free energy change of ATP splitting is about 50 kJ/mol, the mechanical efficiency of an XB is roughly 50 %. The lower efficiency of the whole muscle compared to the XBs is due to additional ATP consumption processes, mainly Ca^{2+} sequestration into the SR by ATP-driven SERCA, sarcolemmal Ca^{2+} pumps, and Na^+/K^+ ATPase (c.f. below).

Biochemical and structural studies lead to the introduction of the steric block model [14, 15]. In the absence of Ca^{2+}, the Tn-Tm complex associated with the thin filament blocks XB binding to the thin filament while allowing some non-force-generating electrostatic XB interactions (off state). Activating Ca^{2+} levels bind to the regulatory sites of TnC located at its N-terminal lobe. This strengthens the TnC-TnI and TnC-TnT interactions, thereby weakening the binding of TnI to actin. The subsequent changes of conformation and location of the Tn-Tm complex on the thin filament relieves myosin-binding sites on actin and turns the thin filament into the on state, which allows force-generating stereospecific interactions with the XBs.

17.6 Excitation-Contraction Coupling of Cardiomyocytes

Compared to nerve fibers or skeletal muscle fibers, the action potential of a cardiomyocyte is long, roughly 200–300 ms, almost the duration of a twitch contraction. Hence, the cardiomyocyte cannot readily generate tetanic contractions.

Action potentials of cardiomyocytes start with a rapid depolarization caused by gating of the voltage-operated Na^+ channels and a subsequent characteristic long-lasting plateau phase due to a slow Ca^{2+} inward current through the α1ca subunit of voltage-operated L-type Ca^{2+} channels (DHPR). Roughly 20–40 DHPR and 100–200 RyR2s are clustered, forming functional couplons. The small Ca^{2+} inward current during the action potential passes the dyadic cleft and triggers the release of large amounts of Ca^{2+} from the SR through RyR2 (Ca^{2+}-induced Ca^{2+} release; CICR; [16]). CICR occurs upon binding of low Ca^{2+} concentration (pCa 6.25) to high-affinity sites of the RyR2; inactivation occurs by high Ca^{2+} (pCa 5.5) through binding of Ca^{2+} to low-affinity site of RyR2. Inactivation of Ca^{2+} release is also facilitated by calmodulin, which increases RyR2 close times. Phosphorylation of RyR2s by Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and by cAMP-dependent protein kinase (PKA), i.e., upon sympathetic stimulation, increases Ca^{2+} release from RyR2.

Activation of the heart by depolarization and generation of action potentials starts in specialized pacemaker cells of the sinoatrial node (SA). SA cells express a specific type of cAMP-gated Na^+ channels which opens at resting membrane potentials ("funny channel", HCN channel) and a T-type Ca^{2+} channel which opens at more negative membrane potentials than the L-type Ca^{2+} channel. Those channel activities slowly depolarize the resting membrane potential up to the threshold potential, thus spontaneously eliciting an action potential. Electrical impulses then propagate between cardiomyocytes by a special class of ion channels, connexones, each made up of 6 connexin molecules (gap junctions or nexus) of the intercalated disks. The heart therefore represents a functional syncytium.

During systole, free cytosolic Ca^{2+} rises to about 600 nM, which only half maximally activates the myofilaments, generating half-maximal force (for ventricular pressure) and shortening (for blood ejection). Thus, there is a large contractile reserve which could be recruited by increasing activating Ca^{2+} concentrations, e.g., via sympathetic stimulation (c.f. below). Activation of the myofilaments of the heart by Ca^{2+} via binding to troponin C, conformational changes of the regulatory troponin-tropomyosin system, chemomechanical energy transformation by the ATP-driven interaction between actin filaments and XBs, and the sliding filament mechanism are similar to that of skeletal muscle [17]. Repolarization of the sarcolemma upon Na^+-channel inactivation and increased K^+-outward conductance inactivates the couplons. Eliminating Ca^{2+} from the myoplasm then elicits diastolic relaxation. In particular, the sarcoplasmic-endoplasmic reticulum Ca^{2+} ATPase (SERCA), a Ca^{2+}-regulated ATP-dependent Ca^{2+} pump, sequesters myoplasmic Ca^{2+} into the SR. In addition, a Na^+/Ca^{2+} exchanger (NCX) in the sarcolemma and sarcolemmal Ca^{2+} pumps (PMCA) eliminates activating Ca^{2+} from the myoplasm into the extracellular space. Decreased free cytoplasmic Ca^{2+} inactivates the myofilaments and the heart relaxes.

The contractile reserve of the myocardium may be recruited by its sympathetic innervation, which originates from the stellate ganglia and cardiac plexus, innervated predominantly from the 4th and 5th thoracic segments of the spinal cord. Binding of norepinephrine to its seven-membrane-spanning Gs-coupled ß1-adrenergic receptor activates adenylyl cyclase which rises the myoplasmic level of the second messenger cyclic adenosine monophosphate (cAMP). cAMP activates cAMP-dependent protein kinase (PKA) which phosphorylates DHPR and RyR, thus increasing their open probabilities and consequently increasing myoplasmic Ca^{2+}. Higher myoplasmic Ca^{2+} levels increase contractile force of the cardiomyocyte (positive inotropy). Increased cAMP levels activate the funny channels of pacemaker cells, thus increasing diastolic depolarization velocity to threshold potential causing an increased frequency of action potentials (positive chronotropy). Higher frequencies can raise myoplasmic free Ca^{2+} and myocardial contraction (frequency inotropy). Activated PKA phosphorylates gap junctions, which increases their open probabilities, thus improving action potential propagation (positive dromotropy). PKA-dependent phosphorylation inactivates phospholamban, a potent SERCA inhibitor, and the cardiac-specific N-terminal extension of TnI. This accelerates Ca^{2+} sequestration from the myoplasm into the sarcoplasmic reticulum and Ca^{2+} dissociation from TnC, respectively, accelerating the relaxation rate (positive lusitropy). The contractile reserve may be conserved by raising the parasympathetic vagal tone. Vagal nerve fibers originate in the dorsal efferent nuclei of the medulla oblongata and principally innervate sinoatrial node cells. Acetylcholine binds to muscarinic G_i-coupled GPCRs of SA cells, thus decreasing myoplasmic cAMP levels via inhibition of adenylyl cyclase activity. Hence funny channel activity is reduced, thus decreasing diastolic depolarization velocity, resulting in a decreased action potential frequency (negative chronotropy) and lower myoplasmic Ca^{2+} activation levels of myocardial cells (negative inotropy).

17.7 Smooth Muscle Cells of Coronary Vessels

Coronary vessels supply the heart with blood. Their tone defines the perfusion rate of the heart and directly corresponds to the contractile state of the smooth muscle cells of the media of the coronary vessels. Smooth muscle cells are spindle shaped, nonstriated, and 5–50 μm wide and 50–500 μm long. They show no cross-striations since the thin and thick filaments of their myofibrils are not arranged in sarcomeres. Instead, actin filaments are fixed by dense bodies, a Z-band analogue within the cells, and anchored to the sarcolemma by dense (adhesion) plaques. The sarcolemma forms small (100 nm) flask-shaped invaginations, called caveolae, which have important functions in signal transduction.

Two classes of smooth muscle, "single-unit" (or "unitary") and "multi-unit" smooth muscle, can be defined on the basis of their membrane properties and innervation [18]. Blood vessels are composed of multi-unit smooth muscle types which have no pacemaker cells and are only sparsely interconnected by gap junctions but are densely innervated by sympathetic nerve fibers. Each smooth muscle cell is activated independently upon nerve stimulation (neurogenic regulation). In contrast, organs composed of single-unit smooth muscle cells (e.g., intestine, urinary bladder, and uterus) have specialized smooth muscle cells which act as pacemaker cells, i.e., they spontaneously and rhythmically generate depolarizations (myogenic regulation). Electrical impulses of single-unit smooth muscle cells then propagate between smooth muscle cells by gap junctions electrically connecting the neighboring smooth muscle cells. Therefore, the single-unit type is syncytial and its contractile state is modulated by sympathetic and parasympathetic nerve fibers.

Contraction of smooth muscle cells generally operates by a Ca^{2+}-dependent sliding filament mechanism but shows distinct activation properties when compared to striated muscles. Thus, smooth muscle activation is elicited by a thick-filament activation mechanism (phosphorylation of the regulatory myosin light chains) rather than by the troponin/tropomyosin-bound thin-filament activation (steric block) mechanism of striated muscle. The maximal shortening velocity of smooth muscle cells is slow, roughly 1/10 of the maximal shortening velocity of striated muscle. Smooth muscle cells contain only 1/3 of the myosin concentration found in striated muscle, but they generate similar forces.

Three myosin heavy chain (MYH) genes are expressed in smooth muscle cells (SM, NMA, NMB) forming a large number of different isoenzymes by alternative splicing mechanisms. All myosin II isoforms expressed in smooth muscle cells bind ADP with stronger affinities than striated muscle myosins. Therefore, the detachment rate, g_{app}, of myosin II isoforms in smooth muscles is lower than those myosin II isoforms expressed in the striated muscle. Smooth muscle myosins thus remain for a longer fraction of their XB cycle time in the force-generating state (the duty cycle is high). Hence, the fraction of actively cycling XBs in the force-generating state increases, explaining the high force generation with little myosin expression. Another consequence of the high ADP affinity and small g_{app} of smooth muscle myosins is that maximal shortening velocity becomes very low.

In contrast to cardiomyocytes which periodically contract and relax, smooth muscle cells of coronary vessels generate a permanent energy-consuming contractile tone. This is facilitated because during sustained activation, smooth muscle cells generate a unique state of high-efficiency contraction, referred to as the "latch state" [19]. Intracellular Ca^{2+}, RLC phosphorylation, ATP consumption, and maximal shortening velocity significantly decrease during latch, while force is maintained. A number of mechanisms, including the formation of slowly cycling dephosphorylated cross-bridges, ADP affinity of smooth muscle myosin, as well as activation of NM-MYH [20], have been proposed as putative latch mechanisms.

17.8 Excitation-Contraction Coupling of Mammalian Smooth Muscle

Smooth muscle cells may be activated by electromechanical and/or pharmacomechanical coupling, which access distinct intracellular pathways for raising activating free myoplasmic Ca^{2+}.

During electromechanical coupling, entry of extracellular Ca^{2+} through the L-type Ca^{2+} channels directly activates the contractile machinery and elicits Ca^{2+} release from the sarcoplasmic reticulum by a Ca^{2+}-induced Ca^{2+} release mechanism which opens the RyRs (predominantly RyR2).

Although a rise of free Ca^{2+} triggers smooth muscle contraction, the Ca^{2+} receptor is calmodulin rather than troponin C (c.f cardiomyocytes). The Ca^{2+}-calmodulin complex activates myosin light chain kinase (MLCK), which specifically phosphorylates Ser19 of the regulatory myosin light chain (RLC) with 20 kDa (MLC_{20}) of the myosin II isoforms expressed in smooth muscle cells. The two heads of smooth muscle myosin with unphosphorylated MLC_{20} show an asymmetrical interaction in which the ATPase and actin-binding domains of one head are blocked, with a very low ATPase activity which is only very weakly activated by actin and adopts a compact, soluble 10S form (S = Svedberg units) ("tonomyosin"). Phosphorylation of Ser19 of MLC_{20} converts the smooth muscle myosin into an open less-soluble 6S conformation with high ATPase activity which assembles into thick filaments and generates contraction. In contrast to striated muscle, which forms end-polar thick filaments, myosins in smooth muscle cells assemble into side-polar thick filaments. Dephosphorylation of MLC_{20} by a specific MLC_{20} phosphatase (MLCP) enforces the relaxation of smooth muscle cells. MLCP is a PP1-type phosphatase composed of three subunits: the 38 kDa catalytic subunit (PP1c), the 110 kDa myosin phosphatase target subunit (MYPT1) which plays an important role in targeting MLCP to myosin filaments, and the 20 kDa (M20) subunit [21, 22].

Pharmacomechanical coupling occurs upon neurohumoral stimulation and comprises distinct mechanisms that regulate myoplasmic Ca^{2+} of smooth muscle independently of the membrane potential. Binding of norepinephrine to its alpha-adrenergic receptor at the sarcolemma of smooth muscle cells activates heterotrimeric Gq/11-proteins which in turn stimulate phospholipase C-ß activity and

phosphatidylinositol turnover. This leads to the formation of the second messengers. IP3 triggers Ca^{2+} release from the sarcoplasmic reticulum by binding to IP3 receptors (IP3R). DAG activates PKC, which phosphorylates a variety of proteins involved in signal transduction, contraction, and membrane polarization. For example, PKC phosphorylates CPI-17, a 17 kDa phosphatase inhibitor, which inhibits the catalytic subunit of MLCP. Inhibition of MLCP activity leads to an increase of the fraction of force-generating myosin with phosphorylated MLC_{20} and therefore increases of force generation. Since inhibition of MLCP activity increases force without affecting Ca^{2+} activation levels, it acts as a Ca^{2+} sensitizing mechanism. Inhibition of MLCP can also be achieved by activation of Rho-kinase through the small monomeric G-protein Rho-A. Active Rho-kinase inhibits MLCP activity by phosphorylation of its regulatory subunit [23].

Conclusion

In conclusion, contraction regulation of cardiomyocytes and smooth muscle cells of the heart represents complex, Ca^{2+}-driven interactomes of a variety of protein complexes in distinct cellular compartments which are still incompletely characterized. Understanding the contractile interaction networks of muscle cells provide the prerequisite for rational therapeutic interventions of cardiac diseases. Thus, familial cardiomyopathies are mainly associated with mutations affecting genes coding for proteins involved in contraction regulation of cardiomyocytes, i.e., myofibrillar, plasmalemmal, or Ca^{2+} handling proteins. Intense future work is required to understand the molecular pathomechanisms of these disease-causing mutations in the genesis of familial cardiomyopathies. Those investigations will eventually lead to the development of novel molecular therapies and biomarkers in patient-based clinical research.

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Technologies to Study Genetics and Molecular Pathways

18

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Abstract

Over the last decades, the study of congenital heart disease (CHD) has benefited from various model systems and the development of molecular biological techniques enabling the analysis of single gene as well as global effects. In this chapter, we first describe different models including CHD patients and their families, animal models ranging from invertebrates to mammals, and various cell culture systems. Moreover, techniques to experimentally manipulate these models are discussed. Secondly, we introduce cardiac phenotyping technologies comprising the analysis of mouse and cell culture models, live imaging of cardiogenesis, and histological methods for fixed hearts. Finally, the most important and latest molecular biotechniques are described. These include genotyping technologies, different applications of next-generation sequencing, as well as the analysis of the transcriptome, epigenome, proteome, and metabolome. In summary, the models and technologies presented in this chapter are essential to study the function and development of the heart and to understand the molecular pathways underlying CHD.

18.1 Introduction

Understanding the genetic alterations and molecular pathways underlying congenital heart disease (CHD) is essential to develop novel therapeutic strategies. Besides genomic mutations, CHD is characterized by multiple changes in epigenetic marks as well as in the expression and modification of RNAs and proteins. Some of these changes have strong effects on the protein structure, function, or localization, while others result in more subtle differences. Over the last decade, advanced high-throughput technologies have been developed that allow the study of CHD from single locus effects to the global level. Here, we describe the most important model systems and techniques to explore the changes in the different regulatory layers affecting cardiac function and development.

18.2 Model Systems

18.2.1 Animal Models

A large variety of animal models are used in cardiovascular research ranging from invertebrates like the nematode *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster* to mammals like the mouse *Mus musculus* and the laboratory rat *Rattus norvegicus*. Although it has no heart or vascular system, *C. elegans* has become a useful model that is easy to culture and manipulate by RNA interference or mutation. Its body wall muscle cells are well suited to gain insights into human cardiomyocytes; as they form, striated muscle and many structures and proteins like sarcomeric components are highly conserved [1]. The fruit fly is characterized by a simple tube-shaped heart, also called the dorsal vessel, which consists of a single layer of cardiomyocytes and pumps the hemolymph around the body. *Drosophila* can be used easily for genetic screens and for crossing experiments, for which several large collections of mutant stocks are available. Due to few genetic redundancies, it is a widely used model of regulatory pathways and essential players of heart development [2, 3]. For example, the important role of the cardiac transcription factor NKX2-5 (tinman) was first demonstrated in *Drosophila* [4]. However, the fly might not be well suited for studies of buffering effects, which are essential for the understanding of human cardiac disease [3].

The zebrafish (*Danio rerio*) has a two-chambered heart, and the transparency of the developing embryo allows the *in vivo* study of heart size, shape, and function [5]. Due to its small size, fecundity, and brief generation time, the zebrafish can easily be used for forward genetic perturbation screens [6] and moreover, allows the study of otherwise lethal disturbances because no functional cardiovascular system is required during embryogenesis [3]. In addition, the zebrafish represents a valuable model for cardiac muscle regeneration, as it has the ability to replace massive sections of damaged myocardium [7]. In contrast to the zebrafish, the African clawed frog (*Xenopus laevis*) has a three-chambered heart with two atria and one ventricle. Its large embryo allows surgical manipulations of the developing heart and moreover, is useful for genetic screens [8]. Like the human, the chicken (*Gallus gallus*) develops a four-chambered heart and is a powerful model for experimental embryology as its imaging during development requires no surgical procedures [9].

The two essential mammalian animal models are the mouse and the laboratory rat. The cardiovascular system of the latter shares a high similarity to human physiology and is widely used for pharmaceutical testing [10]. Recently, the genetic manipulation limits for the rat have been overcome by the development of gene-targeting approaches for gene knockout and replacement [3]. However, the mouse remains the most important model for genetic studies, and transgenic mice have become a valuable human CHD pathology model [11]. The International Knockout Mouse Consortium aims to generate a major resource of knockout mouse embryonic stem cells (ES cells). So far, more than 17,400 mutant murine embryonic stem cell clones have been generated, which provide the opportunity of systematic screens of gene functions [12]. Currently, more than 500 genes are known to be causative for heart defects when

mutated in mice, while only about 50 CHD genes are known in humans [13]. In addition, mouse strains with different genetic backgrounds offer the opportunity to study genetic buffering effects, as recently shown for NKX2-5 [14]. Finally, the mouse is also a useful model for gene expression studies, and several large-scale projects aim to systematically determine gene expression patterns during mouse embryogenesis [15].

18.2.2 Cell Culture Models

The heart comprises a mixture of different cell types including cardiac fibroblasts and cardiomyocytes. *In vitro* studies using largely homogeneous cell populations enable the analysis of spatiotemporal changes and distinct molecular pathways. Moreover, cell culture models are easier to manipulate than animal models, can be sorted for surface markers, and provide the opportunity to produce enough material for downstream experiments.

Isolated cardiomyocytes cultured in primary cell culture are one of the established models to investigate cardiac function and closely reflect *in vivo* physiology. In addition, several stable, immortalized cell lines have been generated. The HL-1 cell line, derived from mouse atrial cardiomyocytes, maintains the ability to contract, shows a gene expression pattern similar to adult cardiomyocytes [16], and can be used in tissue engineering applications (3D cell culture) [17]. In contrast, the H9C2 cell line, which was established from embryonic rat heart tissue, shows cardiac as well as skeletal muscle properties [18]. To study myogenesis, the mouse myogenic cell line C2C12 is frequently used, since it is capable of differentiation into myotubes [19]. Furthermore, embryonic cell lines like the mouse P19 embryonic stem (ES) cells [20] and the human H1 or hES2 ES cells [21] can be differentiated into mesodermal cells including cardiac and skeletal muscle cells and have been used for the quantification of cardiomyocyte differentiation [22]. Finally, the generation of cardiomyocytes from induced pluripotent stem cells (iPSCs) and the direct conversion of fibroblasts into cardiomyocytes have recently been established as additional approaches that enable the study of patient-specific cells and provide novel perspectives for regenerative medicine [23, 24].

18.2.3 Patients with CHD

Patients with CHD and their families are a unique resource to gain insights into cardiac functional properties and molecular pathways. For example, linkage analysis in CHD families has led to the identification of single-gene defects like mutations in *NKX2-5* [25] and *GATA4* (GATA binding protein 4) [26]. Several national registries like the CONCOR registry of the Netherlands or the National Register for Congenital Heart Defects in Germany aim to establish comprehensive collections of biomaterial like blood or cardiac biopsies. While genomic DNA isolated from blood can be used for genetic studies, cardiac biopsies offer the opportunity to study gene expression profiles and epigenetic mechanisms, providing insights into regulatory

relationships. Besides the direct analysis of patient material, the generation of patient-specific iPSCs offers a new and very promising possibility to study human diseases [27]. For example, the power of iPSCs has already been shown for the analysis of congenital arrhythmia and malformations [28, 29].

18.2.4 Techniques to Induce Perturbations

To enable the study of cause-effect relationships in different model systems, *in vivo* gene targeting techniques play an essential role. For example, the generation of designed mouse mutants relies on gene targeting in ES cells. Representing appropriate genetic models of inherited diseases, knockout mice with a null allele in their germline often exhibit embryonic or early postnatal lethality [30]. To study cell type or stage-specific gene functions, a system based on the DNA recombinase Cre and its recognition sites (loxP) has been established [30]. Other faster methods for targeted genome editing, which are also used in cell culture models, include transcription activator-like effector nucleases (TALENs), zinc finger nucleases (ZFN), and the recently established clustered regulatory interspaced short palindromic repeat (CRISPR)/Cas-based RNA-guided DNA endonucleases [31]. Besides the targeted perturbation of selected genes in a reverse genetics approach, random mutation screens enable the discovery of novel gene functions in an unbiased manner. For example, treatment with the supermutagen *N*-ethyl-*N*-nitrosourea (ENU) is the most potent method to induce random point mutations throughout the mouse genome. The offspring of treated animals can then be screened for autosomal dominant or recessive phenotypes and used for the identification of the causal mutation in a forward genetics approach [32].

In addition to the perturbation of the genomic DNA sequence, knockdown of a gene at the transcriptional level provides another valuable approach for studying gene functions and can be used for large genome-scale high-throughput screens. RNA interference (RNAi) is a widely used approach for screens in cell culture systems, *C. elegans* and *Drosophila* [33], while the most common antisense knockdown technique in zebrafish are morpholino oligonucleotides (MOs) [34]. Moreover, the overexpression of genes, for example, by the transfection of expression vectors, not only allows the study of wild-type gene function but also enables the analysis of mutant alleles. Finally, knockdown in combination with overexpression can be applied to perform rescue experiments to demonstrate the specificity of the observed phenotype.

Besides genetic alterations, environmental influences also play an important role in the etiology of CHD. Thus, a variety of stress models have been established to analyze gene–environment interactions. For example, a maternal high fat diet increases the penetrance of CHD in heterozygous *Cited2* knockout mice [35]. Moreover, a commonly used experimental model to induce hemodynamic stress in the mouse heart is the transverse aortic constriction (TAC), which results in a pressure overload-induced cardiac hypertrophy [36]. In cell culture models, hypertrophic agents like phenylephrine (PE) and endothelin 1 (ET1) are commonly used to study signaling pathways involved in cardiomyocyte hypertrophy [37].

18.3 Cardiac Phenotyping

18.3.1 Systematic Phenotyping of Mouse Models

The International Knockout Mouse Consortium has established thousands of mutant mouse models [12], whose characterization, archiving, and distribution are realized by phenotyping centers (mouse clinics) organized in large consortia like the International Mouse Phenotyping Consortium [38] or the European Mouse Disease Clinic (EUMODIC) [39]. The phenotyping pipelines systematically assess the stage of a potential embryonic lethality and a broad range of physiological parameters such as metabolic functions, fertility, behavior, body composition, and immune function in adult animals. Cardiac phenotyping includes the measurement of heart weight, electrocardiography, and echocardiography as well as histological analysis [38, 40].

18.3.2 Imaging Cardiogenesis in Live Animals

Embryogenesis is based on three fundamental processes, namely, growth, differentiation, and organization. Thus, *in vivo* imaging of these processes is important for understanding the structural formation and function of the heart and its molecular background. The main quantitative *in vivo* imaging techniques are optical imaging, ultrasound, micro-computed tomography (micro-CT), and magnetic resonance imaging (MRI) [41].

Confocal microscopy is a routine method to study the transparent zebrafish embryo, and multiple fluorescent transgenic lines have been established to label, for example, cardiac myocytes and endothelial cells [5]. Ultrasound is another inherently real-time imaging technique and is useful to analyze aberrant morphologies and hemodynamic phenotypes in developing embryos [41]. The small size and rapid motions of the embryo make ultrasound imaging challenging in mice and thus the hemodynamics of the dorsal aorta is often used as a surrogate for intracardiac hemodynamics. In contrast, avian embryos allow the direct measurement of chamber-specific blood flow and valve motions [41]. The highest possible imaging resolution is theoretically provided by micro-CT. However, X-rays are attenuated poorly by soft tissues and necessitate the application of contrast agents, which are mostly unsuitable for live embryonic imaging. Nevertheless, recent studies have evaluated different agents with a reduced toxicity [41]. Finally, MRI has mainly been used for the imaging of fixed murine embryos, as technical limitations hinder its application for live embryonic imaging. It offers excellent tissue contrast and the ability of three-dimensional image reconstruction [42], but high field strengths of 7–11 T and long acquisition times (6–24 h) are required for the high spatial resolution needed for embryonic imaging (25–50 μm) [41, 43]. Therefore, multiple embryos are often analyzed at once to increase the throughput of phenotyping. Live imaging applications of MRI with a reduced spatial resolution include the study of mouse embryos in utero and chicken embryos in ovo [41].

18.3.3 Histological Analysis of Fixed Hearts

Classical methods for the histological assessment of fixed hearts are hematoxylin and eosin (HE) staining for morphological inspection, in situ hybridization for transcript expression, and immunohistochemistry for protein expression. For the visualization of subcellular structures, electron microscopy is the gold standard [3].

To identify cell nuclei and cytoplasm, HE staining is the most widely used method and provides a general overview of the sample. Moreover, a large variety of special stains is available to visualize, for example, polysaccharides, glycoproteins, and glycolipids (periodic acid–Schiff; PAS stain) or collagen fibers (trichome staining) [44]. In situ hybridization is based on the binding of an approximately 20-bp-long oligonucleotide probe, usually labeled with digoxigenin or fluorescent dyes, to the complementary mRNA. In contrast, immunohistochemistry is based on the application of labeled antibodies. To reconstruct the 3D structure of embryonic hearts and to visualize cardiac gene expression patterns, techniques like high-resolution episcopic microscopy (HREM) and optical projection tomography (OPT) [45, 46] have proved to be very useful tools and enhance the phenotyping of mouse embryos. Finally, electron microscopes provide the highest resolution in the range of picometers [47], which is needed for studying the ultrastructure of a wide variety of biological and inorganic specimens.

18.3.4 Phenotyping of Cell Culture Models

The easy handling of cell culture models allows the application of various phenotyping techniques. Immunohistochemistry provides the opportunity to detect the subcellular localization of proteins within the cell under defined conditions. Moreover, co-staining with different antibodies allows visualization of protein–protein interactions and thus, is a useful method to study molecular signaling pathways. Fluorescence-activated cell sorting (FACS) combines specific immunostaining and single-cell analysis using flow cytometry. For example, in a heterogeneous cell population like blood, distinct cell types can be distinguished based on their expressed surface markers [48]. Other cell-based assays include the measurement of proliferation, viability, apoptosis, autophagy, production of reactive oxygen species (ROS), mitochondrial function, cell migration, and cytotoxicity of chemical compounds. Finally, cultures of beating cardiomyocytes are an important model for electrophysiology studies, and the patch clamp technique enables the measurement of ionic currents on isolated cells down to the single-channel level [49].

18.4 Molecular Biological Techniques

18.4.1 Genotyping Techniques

One of the first methods to identify disease genes in affected families was the mapping of genes relative to known genetic markers by linkage analysis. This method

is based on the recombination between homologous chromosomes, which occurs randomly. Thus, two genomic positions are less likely to undergo recombination if they are located in close proximity to each other. Different genetic markers including single-nucleotide polymorphisms (SNPs), microsatellites (short repeat sequences of variable length), or restriction fragment length polymorphisms (RFLPs) can be used for linkage analysis. RFLPs are sequence polymorphisms that cause differences in enzymatic cleavage sites between alleles, resulting in DNA fragments of unequal lengths that can be detected by probe hybridization [50]. For the genotyping of SNPs, microsatellites, and other short variations, direct sequencing or denaturing high-performance liquid chromatography (DHPLC) has been frequently used. DHPLC is based on the formation of heteroduplexes between chromosomes and can identify the presence but not the exact position and nature of a mismatch [51].

The gold standard for direct DNA sequencing is the Sanger method, reaching read lengths of up to 1000 bp and a per-base sequencing accuracy as high as 99.999 % [52]. It is based on the incorporation of dideoxynucleotides (ddNTPs) into the DNA that act as specific chain-terminating inhibitors of the DNA polymerase [53]. The introduction of shotgun sequencing, fluorescent labeling, and capillary gel electrophoresis significantly increased the throughput of Sanger sequencing and enabled the deciphering of the complete human genome in 2001 [54, 55]. The sequencing biochemistry is performed in a cycle sequencing reaction, which is stochastically terminated by the incorporation of fluorescently labeled ddNTPs. This results in a mixture of end-labeled products, and the final sequence is determined by electrophoretic separation of the products and laser excitation of the four different fluorescent labels [52] (Fig. 18.1a).

A variety of methods also have been developed for the detection of chromosomal abnormalities. Giemsa staining is a simple and rapid technique for conventional karyotyping and can identify many chromosomal changes including balanced chromosomal aberrations [58]. A higher resolution from tens of kilobases up to several megabases is offered by fluorescence in situ hybridization (FISH), which uses fluorescently labeled probes that hybridize to their complementary chromosomal sequences [58]. As an alternative to these microscopy-based methods, multiplex ligation-dependent probe amplification (MLPA) can be applied, which is based on a multiplexed PCR and can detect copy number changes of up to 50 different loci in parallel [58].

New possibilities for the analysis of genetic variations were provided by microarray-based genotyping, which offers high-resolution genome-wide variation detection and is based on the hybridization of a DNA sample to oligonucleotide probes that have been immobilized on a glass or silicon surface [59]. Array comparative genomic hybridization (array-CGH) is used to identify chromosomal aberrations by comparing a DNA sample to a reference sample. Moreover, DNA microarrays enable the analysis of disease-specific or even genome-wide SNP panels (SNP arrays) [58]. Thus, they allow the detection of known disease-causing mutations in individual patients or the identification of novel associations between SNPs and complex traits in genome-wide association studies (GWAS) [60].

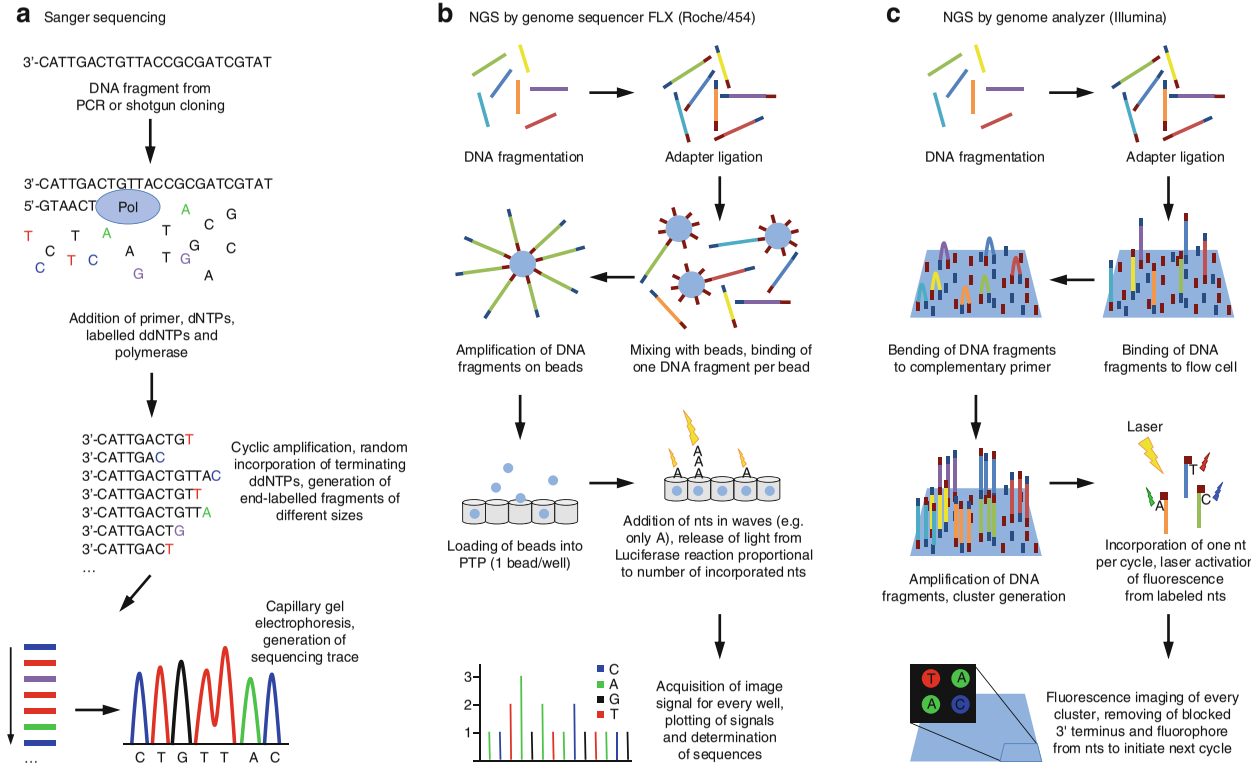

Fig. 18.1 Workflow of Sanger sequencing and two next-generation sequencing platforms. (**a**) Sanger sequencing. (**b**) Genome Sequencer FLX from Roche/454. (**c**) Genome Analyzer from Illumina. *dNTP* deoxynucleotide, *ddNTP* dideoxynucleotides, *NGS* next-generation sequencing, *nts* nucleotides, *Pol* DNA polymerase (Figure adapted from Etheridge [56], Shendure and Ji [52], and Mardis [57])

18.4.2 Next-Generation Sequencing

The development of novel high-throughput sequencing technologies has revolutionized biomedical research. These next-generation sequencing (NGS) technologies, first introduced in 2005 [61, 62], have evolved rapidly, and the costs have been reduced from $1000 per megabase to less than $0.1 in 2014 [63]. Thus, it is much more cost efficient than Sanger sequencing ($500 per megabase) and allows a higher degree of parallelization [52]. In contrast to microarrays, NGS is not dependent on DNA hybridization to preselected probes, enabling the identification of novel variations at a single-base resolution without a priori sequence information.

Different NGS platforms have been established, and the companies Roche/454 (Fig. 18.1b), Illumina (Fig. 18.1c), and Life Technologies have set the standard for high-throughput sequencing [64]. Although their systems vary in their chemistry, they are all based on the principle of cyclic-array sequencing. Here, a dense array of DNA features is iteratively enzymatically sequenced combined with imaging-based data collection [52]. In general, a sequencing run generates reads that randomly cover the genome [65]. The coverage describes the average number of times a single base is read during a sequencing run. A higher number of sequence reads result in greater sequencing depth and thus, in higher sequence confidence. For example, within the 1000 Genomes Project, the coverage ranges from low (2–6×) for whole genome sequencing to high (50–100×) for exome sequencing [66].

For the sequencing of genomic DNA, three basic approaches are available [64]. Whole-genome sequencing allows the determination of all genomic variations but is relatively cost intensive. Here, useful alternatives are provided by whole exome and targeted re-sequencing approaches, which require sequence enrichment technologies such as array-based sequence capturing. Whole-exome sequencing enables the sequencing of almost all protein-coding regions (optionally including untranslated regions or long non-coding RNAs), often combined with a high coverage. When knowledge about possible candidate regions (e.g., genes, promoters, and enhancers) and disease pathways is already available, the targeted re-sequencing of these regions is a promising option. The selection of genomic targets for re-sequencing can be based on data from previous projects like sequencing analyses, GWAS, animal models, as well as publicly available databases [64]. Moreover, disease-specific Web resources like the CHDWiki [67] and the Cardiovascular Gene Annotation Initiative, which has annotated more than 4000 cardiovascular-associated proteins [68], provide useful information for candidate gene selection.

Several large cohorts of CHD patients already are under investigation by NGS [64]. For example, the Congenital Heart Disease Genetic Network Study established by the Pediatric Cardiac Genomics Consortium enrolled more than 3700 patients with a diverse range of CHD [69], and so far, whole-exome sequencing data for a subset of 362 patients and their parents is available [70]. Having a broader focus on undiagnosed children with developmental disorders, the Deciphering Developmental Disorders (DDD) study headed by the Wellcome Trust Sanger Institute aims to recruit 12,000 patients and their parents [71]. Recently, exome

sequencing and array-CGH were performed for 1113 children and their parents, with CHD occurring in 11 % of the patients [72, 73].

18.4.3 Transcriptome and Epigenome Analysis

Both NGS and array-based technologies are extensively used for transcriptome and epigenome analysis. In addition, quantitative real-time PCR is a useful low- to medium-throughput application. The study of gene expression has been revolutionized by RNA sequencing (RNA-seq), which enables the discovery, profiling, and quantification of RNA transcripts across the entire transcriptome without prior knowledge about the probed sequences. Applications of RNA-seq comprise total RNA-seq (coding and non-coding RNA above a certain size), mRNA-seq (including mRNAs and long non-coding RNAs with a poly-A tail), and small RNA-seq (including microRNAs and other small non-coding RNAs). Novel applications of RNA-seq include *de novo* transcriptome assembly [74], single-cell transcriptomics [75], and tomography sequencing to determine spatially resolved transcription profiles in whole embryos or isolated organs [76].

A powerful technique for the genome-wide identification of protein–DNA interactions such as transcription factor binding sites or chromatin histone marks is chromatin immunoprecipitation (ChIP). In ChIP, the protein of interest is cross-linked to the DNA, either in cultured cells or in tissue samples. After cross-linking, the chromatin is sheared and an antibody is used to enrich for DNA fragments bound to the protein. Immunoprecipitation and reverse cross-linking isolate the DNA enriched in the binding sites, and finally, the enriched DNA fragments can further be analyzed by hybridization to microarrays (ChIP-chip) or NGS (ChIP-seq) [77, 78] (Fig. 18.2). If candidate target genes or potential sites are available, ChIP-qPCR represents an alternative strategy. To investigate the co-localization of proteins on the DNA, ChIP-reChIP (sequential ChIP) has been developed using two independent rounds of immunoprecipitation [80]. An alternative method used to map protein–genome interactions is DamID, which does not require the use of antibodies. This technique is based on the fusion of the protein of interest to *Escherichia coli* DNA adenine methyltransferase (dam) and the resulting methylation of adenines in DNA surrounding the native binding sites of the dam fusion partner. In most eukaryotes, adenine methylation does not occur endogenously. Thus, it provides a unique tag to mark protein interaction sites, which can further be identified by array hybridization or NGS [81].

In addition to histone modifications, DNA methylation occurring on cytosine residues in the context of CpG dinucleotides is also an important epigenetic mark. Altered DNA methylation has been shown to play a role in various diseases, including CHD [82]. Three methods are commonly used to detect genome-wide DNA methylation levels. Two techniques are based on the isolation of methylated DNA fragments by methylated DNA immunoprecipitation (MeDIP) or methyl-CpG binding domain-based (MBD) proteins. Subsequently, the enriched DNA fragments can be detected by arrays or NGS [83]. The third technique applies the treatment of

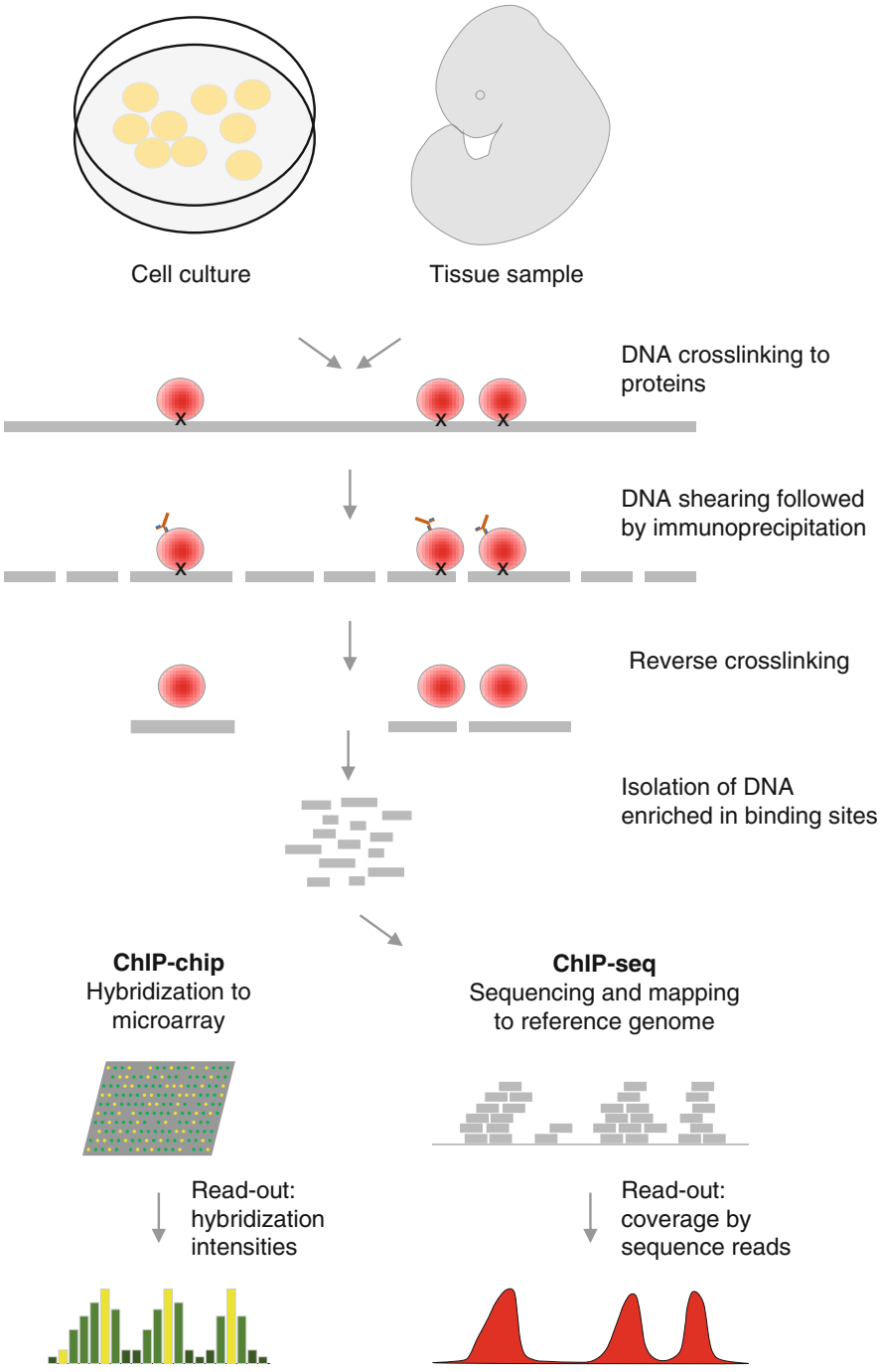

Fig. 18.2 Schematic representation of a chromatin immunoprecipitation (ChIP) experiment followed by microarray detection (ChIP-chip) or next-generation sequencing (ChIP-seq) (Figure adapted from Visel et al. [79])

DNA with sodium bisulfite, which converts all non-methylated cytosines to uracil. These will finally be detected as thymine residues, analogous to a C to T SNP, by, for example, pyrosequencing [84] or NGS.

Several techniques are available to assess chromatin structure and regulatory interactions. Chromatin that has lost its condensed structure is sensitive to cleavage by the DNase I enzyme (DNase I hypersensitive sites). Thus, the enzymatic degradation of DNA can be used to identify regions of open chromatin, representing cis-regulatory elements including promoters, enhancers, insulators, and silencers [85]. An alternative method to DNase-seq is the assay of transposase-accessible chromatin (ATAC-seq), which uses an engineered Tn5 transposase to cleave DNA in open chromatin and to integrate primer DNA sequences into the cleaved genomic DNA. Furthermore, a commonly used method to identify the exact positions of nucleosomes is the treatment with micrococcal nuclease (MNase), an endo-exonuclease that processively digests DNA until it is blocked, for example, by a nucleosome [86]. To study interactions between regulatory elements, including long-range interactions between different chromosomes, the chromosome conformation capture (3C) and various derivatives (4C, 5C, and Hi-C) have been developed. They are all based on the cross-linking of interacting DNA fragments and their subsequent restriction digest [87]. Using an additional ChIP step, chromatin analysis by paired-end tag sequencing (ChIA-PET) allows the identification of long-range interactions mediated by target proteins of interest [88].

The interaction of RNAs and proteins is also an important layer for the co-transcriptional and posttranscriptional regulation of gene expression. Genome-wide protein–RNA interaction can be identified based on ultraviolet cross-linking and immunoprecipitation (CLIP). To reach a base-pair resolution, this method was further developed to photoactivatable ribonucleoside-enhanced CLIP (PAR-CLIP), which relies on the incorporation of photoactivatable nucleotide analogues into the RNA. Here, reverse transcription results in a T to C base transition at the cross-link site, detectable as SNPs in the subsequent NGS analysis. However, the need to incorporate photoactivatable nucleotides restricts PAR-CLIP to cultured cells [89]. Finally, a highly sensitive method for the general profiling of RNA-induced silencing complexes (RISC) and individual microRNA target identification is RISC-seq [90].

18.4.4 Proteome and Metabolome Analysis

The quantitative and qualitative large-scale study of proteins (proteomics) and small-molecule metabolites such as alcohols, amino acids, and nucleotides (metabolomics) has undergone great developments over recent years. However, these new technologies have only begun to be applied in CHD research [91, 92], where they have the potential to boost our knowledge of molecular mechanisms underlying heart disease from the pharmacological viewpoint and to enable the discovery of novel biomarkers.

The core technologies for both proteome and metabolome studies are mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy. Most

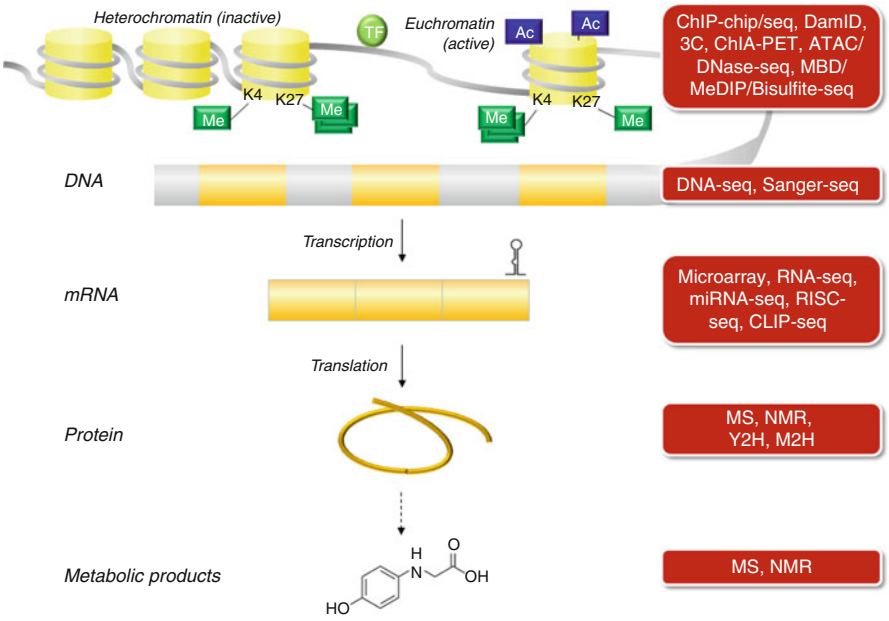

Fig. 18.3 Overview of various molecular biological techniques to study the different regulatory layers controlling gene and protein expression (Figure adapted from Lara-Pezzi et al. [104])

approaches are based on the analysis of peptides, which are frequently generated by enzymatic digestion of proteins. A key step for MS analysis is the selection and enrichment of the proteins/peptides of interest, which can be achieved by subcellular fractioning (e.g., membrane enrichment, nucleus precipitation, or mitochondria separation), by co-immunoprecipitation (e.g., for a protein and its interaction partners), or by enrichment for proteins with particular modifications (e.g., phosphorylation). Furthermore, the development of stable isotope labeling enabled the generation of relative quantitative information [93]. An important technique that can be applied to cell culture studies and more recently, also to studying mouse and drosophila models [94, 95] is stable isotope labeling by amino acids in cell culture (SILAC). Here, two cell populations are cultured in the presence of heavy or light amino acid (e.g., lysine or arginine, respectively) and are further combined for MS analysis [96]. In addition to the metabolic labeling used in SILAC, other methods have been established, including chemical (ICAT and iTRAQ) [97, 98] or enzymatic labeling (^{18}O) [99].

A common method in metabolomics is NMR, which in contrast to MS does not require analyte separation and allows the recovery of the sample for further analyses. It can provide detailed information on the molecular structure of compounds found in complex mixtures like biofluids as well as cell and tissue extracts. NMR offers a high analytical reproducibility and easy sample preparation but is relatively insensitive in comparison to MS [100].

Methods suitable for the high-throughput analysis of protein–protein interactions are the yeast-two-hybrid (Y2H) and the mammalian-two-hybrid (M2H) systems.

Both are based on the expression of the two proteins of interest, one fused to the DNA-binding domain and the other to the transactivation domain of a transcription factor, typically Gal4. The binding of the two proteins leads to the complementation of the TF, which activates the expression of a reporter gene (e.g., LacZ). For example, Y2H experiments have been used to identify a large and highly connected network comprising over 3000 interactions between 1705 human proteins [101]. Moreover, a M2H study provided a map of physical interactions within 762 human and 877 mouse DNA-binding transcription factors [102]. In addition to the two-hybrid systems, peptide microarrays have been employed to study protein–protein interactions [103]. However, they have been implemented much slower than DNA arrays due to technical challenges including the high-throughput and economic synthesis of peptides.

An overview of the various molecular biological techniques to study the different regulatory layers that control the gene and protein expression is given in Fig 18.3.

Conclusion

In this chapter, we described various model systems and biotechniques to study the different regulatory levels affecting congenital heart defects. In particular, the application of NGS techniques has revolutionized biomedical research and is still rapidly developing, enabling its application to a wide range of scientific questions. Thus, these high-throughput techniques will enhance our understanding of CHD and will hopefully accelerate the development of novel therapeutic and preventive strategies.

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Part IV

Atrial Septal Defect

Clinical Presentation and Therapy of Atrial Septal Defect

19

David J. Driscoll

Contents

19.1 Introduction

Atrial septal defects (ASDs) occur in 1 of 1500 live births and constitute 6–10 % of congenital cardiac defects. There is a female-to-male predominance of 2 to 1. There are four types of ASDs (see Fig. 19.1):

1. Ostium secundum
2. Ostium primum
3. Sinus venosus
4. Unroofed coronary sinus

The most common form of ASD is the *ostium secundum ASD*. This occurs in the region of the fossa ovalis. It results from excessive absorption of the septum primum or insufficient development of the septum secundum or both.

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DOI 10.1007/978-3-7091-1883-2_19

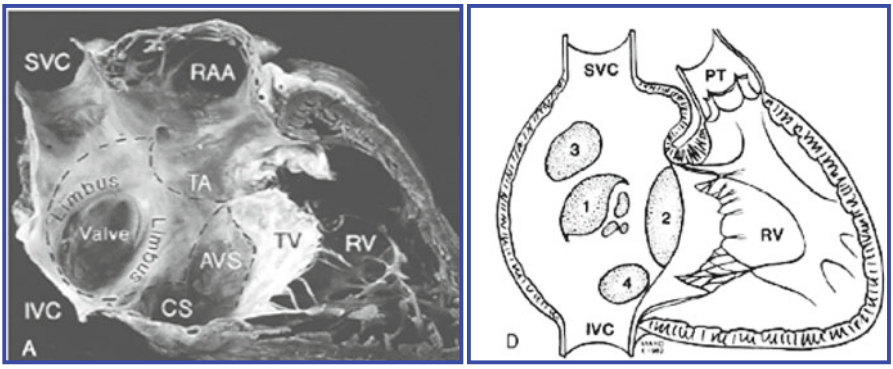

Fig. 19.1 An illustration of the location of atrial septal defects (ASD). The *left panel* is a pathological specimen of a normal heart with the anterior surface of the heart removed. Viewed from the right atrium the atrial septum is enclosed within the *dotted line*. The *right panel* is a diagrammatic representation of the pathological specimen showing the relative locations of the four types of ASD. Abbreviations: *1* ostium secundum ASD, *2* ostium primum ASD, *3* sinus venosus ASD, *4* coronary sinus ASD, *SVC* superior vena cava, *IVC* inferior vena cave, *RAA* right atrial appendage, *CS* coronary sinus, *TV* anterior tricuspid valve leaflet, *RV* right ventricle, *TA* tricuspid valve annulus, *AVS* atrioventricular septum, *valve* the valve of the inferior vena cava (Eustachian valve), *PT* pulmonary trunk (pulmonary artery) (Reproduced or adapted from Driscoll, David (2006) Fundamentals of pediatric cardiology. Lippincott Williams & Wilkins, with permission of the author and publisher)

Ostium primum ASD is a type of atrioventricular sepal defect (formerly known as "endocardial cushion defects"). It is located in the inferior aspect of the atrial septum contiguous with the tricuspid and mitral valves and will be discussed further in Chap. 25.

Sinus venosus ASDs occur in the posterosuperior aspect of the atrial septum. Frequently there is associated partial anomalous drainage of the right upper pulmonary vein. This vein can drain into the superior vena cava or the right atrium. Resorption of the wall between the vena cava and pulmonary vein results in this type of atrial septal defect. This also explains the anomalous drainage of the right upper pulmonary vein into the right atrium or superior vena cava (SVC) commonly associated with sinus venous atrial septal defects. In fact, technically the atrial septum is not defective, but rather the roof of the pulmonary vein is defective.

The most uncommon form of ASD is *the unroofed coronary sinus*. The coronary sinus is apposed to the posterior aspect of the left atrium, but the orifice is in the right atrium. If a hole exists in the roof of the coronary sinus, the coronary sinus and the left atrium will be in continuity, and, therefore the right and left atria will be in communication with each other (Fig. 19.2).

19.2 Pathologic Physiology

In uncomplicated atrial septal defects, the flow of blood is from the left to the right atrium through the defect. The flow is in this direction because the right atrium and right ventricle are more compliant than the left atrium or the left ventricle. This

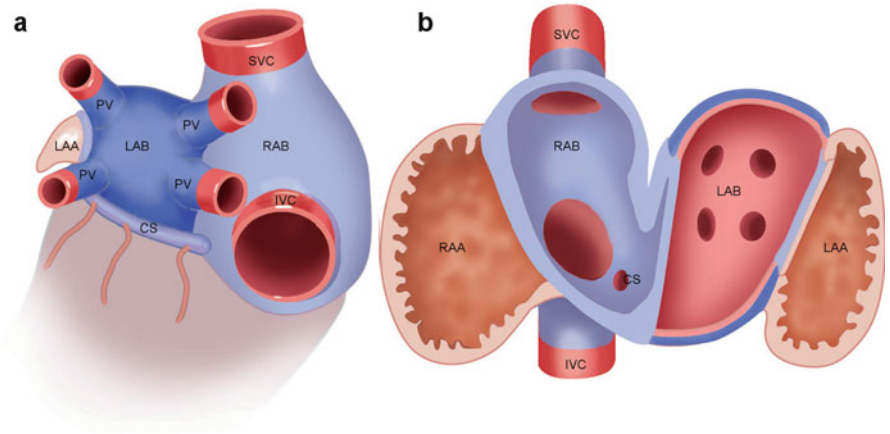

Fig. 19.2 (**a**) Schematic representation of the outside of the atrial chambers with the pulmonary veins (*PV*) and systemic veins (superior caval vein (*SCV*), inferior caval vein (*IVC*), coronary sinus (*CS*). The left atrial body (*LAB*) and right atrial body (*RAB*) are covered with a smooth-walled myocardium (*blue*), extending to the extracardiac segments of the PV and over a small peripheral part of the systemic veins. The left atrial appendage (*LAA*) and right atrial appendage (*RAA*) consist of trabeculated myocardium (*brown*). A small area of myocardium at the junction of the LAB to the LAA resembles the right-sided sinus venosus myocardium. (**b**) Schematic representation of different tissue types in the left and right atria as observed from the inside of the atria. Vessel wall tissue (*red*), myocardial tissue with a smooth-walled inner aspect (*blue*), primary atrial segment tissue (*brown*), sinus venosus-like tissue (*light blue*, between LAA and LAB), which is smooth-walled, lacking vessel wall tissue. *CS* coronary sinus, *IVC* inferior caval vein, *SVC* superior caval vein (Source: Reproduced with permission (Douglas YL, Jongbloed MR, Gittenberger-de Groot AC, Evers D, Dion RA, Voigt P, Bartelings MM, Schalij MJ, Ebels T, DeRuiter MC (2006) Histology of vascular myocardial wall of left atrial body after pulmonary venous incorporation. Am J Cardiol 97:662–670))

results in an excess volume of blood transiting the right atrium, the tricuspid valve, the right ventricle, the pulmonary valve, and the pulmonary circulation. This explains why a systolic murmur is present from excess flow through the pulmonary valve and a diastolic murmur may be present from excess flow through the tricuspid valve.

Anything that results in decreased compliance of the right ventricle can alter the direction of flow through the atrial septal defect, such as development of pulmonary hypertension. In this case, the patient may exhibit cyanosis.

19.3 Clinical Presentation

Most patients with an ASD are asymptomatic and come to medical attention because a heart murmur is detected. In fewer than 10 % of patients with ASD, symptoms of congestive heart failure and growth failure can occur in infancy

19.4 Physical Examination

In patients with an ASD, the right ventricular impulse felt along the lower left sternal border or the subxiphoid area may be more forceful than normal. The first heart sound will be normal. The second heart sound will be more widely split than normal and does not become single with expiration; the so-called "fixed splitting" of S2. Patients with an ASD have a systolic ejection murmur which is best heard along the left sternal border and is loudest at the upper left sternal border. This murmur is created by excessive blood flow through the pulmonary valve. This murmur is similar to an "innocent pulmonary flow murmur" and probably explains why many patients with ASD are not diagnosed until several years of age. With moderate- and large-size defects, a mid-diastolic murmur can be heard along the lower right or left sternal border. In some patients, this murmur is best heard over the xiphoid. This murmur is created by excessive blood flow through the tricuspid valve.

Patients with ostium primum ASD, in addition to the murmurs described above, may have a murmur of mitral insufficiency because of the cleft in the septal leaflet of the mitral valve associated with this defect.

19.5 Echocardiographic and Cardiac Catheterization Issues

The diagnosis of ASD and the type of ASD can be confirmed by echocardiography. In addition, the degree of right atrial and right ventricular enlargement and hypertrophy can be assessed. Elevation of pulmonary artery pressure can be approximated using Doppler techniques. It can be difficult to diagnose sinus venosus defects with transthoracic echocardiography, and, for some patients, transesophageal echocardiography may be necessary to confirm this diagnosis. In the era of echocardiography, it rarely is necessary to perform cardiac catheterization for the diagnosis of ASD. However, now cardiac catheterization is used to deliver and implant devices to close secundum ASDs without the need for open-heart surgery.

19.6 Treatment

The treatment of ASD is surgical or device closure. For secundum ASD, this can be accomplished surgically by direct suture or patch closure or by device closure using cardiac catheterization techniques. Currently, device closure is the preferred method to close secundum ASD in appropriately selected patients. However, the long-term outcome of such treatment remains unknown.

For ostium primum ASD, patch closure is invariably used, and in most cases, the cleft in the mitral valve leaflet is repaired. For sinus venosus ASD, the anomalous drainage of the right upper pulmonary vein is corrected and the ASD is closed.

The usual age for closure of an uncomplicated ASD is 2–4 years. In the rare infant with ASD and heart failure, surgery should be performed in infancy.

19.7 Outcome

The periprocedural mortality for device or surgical closure of a secundum atrial septal defect is 1 % or less, and the long-term outcome is excellent. Rarely patients will require reoperation for a residual atrial septal defect or to deal with a prior surgical misadventure.

The periprocedural mortality for closure of an ostium primum atrial septal defect also is low, but there is an 8–10 % chance that a future reoperation will be need to deal with mitral insufficiency or left ventricular outflow tract obstruction.

Human Genetics of Atrial Septal Defect

20

Rabia Khan and Patrick Y. Jay

Contents

Abstract

Atrial septal defects and other interatrial communications share the physiologic phenotype of shunting blood from the left to the right atrium, but their genetics and development have important differences. Secundum ASDs are the most common and usually the type implied by clinicians when not otherwise specified. The distinction is important because the development and genetics of ostium primum ASDs, the second most common type, are related to atrioventricular septal defects. Sinus venosus and coronary sinus defects are rare. Their anatomy and development are more properly considered as an interatrial communication rather than a true septal defect. Little is known about their genetics. This chapter focuses on the human genetics of secundum ASD and sinus venosus and coronary sinus defects.

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20.1 Introduction

A genetic basis for congenital heart disease was probably suspected more than a century ago in a case of two, cyanotic siblings [1], but coincidence or environmental causes were impossible to exclude in early reports of sibling or parent-child combinations. In 1960, Zetterqvist provided the first compelling evidence for a heritable basis, documenting atrial septal defects (ASD) in multiple generations of a large, extended Swedish family [2, 3]. Reports soon followed of families who had secundum ASDs associated with atrioventricular conduction defects [4–6] or hand defects [7]. These two classic ASD phenotypes are now known to be caused by mutations of NK2 homeobox 5 (*NKX2-5*) [8] or T-box protein 5 (*TBX5*) in Holt-Oram syndrome [9, 10]. Although only ASDs were diagnosed in the family, Zetterqvist intuited that other congenital heart defects were also heritable but not more frequently observed in families, especially parents, because they were incompatible with survival [2]. He was prescient, as mutations of the transcription factors NKX2-5 and TBX5 have since been recognized to cause a wide variety of simple and complex heart defects [11–13]. Of course, the chromosomal aneuploidy syndromes, e.g., trisomy 13, 18, and 21, provide clear, direct evidence for a genetic basis of congenital heart disease.

Almost 300 genes have known or suspected associations with congenital heart disease or cardiac development (for a comprehensive list see Table S12 in Ref. [14]). A search of PubMed and Online Mendelian Inheritance in Man identified genes and chromosomal anomalies that have been reported in association with a secundum ASD or sinus venosus defect in humans (Tables 20.1 and 20.2); genes reported in an animal model only are not included. Often, the gene or genes that underlie a chromosomal anomaly or structural variant are not well defined. Most of the genes listed in Table 20.2 are candidates based on experimental evidence; a few have been described in the context of human, single gene mutations.

Two emerging classes of genetic variation – copy number variations (CNVs) and common single nucleotide polymorphisms (SNPs) – have been associated with ASDs but are not listed in Tables 20.1 and 20.2. CNVs are chromosomal segments that are duplicated or deleted such that there will be more or fewer than two copies of the genes contained within the interval. In general the genes in CNVs that are relevant to their association with congenital heart disease are poorly defined, although a significant fraction contains known or suspected cardiac developmental genes [60, 65]. Two genome-wide association studies (GWAS) of ASD in European and Chinese populations have reported common SNPs associated with ASD risk. The GWAS of European Caucasians detected three SNPs near *MSX1* and *STX18* on chromosome 4p16 [66]. The 4p13 SNPs were also associated with risk in two different Chinese populations in studies that genotyped just those SNPs [67, 68]. The original GWAS on one of the two Chinese populations did not detect the 4p16 locus; instead, two SNPs near *TBX15* at 1p12 and *MAML3* at 4q31.1 were reported [69]. None of the genes near the SNPs has a known function in atrial septation, although there are some studies of the role of *MSX1* in cardiac development. Much more investigation is necessary to determine the biological meaning of statistically significant CNVs and SNPs.

Table 20.1 Genes in which human mutations have been associated with secundum ASD or sinus venosus defect

Gene symbol	Protein	Extracardiac phenotypes	Reference
	Sarcomeric protein		
ACTC1	Alpha-cardiac actin	No	[15]
MYH6	Alpha-cardiac myosin heavy chain	No	[16]
MYH7	Beta-myosin heavy chain	No	[17]
	Signaling pathway		
BRAF	v-raf murine sarcoma viral oncogene homolog B	Noonan syndrome	[18, 19]
DHCR7	7-dehydrocholesterol reductase	Smith-Lemli-Opitz syndrome	[20–22]
JAG1	Jagged 1	Alagille syndrome	[23]
KRAS	Kirsten rat sarcoma viral oncogene homolog	Noonan syndrome	[18, 19]
NOTCH2		Alagille syndrome	[23]
PTPN11	Protein-tyrosine phosphatase, nonreceptor-type 11	Noonan syndrome	[24]
RIT1	Ras-like without CAAX 1	Noonan syndrome	[25]
SOS1	Son of sevenless homolog 1	Noonan syndrome	[26]
STRA6	Stimulated by retinoic acid 6	Brain, eyes, diaphragm, lung	[27]
	Transcription factor		
CITED2	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	None reported in human	[28]
FOXC1	Forkhead box C1	Axenfeld-Rieger syndrome	[29]
GATA4	GATA binding protein 4	Diaphragm, gonad, pancreas	[30]
GATA6	GATA binding protein 6	Pancreas	[31]
NKX2-5	NK2 homeobox 5	Pylorus, spleen	[8]
PITX2	Paired-like homeodomain 2	Axenfeld-Rieger syndrome	[32]
SALL1	Spalt-like transcription factor 1	Townes-Brocks syndrome	[33]
SALL4	Spalt-like transcription factor 4	Okihiro/Duane-radial ray syndrome	[34, 35]
TBX1	T-box 1	DiGeorge syndrome	[36]
TBX5	T-box 5	Holt-Oram syndrome	[9, 10]
TBX20	T-box 20	No	[37]
ZIC3	Zic family member 3	Heterotaxy	[38]
	Other gene regulatory mechanisms		
BCOR	BCL6 corepressor	Oculofaciocardiodental and Lenz microphthalmia syndrome	[39, 40]
CHD7	Chromodomain helicase DNA-binding protein 7	CHARGE syndrome	[41]
CREBBP	CREB binding protein	Rubinstein-Taybi syndrome	[42, 43]

(continued)

Table 20.1 (continued)

Gene symbol	Protein	Extracardiac phenotypes	Reference
EHMT1	Euchromatic histone methyltransferase 1	Brain, craniofacial, urogenital	[44]
KDM6A	Lysine (K)-specific demethylase 6A	Kabuki syndrome	[45]
KMT2D	Lysine (K)-specific methyltransferase 2D (commonly referred to as *MLL2*, myeloid/lymphoid or mixed-lineage leukemia 2)	Kabuki syndrome	[46]
NSD1	Nuclear receptor binding SET domain protein 1	Sotos syndrome	[47, 48]
RBM10	RNA binding motif protein 10	Brain, craniofacial, palate, lung, limb	[49]
STK4	Serine/threonine kinase 4	Immunodeficiency	[50]
	Other or unknown function		
CYR61	Cysteine-rich, angiogenic inducer, 61		[51]
KIAA0196	Strumpellin	Ritscher-Schinzel/cranio-cerebello-cardiac syndrome	[52, 53]

Some of them are associated with extracardiac phenotypes

Table 20.2 Chromosomal abnormalities that have been associated with ASD. Candidate genes are suggested by experimental evidence or human mutations that cause congenital heart disease

Syndrome	Candidate genes	Reference
2q31.1 deletion		[54]
4p16.3 deletion (Wolf-Hirschhorn)	*WHSC1*, *FGFRL1*	[55]
4q32.2-34.3 deletion	*HAND2*, *TLL1*	[56]
5p deletion (Cri du chat)		[57]
9q subtelomeric deletion	*EHMT1*	[44]
Distal 11q deletion (Jacobsen)	*ETS1*	[58]
Trisomy 13 (Patau)		[59]
15q11.2 deletion		[60]
16p13.3 deletion (Rubinstein-Taybi)	*CREBBP*	[42, 43]
17q23.1-q23.2 deletion	*TBX2*, *TBX4*	[61]
Trisomy 18 (Edward)		[59]
Trisomy 21 (Down)		[62]
22q11 deletion (DiGeorge)	*TBX1*, *CRKL*, *MAPK1*	[63, 64]

As a rule, no gene is associated with just one type of defect. Possible exceptions to the rule may offer clues into the mechanisms that lead specifically to ASD. The functional classification of genes in Table 20.1 helps to explain why the rule is generally true. Genes in three of the five categories – signaling pathway, transcription factor, and other mechanisms of gene regulation such as chromatin modification or

RNA splicing – affect the expression of many downstream genes, which in turn act in diverse developmental pathways. The specific phenotype expressed depends not only on the causal mutation but also genetic or environmental factors that modify the susceptibility of particular developmental pathways to the mutation [70].

20.2 Secundum ASD Genes

Mutations of some ASD genes are preferentially associated with other classes of defects. For example, mutations of *TBX1* are associated with outflow tract defects like truncus arteriosus, tetralogy of Fallot, and interrupted aortic arch, all of which commonly are observed in the 22q11.2 deletion or DiGeorge syndrome [36]. The disease phenotype is consistent with the well-established role of *TBX1* in regulating second heart field development at the anterior or arterial pole [71]. Atrial septal development receives contributions from the posterior but not the anterior second heart field [72, 73]. *TBX1* is expressed in the entire second heart field [74, 75], so the few cases of ASD may represent a lesser dependence in the posterior second heart field on its activity [76].

Mutations of cardiac transcription factors may be associated preferentially with ASD, but a large fraction of cases involve other simple and complex heart defects. In the cases of *NKX2-5* and *GATA4*, the associations with ASD may be overestimated because of ascertainment bias. Mutations were first described in families in whom more than half and sometimes all the affected members had an ASD, although a few relatives had other types of defects [8, 30]. The incidence of ASD among unselected *NKX2-5* or GATA binding protein 4 (*GATA4*) mutation carriers has not been assessed systematically, but it is probably not as high as in the families who came to attention because of their striking presentations. *NKX2-5* mutations are found in only up to 4 % of unselected ASD cases [12, 77, 78]. GATA4 mutations are found in only 1–2 % of unselected ASD cases [78–82]. The incidences of *NKX2-5* or *GATA4* mutations in other types of defects such as tetralogy of Fallot are similar, which suggests no predilection for ASD [12, 82]. Likewise, pleiotropic heart defects are found in mice that are haploinsufficient for either gene [70, 83, 84].

Mutations of the transcription factor *TBX5* cause pleiotropic heart defects as well [13, 85], but a predilection for ASD is well documented. The incidence of ASD ranges from 35 to 60 % in case series of Holt-Oram syndrome patients, which exceeds the incidences of other defects [86–88]. *TBX5* genetically interacts with the spalt-like transcription factor 4 (*SALL4*), the gene involved in Okimoto or Duane-radial ray syndrome [89, 90]. Mutations of *SALL4* and *SALL1* cause heart and radial limb defects that resemble the Holt-Oram phenotype but without the predilection for ASD [33–35]. Thus, the pathogenesis of ASD in *TBX5* mutation may involve a *SALL1-SALL4* independent pathway, or *SALL1*, *SALL4*, or another gene may serve redundant functions for each other in atrial septal development.

Most genes that cause congenital heart disease sit atop regulatory or signaling hierarchies. Thus, the roles of cardiac alpha-actin (*ACTC1* [15, 91, 92]) and alpha- and beta-myosin heavy chains (*MYH6* [16, 93, 94] and *MYH7* [17], respectively) came as a surprise. Sarcomere mutations have long been known to cause

cardiomyopathies that are usually not associated with heart defects. Conversely, the first families discovered to have ASD caused by mutations of *ACTC1* or *MYH6* showed no evidence for cardiomyopathy [15, 16, 91]. Affected members in subsequent families have hypertrophic cardiomyopathy or ventricular noncompaction that may or may not be associated with a heart defect [17, 92]. No genotype-phenotype correlation or general molecular mechanism explains why a mutation would cause a heart defect, cardiomyopathy, or both. For example, the p.Glu101Lys mutation of *ACTC1* can cause hypertrophic, dilated, restrictive, or noncompaction cardiomyopathy or ASD [92].

Unlike most other genes, mutations of *ACTC1* and *MYH6* seem to be preferentially or exclusively associated with ASD as the phenotypic manifestation of congenital heart disease. No congenital heart defect beside ASD has been diagnosed in large families who have *ACTC1* mutations, including the descendants of the family originally described by Zetterqvist [15]. Selection bias seems unlikely in the setting of diagnostic evaluations that span multiple generations up to the modern era [15, 91]. Among the sarcomeric genes, *MYH6* mutations appear to be the most common cause of familial ASD [94]. In three studies that identified 54 *MYH6* mutation carriers from 12 families, ASD was most common defect (19/54). Certain defects, however, occurred more frequently than might be expected, including tricuspid atresia (2/54), atrioventricular septal defect (2/54), and left-sided obstructive lesions, i.e., aortic or subaortic stenosis or coarctation (7/54) [93–95]. This suggests that the defects share an unknown developmental basis.

Mutations of *MYH7* cause ASD, but Ebstein anomaly and left ventricular noncompaction are more prominent developmental phenotypes [17, 96, 97]. Ebstein malformation of the tricuspid valve frequently coexists with an ASD, which is presumed to be secondary to the right-to-left shunt induced by regurgitation from the defective tricuspid valve. This may not always be true, however, as one *MYH7* mutation carrier had an ASD without an Ebstein malformation [17]. Independent of the cause, left ventricular noncompaction in children commonly is associated with a wide variety of congenital heart defects [98]. The mechanical function of sarcomeric proteins likely plays dual roles in the formation of the compact myocardium and cardiac morphogenesis [99].

20.3 Sinus Venosus Defect Genes

Only three human genetic mutations have been reported with isolated sinus venosus defects and none with coronary sinus defects. One patient who had a sinus venosus defect with partial anomalous pulmonary venous return had a mutation of *CITED2* (Cbp/P300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain 2) [28]. Partial anomalous pulmonary venous return is a feature of sinus venosus defects. Typically, the right upper pulmonary vein communicates with the superior vena cava but still connects to the left atrium [100]. The interatrial shunt is across the abnormal venovenous communication and not a defect in the atrial septum per se. Genetic mutations that cause total anomalous pulmonary venous return or

anomalous connections to form between the systemic and pulmonary venous circulations could explain some cases of sinus venosus defects [101], but none has been described yet.

A truncation mutation of *NKX2-5* that lacked the homeodomain was found in three members of a family who had a sinus venosus defect and atrioventricular block [102]. *NKX2-5* loss-of-function mutations are associated with conduction defects [103]. One of the three also had pyloric stenosis; common variants of *NKX2-5* have been associated with infantile hypertrophic pyloric stenosis [104]. Finally, a mutation in the Kozak sequence of *GATA4*, which disrupted mRNA translation to protein, was discovered in a patient who had a sinus venosus defect and partial anomalous pulmonary venous return. Her mother had a secundum ASD [105].

Conclusion

In general, human genetic mutations that have been associated with ASD or sinus venosus defects cause a wide variety of cardiac malformations. The pleiotropy exists probably because the genes act in regulatory or signaling pathways that affect the development of multiple cardiac structures and other organs. Additional, undefined genetic, environmental, or stochastic factors must determine the phenotypic expression of an ASD. Thus, although high-throughput DNA sequencing has rapidly accelerated gene discovery, most research has illuminated the causes of congenital heart disease in general but not ASD specifically. Some genetic mutations show a predilection for ASD, sometimes in association with other specific defects or cardiomyopathy. These mutations may offer clues into the pathways that are more specific to atrial septation. With these observations in mind, one may design human or animal studies to delineate the genes and pathways pertinent to specific defects, say, by the analysis of genetic polymorphisms that modify the risk of a defect in the setting of a known, causative mutation.

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Molecular Pathways and Animal Models of Atrial Septal Defect

21

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Contents

Abstract

The seeming simplicity of the clinical presentation and management of an atrial septal defect belies the complexity of the developmental pathogenesis. Here, we describe the anatomic development of the atrial septum and the venous return to the atrial chambers. Recent experimental results suggest where genetic mutations could disrupt developmental steps to cause a defect within the oval fossa, the so-called secundum defect or other interatrial communication, such as the sinus venosus defect.

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21.1 Introduction

The term "atrial septal defect" (ASD) is commonly used to describe any abnormal communication between the atrial chambers. The hemodynamic consequences of "atrial septal defects" as loosely defined are similar, but the phenotypic ambiguity makes it difficult to discuss their developmental pathogenesis. To be clear, we define a component of the atrial septum as one in which a defect through it will produce a direct communication between the two atria [1]. By this definition, the primary atrial septum and its ventral buttress are the only structures that comprise the atrial septum. The primary atrial septum acts as a flap valve over the foramen ovale. An ostium secundum defect, often referred to as a secundum ASD but perhaps better considered an oval fossa defect, is a direct communication in which deficiency of the primary atrial septum permits shunting across the oval fossa. The ostium primum defect represents the persistence of the primary atrial foramen, a direct communication that existed in the embryo. This defect is more appropriately discussed in the context of atrioventricular septal defects with common atrioventricular junction. Indirect communications involve anomalous venous connections. Sinus venosus defects involve an anomalous pulmonary venous connection to the superior or inferior vena cava. Coronary sinus defects involve an anomalous connection to the left atrium. Anomalous venous connections are outside the true atrial septum.

This chapter provides an overview of the mechanisms pertinent to the secundum ASD and anomalous venous connections. The discussion focuses on mouse models, in which most all experimental manipulations have been performed. Some experiments have been done in chick [2], but the system is not as amenable to genetic dissection. Frogs appear to have a rudimentary primary atrial septum and could become a useful model for visualizing atrial septation in a living embryo [3, 4]. There are some anatomic differences between the human and mouse, but the similarities of normal development and of human and mouse phenotypes suggest that the genetic pathways are conserved.

21.2 Synopsis of Normal Atrial Septation

In the mouse on embryonic day (E) 8, the heart is a linear tube, oriented along the anterior-posterior or rostral-caudal axis. (Developmental biologists commonly refer to the embryonic head-tail axis by the former, whereas human anatomists prefer the latter. "Anterior-posterior" in the human anatomic frame of reference is synonymous with "ventral-dorsal.") The arterial and venous poles are rostral and caudal, respectively. Each pole is connected to the splanchnic mesoderm by the dorsal mesocardium. Second heart field progenitors from the mesoderm migrate through the dorsal mesocardium to contribute to the right ventricle, outflow tract, and ventricular septum at the arterial pole and to the atrial chambers, including the primary atrial septum and vestibular spine, at the venous pole [5].

At E9, the right and left sinus horns can be seen entering the common atrium on either side of the midline dorsal mesocardium. Just rostral to the confluence of the sinus horns, the pulmonary pit can be seen at the site of the dorsal mesocardium. The sinus horns are the precursors of the systemic venous return to the heart. The pulmonary pit provides the eventual site of entrance of the pulmonary venous return and is flanked at this early stage by the right and left pulmonary ridges (Fig. 21.1a). Second heart field progenitors migrate through the dorsal mesocardium to contribute to the growth of the right pulmonary ridge, also referred to as the dorsal mesenchymal protrusion, which ultimately forms the vestibular spine (Fig. 21.1b). At E10, growth at the right pulmonary ridge partitions the pulmonary vein to the left atrium, while the sinus horns rotate to the right atrium (Fig. 21.1a).

The right superior and inferior vena cava will drain to the right sinus horn, while the left superior vena cava will drain through the left sinus horn, opening to the right atrium as the coronary sinus. The left superior vena cava persists in the mouse but normally regresses in humans. Rotation of the sinus horns ultimately places the coronary sinus in the dorsal atrioventricular groove. Although the coronary sinus runs adjacent to the left atrium, each has its own wall from the outset of development (Fig. 21.1a) [6].

In humans, the solitary pulmonary vein that initially opens through the pulmonary pit becomes incorporated into the roof of the left atrium, eventually achieving four discrete orifices [7]. This does not occur in the mouse: the right and left pulmonary veins connect to a common vein to the left atrium adjacent to the dorsal atrioventricular groove [8].

At E10, the primary atrial septum, which develops from second heart field progenitors, is first seen growing from the dorsal roof of the common atrium toward the endocardial cushions of the atrioventricular canal (Fig. 21.1c). Its leading edge has a mesenchymal cap, which is derived from the endocardium [9]. At the caudal end of the primary atrial septum, the mesenchymal cap is continuous with the developing vestibular spine (Fig. 21.1d). The primary atrial foramen, or ostium primum, is thus bounded by the edge of the primary atrial septum with its mesenchymal cap, the vestibular spine, and the atrial surface of the endocardial cushions. By E12, fusion of the mesenchymal cap, vestibular spine, and endocardial cushions closes the primary atrial foramen. Persistence of the primary atrial foramen, in other words an ostium primum defect, results from failure of the development of the vestibular spine (Fig. 21.1e, f) [9–11].

At E10.5, fenestrations appear in origin of the primary atrial septum at the atrial roof as the septum grows toward the atrioventricular canal. The fenestrations coalesce to form the secondary atrial foramen, which is essential during fetal life for right-to-left shunting of oxygenated blood after closure of the primary atrial foramen (Fig. 21.1c). The primary atrial septum thus becomes the flap valve of the foramen ovale (Fig. 21.1e). At E13, the dorsal rim of the foramen ovale forms by the infolding of the atrial wall between the pulmonary vein and left venous valve of the right atrium (Fig. 21.1g). The cranial rim is a muscular ridge that remains at the site

of breakdown at the atrial roof of the primary septum (Fig. 21.1h). Infolding is a consequence of the incorporation of the pulmonary vein into the left atrium. The infolded rim of the foramen is commonly referred to as the secondary atrial septum

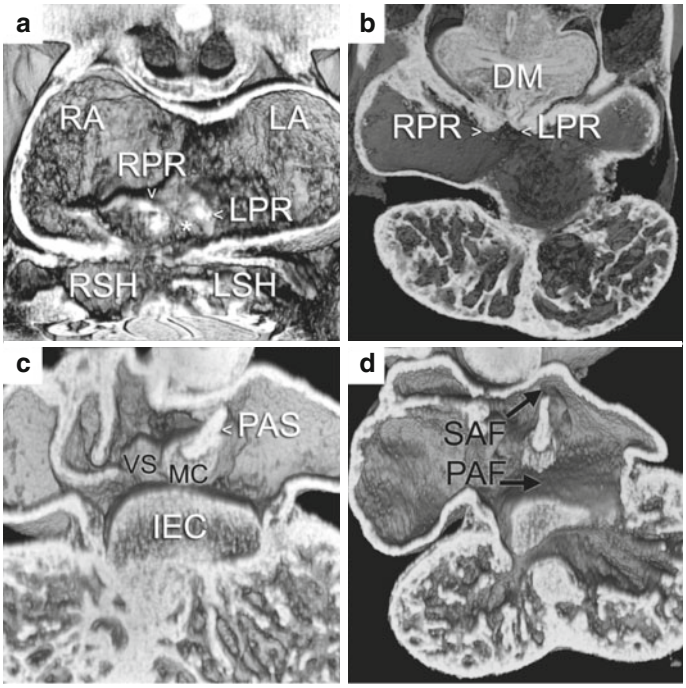

Fig. 21.1 Details of atrial septation and venous return in the developing mouse heart are shown in a series of episcopic images. (**a**, **b**) Images of an E10.5 heart are shown in the frontal (**a**) and transverse (**b**) planes. The right and left sinus horns (*RSH*, *LSH*) drain into to the right atrium (*RA*). The left sinus horn, which gives rise to the coronary sinus, never shares a common wall with the left atrium (*LA*) during development. The pulmonary pit (*) is situated between the right and left pulmonary ridges (*RPR*, *LPR*). Second heart field progenitors migrate through the dorsal mesocardium (*DM*) into the right pulmonary ridge to form the vestibular spine. (**c**, **d**) In two E11.5 hearts, the relationships are shown between the primary atrial septum (*PAS*), its mesenchymal cap (*MC*), the developing vestibular spine (*VS*), and the inferior endocardial cushion (*IEC*). The primary and secondary atrial foramina are noted (*PAF*, *SAF*). (**c**) is cranial to (**d**). (**e**) In this E14.5 heart, fusion of the mesenchymal cap of the primary atrial septum, the vestibular spine, and the endocardial cushions has closed the primary atrial foramen. The primary atrial septum acts as a flap valve on the foramen ovale (*FO*). (**f**) An ostium primum defect (*arrow*) in this E18.5 heart results from persistence of the primary atrial foramen. (**g**, **h**) In this E18.5 heart, the atrial septum is shown (**g**) at the level of the pulmonary vein and (**h**) more cranially. Incorporation of the pulmonary vein (PV) into the left atrium causes infolding of the dorsal atrial wall (*arrowhead*, **g**). A muscular rim, not present in humans, exists at the site of breakdown at the atrial roof of the primary septum (*arrowhead*, **h**) (The episcopic image datasets were prepared by Dr. Timothy Mohun of the Francis Crick Institute, London)

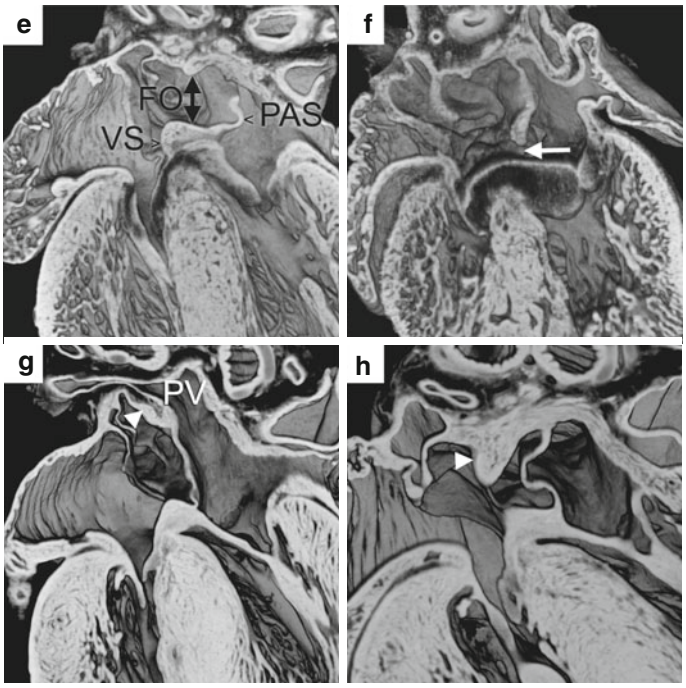

Fig. 21.1 (continued)

or septum secundum, but it is not a true septum, since a hole through it will enter a potential space outside the heart. The ventral-caudal rim of the foramen, which buttresses the flap valve to the atrioventricular junctions, is formed by muscularization of the mesenchymal cap of the primary atrial septum and vestibular spine (Fig. 21.1e–h) [9–11]. This rim is a true septum, as illustrated by a rare vestibular defect in an *Nkx2-5*$^{+/-}$ mouse (Fig. 21.2a) [12].

The embryologic relationship of the systemic and pulmonary venous return was hotly debated in the past, but multiple studies in human and mouse now clearly establish their separate development. A recent analysis on the chick embryo demonstrates this elegantly with three-dimensional reconstructions. The pulmonary veins in the lungs develop from a plexus within the splanchnic mesoderm. They subsequently connect to the main pulmonary vein that develops at the pulmonary pit. The systemic venous sinus originates from the junction between the splanchnic and somatic mesoderm. The systemic veins develop in the splanchnic mesoderm caudal to the pulmonary venous plexus. There is no evidence for anastomoses between the two venous systems in the normal embryo [13]. Studies of developing human hearts also support the separate developmental origins of the systemic and pulmonary veins [14].

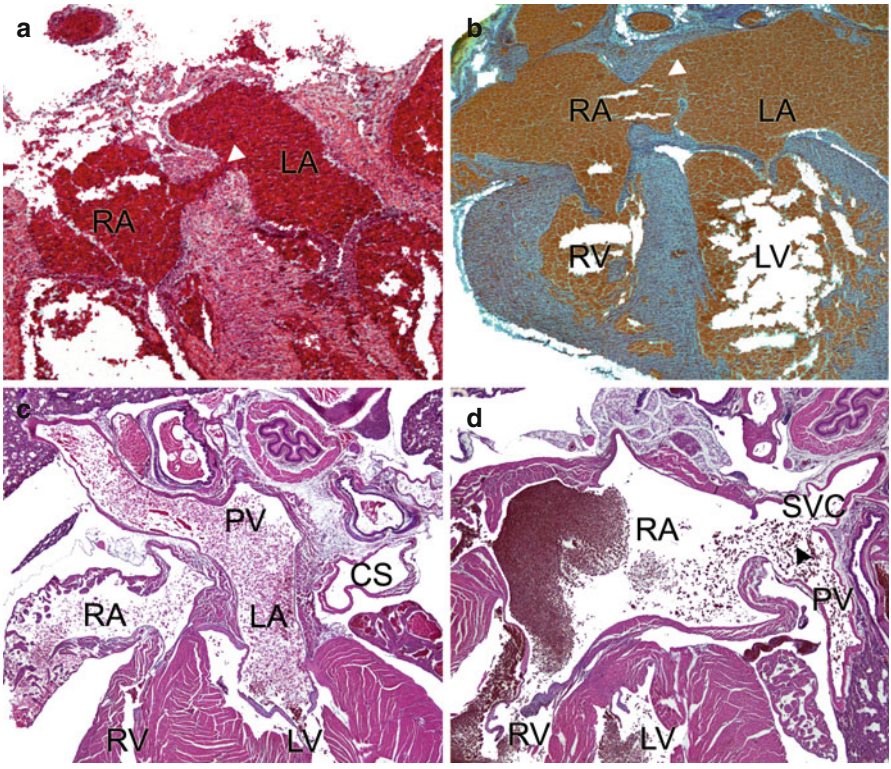

Fig. 21.2 Examples of atrial septal defects and an anomalous venovenous communication. (**a**) A vestibular defect and (**b**) a secundum ASD in newborn *Nkx2-5*$^{+/-}$ mice. (**c**) Normal pulmonary venous drainage is shown into the left atrium of a mouse. (**d**) The pulmonary vein communicates anomalously with the superior vena cava in a *Sema3d*$^{-/-}$ mouse. *Arrowheads* indicate the defects (Mr. Suk Dev Regmi prepared the sections shown in **a**, **b**. Mr. Ehiole Akhirome discovered the vestibular defect. Both are at Washington University School of Medicine, St. Louis) *CS* coronary sinus, *PV* pulmonary vein, *RA*, *LA* right, left atrium, *RV*, *LV* right, left ventricle, *SVC* superior vena cava

21.3 Pathways to a Defect within the Oval Fossa (Secundum ASD)

Most experimental studies of atrial septation to date pertain to the pathogenesis of the primum rather than secundum defect. Unfortunately, developmental biologists often do not distinguish the two defects, describing mutant phenotypes merely as "ASDs." A secundum ASD is the consequence of deficiency of the primary atrial septum, such that it cannot cover the complete area of the foramen ovale (Fig. 21.2b). In the usual case, the primary atrial septum does not overlap the infolded rim of the foramen. Fenestrations in the primary atrial septum also occur but are less common. Of note, the vestibular spine and the mesenchymal cap, forming the buttress of the foramen, have developed and

muscularized normally, which is not the case in the primum defect, which is an atrioventricular septal defect [9–11, 15].

An appreciation of normal development and the morphology of defects within the oval fossa can help to circumscribe the mechanisms of pathogenesis. Mutations that cause secundum defects likely relate to the development of the secondary atrial foramen. On the other hand, deficient growth of the primary atrial septum from the atrial roof to the atrioventricular canal is unlikely to explain the secundum defects. Experiments with *Tbx5* (T-box 5) and *Nos3* (nitric oxide synthase 3) mutants offer a glimpse into a specific cellular and molecular mechanism.

Human and mouse *TBX5* mutations are commonly associated with secundum ASD, although other malformations, including atrioventricular septal defects, are also seen [16–19]. Independent functions of *Tbx5* in the endocardium and the second heart field can explain the pathogenesis of both secundum and primum defects, respectively. These functions have been explored in a series of conditional deletion experiments, in which one or both copies of a gene are deleted in a specific cell type or developmental period. In the embryo, *Tbx5* is expressed by the endothelial cells lining the primary atrial septum. Conditional knockout of *Tbx5* in endothelial cells increased the number of apoptotic cells in the developing primary septum. The adult conditional knockout mice had near-complete absence of the primary atrial septum, while the conditional heterozygotes had a high incidence of patency of the foramen ovale. Endothelial knockout of *Tbx5* did not affect the vestibular spine [20]. The diminished activity of Tbx5 in the endothelium thus causes defects within the oval fossa, or the forme fruste as seen in persistent patency of the foramen ovale. On the other hand, conditional deletion of *Tbx5* in the second heart field prevented the development of the vestibular spine, resulting in primum defects [21].

The molecular pathways that lead to a secundum ASD are mostly undefined, but one has been suggested for *Tbx5* and *Gata4* (GATA binding protein 4). Mutations of the latter also cause secundum defects in both man and mouse [22–24]. In an endocardial cell culture model, TBX5 upregulates the expression of nitric oxide synthase 3 (*Nos3*, also known as eNOS or endothelial NOS). In the same cell culture system, TBX5 and GATA4 independently and cooperatively transactivate a *Nos3* promoter construct [20]. *Nos3* null mutant mice have a high incidence of defects within the oval fossa. The thinness of the primary atrial septum may make it more susceptible to apoptosis caused by the loss of *Nos3* [25]. Furthermore, compound heterozygotes, in which one allele of *Tbx5* is deleted in the endothelium and one allele of *Nos3* in the germline, have a higher incidence of secundum defects than either single heterozygote. *Nos3* is expressed in both the endocardial cells and myocytes, so its activity in one or both cell types may be necessary to prevent myocyte apoptosis. Taken together, the results suggest that *Tbx5*, *Gata4*, and *Nos3* operate in a molecular pathway involving an endocardial signal that inhibits myocyte apoptosis.

Genetic models that perturb the posterior second heart field have consistently produced atrioventricular septal defects [21, 26, 27], but it is worth exploring the hypothesis that subtler perturbations cause secundum defects. The expression patterns of the Hox (homeobox) transcription factors *Hoxb1*, *Hoxa1*, and *Hoxa3* define

subdomains of the second heart field. A *Hoxb1*$^{+}$/*Hoxa1*$^{-}$/*Hoxa3*$^{-}$ subdomain contributes to both poles of the heart, including the subpulmonary myocardium, primary atrial septum, and vestibular spine [28]. A primary defect of cells in this subdomain has been postulated to explain the co-occurrence of tetralogy of Fallot and atrioventricular septal defects in persons who have Down syndrome [29]. Isolated tetralogy of Fallot, atrioventricular septal defect, and defects within the oval fossa occur in a significant fraction of patients with Down syndrome, so one may wonder whether trisomy 21 affects a common progenitor population even before the cells have entered the developing heart or started differentiation. Modifying factors, whether genetic, environmental, or stochastic, would determine the ultimate phenotype that results from a "hit" at a single location in the embryo. One can speculate about the specific mechanisms, but the model parsimoniously explains the pleiotropic effects of every genetic mutation that causes a secundum defect in some but not all individuals.

21.4 Pathways to Sinus Venosus and Coronary Sinus Defects

With regard to the developmental pathogenesis of sinus venosus and coronary sinus defects, we assert that the salient anatomic feature is the anomalous communication between a vein and an adjacent structure, with the vein itself continuing on its normal course. In a sinus venosus defect, a pulmonary vein communicates with the superior or inferior vena cava. The most common defect is between the right upper pulmonary vein and the superior vena cava. The normal connections of the vena cava and the pulmonary vein to their respective atrial chambers permit the interatrial shunting of blood. In a coronary sinus defect, the anomalous communication is with the left atrium as the vessel courses toward the right atrium. The shunt flows from the cavity of the left atrium to the right atrium through the mouth of the coronary sinus.

The morphology of sinus venosus defects and the separate development of the systemic and pulmonic venous return make certain pathogenic mechanisms unlikely. The anomalous communication cannot be due to the persistence of a transient anastomosis in the embryo. The failure of development of a common wall, or its dissolution between two veins, is also implausible. On the other hand, a semaphorin 3d (*Sema3d*) knockout mouse model of anomalous pulmonary venous return suggests that the basis is the development of an ectopic communication [30].

Semaphorins are secreted ligands. First discovered as mediators of axonal guidance by repulsion or attraction, semaphorins and their receptors, the plexins and neuropilins, have broad functions in cardiovascular development [31, 32]. In the embryonic mouse heart, *Sema3d* expression marks an avascular border region in the splanchnic mesoderm between the pulmonary and systemic venous plexuses, with the latter developing more caudally in the splanchnic mesoderm. Pulmonary venous endothelial cells express the neuropilin-1 receptor for *Sema3d*. In *Sema3d* knockout embryos, pulmonary venous endothelial cells cross the border and form connections with the systemic veins, while the main pulmonary vein still uses the pulmonary pit to enter the atrial cavity to the left of the primary atrial septum [30]. In the

Sema3d knockout mutant, abnormal venovenous communications arise because of a failure of repulsion. The mechanism that directs the main pulmonary vein to the left atrium remains intact. We hypothesize that sinus venosus defects arise by a similar failure of repulsion. As a pulmonary vein passes by the superior or inferior sinus horn on its way to the left atrium, it forms the anomalous communication (Fig. 21.2c, d).

The morphology of a coronary sinus defect and normal development also makes certain mechanisms unlikely. There is never a common wall between the coronary sinus and left atrium in the embryo, so the failure of its development or its dissolution is implausible. The development of an anomalous venoatrial communication, similar to the mechanism hypothesized for a sinus venosus defect, seems more plausible, but affirmative evidence remains lacking. No gene has been associated with isolated coronary sinus defect in humans or mice. In the only report of such a defect in the mouse, the authors suggest that a knockout of *mahogunin ring finger*-1 (*MGRN1*) caused a coronary sinus defect, albeit in association with a cardiac phenotype suggestive of isomerism, or heterotaxy [33]. Genes that affect embryonic left–right patterning might be candidates for screening in isolated coronary sinus defects.

Conclusion

Secundum ASD and sinus venosus and coronary sinus defects are simple from the clinician's perspective. From a developmental and genetic perspective, however, they are just as complicated and arguably less well understood than defects like tetralogy of Fallot. What little is known suggests that investigation of ASDs and anomalous venous communications in animal models will yield novel insights into second heart field development, interactions between the endocardium and myocytes, and vascular patterning. Finally, we suggest that a clear understanding of the developmental morphogenesis and methods that clearly depict the complex anatomy will be as important as any advances in experimental genetics to elucidate the mechanisms.

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Part V

Ventricular Septal Defect

Clinical Presentation and Therapy of Ventricular Septal Defect

22

David J. Driscoll

Contents

22.1 Introduction

Ventricular septal defects (VSDs) occur in 1.5–3.5 of 1000 live births and constitutes 20 % of congenital cardiac defects. There is no gender predominance. There are four types of VSD:

1. Perimembranous
2. Supracristal (or subpulmonary or subaortic)
3. Inlet (VSD of the atrioventricular canal type)
4. Muscular

A *perimembranous VSD* is in the area of the membranous septum. This places it in the outflow tract of the left ventricle and immediately under the aortic valve. A *supracristal VSD* is located superior to the crista supraventricularis which places it in the right

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however, determines the size of the left-to-right shunt. If the VSD itself is unrestrictive to blood flow, then the downstream resistance of the right ventricular outflow tract or the pulmonary microcirculation will determine the volume of pulmonary blood flow. The type of VSD may influence the clinical presentation. For example, a patient with a supracristal VSD may present primarily with aortic valve insufficiency.

Patients with a small- or moderate-size VSD present with a cardiac murmur. Infants with a large VSD may, initially, present only with a murmur. As pulmonary resistance declines, however, they will develop signs and symptoms of pulmonary edema, pulmonary venous hypertension, and congestive heart failure. This is manifested by tachypnea, tachycardia, pallor, poor feeding, and poor weight gain.

22.4 Physical Examination

The physical findings for a patient with a VSD depend upon the size of the VSD, the magnitude of the left-to-right shunt, and the level of right ventricular and pulmonary artery hypertension.

A small VSD and normal or only slightly elevated right ventricular and pulmonary artery pressure is characterized by normal precordial impulses and normal first and second heart sounds. The murmur is holosystolic. For moderate and large VSDs, the right ventricular impulse, felt at the lower left sternal border or the subxiphoid region, is prominent. In addition, the left ventricular impulse will be displaced laterally and have increased activity. The first heart sound will be normal. As the degree of pulmonary hypertension increases, the intensity of the pulmonary component of S2 will increase. There will be a holosystolic mid-frequency murmur. In patients with Qp/Qs >2 (ratio of pulmonary to systemic blood flow greater than 2), in addition to the systolic murmur, there will be a mid-diastolic mitral flow murmur (this produces the so-called "gallop rhythm") as a result of increased volume of blood flowing from the left atrium to the left ventricle.

22.5 Echocardiographic and Cardiac Catheterization Issues

The diagnosis of a VSD can be confirmed using echocardiography. The location and size of the defect can be defined, and associated anomalies can be identified. Using Doppler techniques, right ventricular and pulmonary artery pressures can be estimated.

Cardiac catheterization rarely is necessary for patients with uncomplicated VSD and without evidence for pulmonary vascular obstructive disease. In patients suspected of having pulmonary vascular obstructive disease, cardiac catheterization is essential to accurately measure pulmonary vascular resistance to know if the patient is a candidate for closure of the VSD.

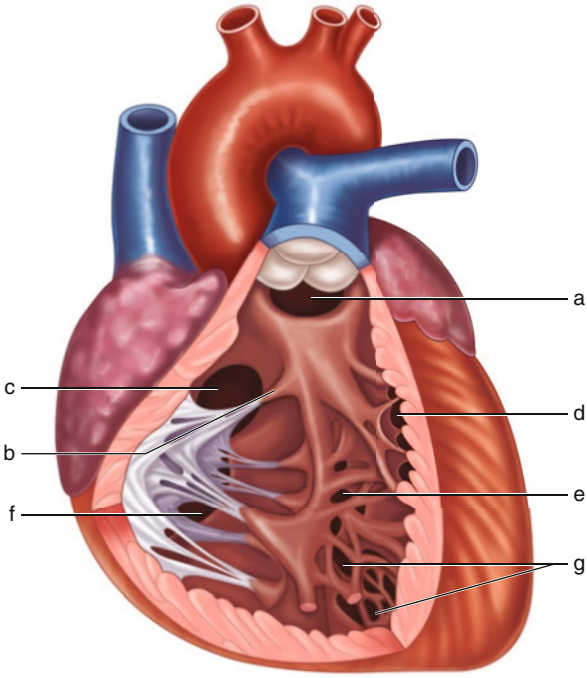

Fig. 22.1 Diagrammatic representation of the types and locations of VSDs. The anterior surface of the heart has been removed, revealing the ventricular septum as viewed from the right side. *Abbreviations*: *a* outlet or supracristal VSD, *b* septal papillary muscle of the tricuspid valve, *c* perimembranous VSD, *d* right ventricular muscle bundle, *e* muscular VSD, *f* inlet VSD, *g* muscular VSDs

ventricular outflow tract and immediately below the right cusp of the aortic valve. An *inlet VSD* is located posteriorly in the septum just inferior to the tricuspid and mitral valves. This is a type of endocardial cushion defect. A *muscular VSD* is located in the muscular ventricular septum. Patients can have more than one muscular VSD Fig. 22.1.

22.2 Pathologic Physiology

In uncomplicated VSD, the flow of blood is from the left ventricle through the VSD traversing the right ventricle and into the pulmonary artery. This results in a volume overload of the pulmonary circulation, the left atrium, and the left ventricle. A systolic murmur is created by blood flowing through the VSD (for restrictive VSDs) or through the pulmonary valve (for unrestrictive VSDs). If the patient develops pulmonary vascular obstructive disease, this direction of flow of blood can reverse such that desaturated blood from the right ventricle will traverse the VSD into the left ventricle and out the aorta. These patients may exhibit cyanosis.

22.3 Clinical Presentation

The clinical presentation of patients with VSD depends primarily upon the size of the VSD and, to a much lesser extent, upon the type of VSD. If the VSD is as large as the aorta, it is considered to be a large VSD. More than the size of the VSD,

22.6 Treatment

The goals of treatment are to insure adequate growth of the patient, prevent the development of pulmonary vascular obstructive disease, prevent chronic right and left ventricular dysfunction, and prevent bacterial endocarditis.

Small VSDs do not require surgical closure unless they are supracristal.

Infants with moderate to large VSDs associated with congestive heart failure are treated with medication such as afterload reducing agents and diuretics. High caloric formula can be used to optimize growth if growth failure results from the congestive heart failure. If, despite these measures, growth failure continues, the defect should be closed surgically. For infants with pulmonary hypertension, the VSD should be closed before 6–9 months of age to prevent the development of irreversible pulmonary vascular obstructive disease.

22.7 Outcome

Provided that operation is done prior to the onset of pulmonary vascular obstructive disease, the surgical mortality is less than 5 % and the long-term outlook is excellent.

Human Genetics of Ventricular Septal Defect

23

Katherina Bellmann, Andreas Perrot, and Silke Rickert-Sperling

Contents

Abstract

Ventricular septal defects (VSDs) are recognized as one of the commonest congenital heart diseases (CHD), accounting for up to 40 % of all cardiac malformations, and occur as isolated CHDs as well as together with other cardiac and extracardiac congenital malformations in individual patients and families. The genetic etiology of VSD is complex and extraordinarily heterogenous. Chromosomal abnormalities such as aneuploidy and structural variations as well as rare point mutations in various genes have been reported to be associated with this cardiac defect. This includes both well-defined syndromes with known

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genetic cause (e.g., DiGeorge syndrome and Holt–Oram syndrome) and so far undefined syndromic forms characterized by unspecific symptoms. Mutations in genes encoding cardiac transcription factors (e.g., *NKX2-5* and *GATA4*) and signaling molecules (e.g., *CFC1*) have been most frequently found in VSD cases. Moreover, new high-resolution methods such as comparative genomic hybridization enabled the discovery of a high number of different copy number variations, leading to gain or loss of chromosomal regions often containing multiple genes, in patients with VSD. In this chapter, we will describe the broad genetic heterogeneity observed in VSD patients considering recent advances in this field.

23.1 Introduction

Ventricular septal defects are recognized as one of the commonest congenital heart defects, accounting for up to 40 % of all cardiac malformations [1]. They can be classified according to their location, either within the muscular septum (muscular defects) or at its margins (perimembranous and supracristal defects) (see Chap. 22) [1]. Further, ventricular septal defect (VSD) is not only a common isolated congenital heart disease (CHD) but is often associated with other congenital cardiac defects, either in an individual or within a family. This heart defect also exists as an intrinsic component of several complex malformations, including tetralogy of Fallot (see Chap. 31) and univentricular heart (see Chap. 49) [1].

Epidemiologic studies strongly suggest that genetic factors play an important role in CHD etiology, although environmental exposures are also relevant (see Chap. 16) [2]. In a nationwide population-based study, Oyen et al. reported that CHDs in general show highly variable familial clustering in first degree relatives and indicated a threefold recurrence risk for isolated VSD [3]. In the following chapter, we will focus on the role of genetic factors associated with ventricular septal defects and describe their various genetic causes including chromosomal aberrations, structural variants, and single disease genes and mutations.

23.2 Isolated VSD

A number of studies have shown that isolated VSDs (without further cardiac and/or extracardiac congenital defects) can be associated with copy number variations as well as single gene mutations in patients and related families. Of note, the affected families often comprise individuals showing isolated VSDs as well as individuals with other CHDs (the latter will be presented in Sect. 23.3).

23.2.1 Copy Number Variation in Isolated VSD

A copy number variation (CNV) is a structural genomic variant that results in confined copy number changes in a specific chromosomal region that often contains multiple contiguous genes [4]. Commonly, CNVs are defined as any submicroscopic

chromosomal changes affecting more than 1000 bases [5]. Frequently, microdeletions and microduplications are identified by high-resolution comparative genomic hybridization (array CGH) as changes of DNA quantity (see Chap. 18) [4].

Studies analyzing CNVs commonly used cohorts of patients with a broad range of different CHDs [6–10]. To date, in five studies analyzing a range of CHDs including isolated VSDs, a total of eight affected loci have been identified with the chromosomal region 22q11.2 and 8p23.1 being most frequently affected. At the 22q11.2 locus, the *TBX1* gene (transcription factor T-box 1) known to be implicated in DiGeorge syndrome (see Sect. 23.4.2) was duplicated in one familial case [6], while the protein kinase *CRKL* gene (V-Crk avian sarcoma virus CT10 oncogene homolog-like) was affected in two independent patients [9, 10]. The locus 8p23.1 contains the candidate genes *GATA4* (transcription factor GATA binding protein 4), playing an important role in cardiac development (see Sect. 23.2.2.1), and *SOX7* (SRY (sex determining region Y)-box 7) [8, 9]. Rare copy number gains at the locus 11q25 were found in two patients but not associated with known risk genes [6, 7]. A summary of all CNVs found in isolated VSD cases is given in Table 23.1.

23.2.2 Single Gene Defects in Isolated VSD

In patients with isolated VSD, a number of different mutations have been found in genes encoding for transcription factors, signaling molecules, and proteins of other functions (see Table 23.2).

23.2.2.1 Transcription Factors

The development of the heart is orchestrated by transcription factor (TF) networks including members of the NK2 homeobox, T-box, and GATA binding families (see Chap. 12) [26]. GATA binding protein 4 (GATA4) is a transcriptional activator found to be affected in sporadic and familial cases of isolated VSD. Three different missense mutations in the *GATA4* gene (p.Pro407Val, p.Ser175Cys, and p.Ala411Val) have been reported in sporadic cases [12–14]. In addition, two families carrying *GATA4* missense mutations (p.Arg43Trp and p.Gly296Arg) were discovered in a follow-up analysis of index patients that participated in a screen of unrelated individuals (see also Sect. 23.3.2.1) [15, 16]. In the follow-up of affected family members, two and four additional cases of isolated VSD were found in the studies by Wang et al. [15] and Yang et al. [16], respectively.

So far, mutations affecting three members of the T-box family, namely, TBX1, TBX5, and TBX20, have been associated with isolated VSDs. Pan et al. screened 230 CHD cases and observed a heterozygous nonsense mutation (p.Gln277X) in the DNA-binding domain of *TBX1* in one patient with double outlet right ventricle who had one affected relative with isolated VSD carrying the same mutation (see also Sect. 23.3.2.1) [19]. A missense mutation (p.Ile152Met) in *TBX20* causing impaired DNA binding was found in a family affected by multiple septal defects including isolated VSD, atrial septal defect (ASD), and a large patent foramen ovale in different relatives [21]. In the case of *TBX5*, a non-coding variant in one of its enhancers was suggested to impact on the development of VSD [20]. The variant was found homozygous in a case of isolated VSD with unaffected heterozygous parents and

Table 23.1 Copy number variation in isolated VSD

Cytoband	CNV start (hg18)	CNV end (hg18)	Size	Copy number	Candidate genes	References
1q21.1 22q11.2	144723763 19389671		1574 kb 406 kb	Deletion Duplication	*ACP6, BCL9, CHD1L, FMO5, GJA5, PRKAB2 CRKL*	[9]
2p22.3	32.51 Mb	33.21 Mb	0.70 Mb	Duplication	*LTBP1*	[6]
3p14.2				Deletion		[8]
3p22.1				Deletion		[8]
3q25				Deletion		[8]
3q29				Duplication		[8]
5q31.3				Deletion		[8]
6p12.1	55356489	55493937	137 kb	Deletion		[10]
6q24.1	142187041	142290373	103 kb	Deletion		[10]
8p23.1				Deletion	*GATA4*	[8]
8p23.1				Duplication		[8]
8p23.1	8027361		4456 kb	Deletion	*GATA4, SOX7*	[9]
9p24.1	6770364	6953533	183 kb	Deletion		[10]
9q33.2	124774046	125024684	251 kb	Duplication		[10]
11p13	34458230[a]	34460862[a]	2.6 kb	Deletion		[7]
11p15.4				Deletion		[8]
11q25	134598043[a]	134617838[a]	19.8 kb	Duplication		[7]
11q25	132.23 Mb	132.76 Mb	0.53 Mb	Duplication		[6]
14q32.12	92475603	92709736	234 kb	Duplication		[10]
15q11.2	20384417		251468 bp	Deletion		[8]
15q13.3				Duplication		[8]
16p13.11				Duplication		[8]
17q12				Duplication		[8]
18q11.1-11.2	16795645		6118 kb	Duplication	*GATA6*	[9]
18q22.1				Duplication		[8]
18q23	75996798	75076224	921 kb	Duplication	*NFATC1*	[10]
20p12.3				Duplication		[8]
20q13.2				Duplication		[8]
22q11.2	17.39 Mb	19.74 Mb	2.35 Mb	Duplication	*TBX1, CRKL*	[6]
22q11.2				Deletion		[8]
22q11.2	19051034	19825156	774 kb	Deletion		[10]
Xq28	153436333	154895334	1.5 Mb	Deletion		[10]

Each row refers to one case
hg18 human reference genome (full sequence) version 18, *kb* kilobases, *Mb* megabases, *bp* basepairs
[a]Genomic coordinates refer to hg19

Table 23.2 Single gene defects in isolated VSD

Gene	Protein function	References
Transcription factors (TF)		
CITED2	Transcriptional coactivator	[11]
GATA4	GATA binding TF	[12–16]
IRX4	Iroquois homeobox TF	[17]
NKX2-6	Homeobox TF	[15]
PITX2	Homeodomain TF	[18]
TBX1	T-box TF	[19]
TBX5	T-box TF	[20]
TBX20	T-box TF	[21]
Signaling molecules		
CFC1	Ligand (TGFβ signaling)	[22]
GDF3	Ligand (TGFβ signaling)	[23]
TDGF1	Co-receptor (TGFβ signaling)	[24]
Other genes		
HAS2	Hyaluronan synthase	[25]

supported by functional evidence based on transgenic expression studies in mouse and zebrafish [20].

A study focusing on the analysis of *PITX2* (paired-like homeodomain 2) in a cohort of 170 unrelated neonates with CHD found two missense mutations (p.Arg91Gln and p.Thr129Ser) in two affected families [18]. Four mutation carriers presented with isolated VSD whereas two other relatives showed transposition of the great arteries (TGA) with VSD (see Sect. 23.3.2.1) [18].

Mutations have also been identified in the TF genes *NKX2-6*, *CITED2*, and *IRX4* [11, 17, 27]. Screening a CHD cohort including 66 isolated VSD cases, Wang et al. identified a missense mutation (p.Lys152Gln) in the homeodomain of *NKX2-6* (NK2 homeobox 6) [27]. Subsequent analysis identified the mutation in two further family members with isolated VSDs [27]. In a cohort of nearly 400 sporadic CHD cases, a nine amino acid deletion (p.Ser170_Gly178del) in *CITED2* (Cbp/P300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2), resulting in impaired activity, was detected by us in one patient with isolated perimembranous VSD [11]. For the ventricle-specific TF *IRX4* (Iroquois homeobox 4), two missense mutations (p.Asn85Tyr and p.Glu92Gly) were reported in two unrelated patients with isolated VSD by direct sequencing of the gene in a cohort of about 700 CHD patients [17]. The two mutations affected the interaction with retinoid X receptor alpha, a nuclear receptor of the vitamin A signaling pathway important in cardiac morphogenesis.

23.2.2.2 Signaling Molecules

Various cellular processes in both the embryonic and adult organism are regulated via transforming growth factor beta (TGFβ) signaling pathways. Important

developmental steps, such as the establishment of left–right asymmetry, are driven by the NODAL signaling pathway, which is named after the TGFβ superfamily member of the same name (see Chap. 7). Two genes encoding cofactors of the NODAL signaling pathway, *TDGF1* (teratocarcinoma-derived growth factor 1 also known as CRIPTO) and *CFC1* (Cripto, FRL-1, Cryptic family 1 also known as CRYPTIC), have been analyzed in a cohort of 500 CHD cases [22, 24]. Three missense mutations, p.Arg41Gly in *TDGF1* [24] as well as p.Leu219Phe and p.Gly169Val in *CFC1* [22], were identified in three patients with isolated VSD (see also Sect. 23.3.2.2). Another member of the NODAL signaling pathway, *GDF3* (growth differentiation factor 3), was analyzed by Xiao et al. [23]. Direct sequencing of *GDF3* in a cohort of 200 CHD patients led to the identification of a missense mutation (p.Ser212Leu) in a patient with isolated muscular VSD [23].

23.2.2.3 Other Genes

The gene *HAS2* encodes hyaluronan synthase 2, an enzyme that synthesizes hyaluronic acid (a major component of the extracellular matrix) during embryogenesis [25]. Among 100 non-syndromic VSD cases, Zhu et al. detected a *HAS2* missense mutation (p.Glu499Val) in one patient. The synthesis of hyaluronic acid was significantly impaired in the mutant enzyme as shown by *in vitro* assays [25].

23.3 Non-syndromic VSD

VSDs do not only occur as isolated malformations but most frequently are part of a more complex malformation. In the absence of extracardiac malformations, these VSDs are classified as "non-syndromic" (in contrast to syndromic VSD; see Sect. 23.4). Of note, VSD is an intrinsic component of complex malformations such as tetralogy of Fallot and double outlet right ventricle as well as univentricular heart (see Chaps. 31 and 49, respectively).

23.3.1 Copy Number Variation in Non-syndromic VSD

Two microduplications and two microdeletions were described in four cases of VSD with additional cardiac malformations (see Table 23.3) [6, 9]. Tomita-Mitchell et al. analyzed a cohort of several hundred CHD cases and identified among others one patient with VSD and pulmonary atresia who carried a duplication at the locus 1q21.1 comprising candidate genes such as *CHD1* (chromodomain helicase DNA binding protein 1) and *GJA5* (gap junction protein alpha 5, 40 kDa; also known as connexin 40) [9]. Screening a cohort of 105 CHD patients by array CGH, Erdogan et al. found a deletion at the 22q11.2 locus (including *TBX1*) in a case of VSD and aortic coarctation [6]. Further, they detected a duplication at chromosome 4q32.3 in one patient with VSD and PDA and a large 4 megabase deletion at chromosome 17p11.2 in a patient with VSD and ASD. No evident candidate genes have been identified in either of these regions [6].

Table 23.3 Copy number variation in non-syndromic VSD

Cytoband	CNV start (hg18)	CNV end (hg18)	Size	Copy number	Candidate genes	References
1q21.1	144812585		1480 kb	Duplication	*ACP6, BCL9, CHD1L, FMO5, GJA5, PRKAB2*	[9]
4q32.3	167.52 Mb	169.28 Mb	1.76 Mb	Duplication		[6]
17p11.2	16.47 Mb	16.47 Mb	3.98 Mb	Deletion		[6]
22q11.2	17.39 Mb	20.00 Mb	2.61 Mb	Deletion	*TBX1*	[6]

Each row refers to one case
hg18 human reference genome (full sequence) version 18, *kb* kilobases, *Mb* megabases

Table 23.4 Single gene defects in non-syndromic VSD

Gene	Protein function	References
Transcription factors (TF)		
GATA4	GATA binding TF	[15, 16, 28]
NKX2-5	Homeobox TF	[12, 29–32]
PITX2	Homeodomain TF	[18]
TBX1	T-box TF	[19]
ZIC3	Zink finger TF	[33]
Signaling molecules		
NF1	Negative regulator (RAS signaling)	[34]
Sarcomere genes		
MYH7	Thick filament	[35, 36]
TNNI3	Thin filament	[37]

23.3.2 Single Gene Defects in Non-syndromic VSD

Mutations have been reported in transcription factors, signaling molecules, and sarcomeric proteins in patients and families with non-syndromic VSD (see Table 23.4).

23.3.2.1 Transcription Factors

The evolutionary highly conserved homeobox factor NKX2-5 controls the expression of various cardiac genes during heart development [26]. Frameshift and missense mutations in the DNA-binding domain of *NKX2-5* have been described in six families mainly affected by ASD and atrioventricular conduction block, but also showing other CHDs [29–32]. In total, 11 affected mutation carriers from those families showed additional VSDs [29–32]. Moreover, screening of 135 sporadic CHD cases revealed an *NKX2-5* missense mutation (p.Pro283Gln) in a patient characterized by VSD, ASD, and PDA [12].

Direct interaction partners of NK2 homeobox 5 include the cardiac TFs T-box 5 and GATA binding protein 4. In a large pedigree with familial ASD, Garg et al. described a *GATA4* missense mutation (p.Gly296Ser) located between the nuclear localization sequence and one of two *GATA4* zinc fingers, which altered the interaction between *GATA4* and *TBX5* [28]. Three affected mutation carriers in that family presented with an additional VSD [28]. Furthermore, sequencing of *GATA4* in two VSD cohorts revealed two missense mutations (p.Arg43Trp and p.Gly296Arg) in two families [15, 16]. Besides isolated VSDs, affected family members presented VSDs in combination with ASD in three cases and with PDA in one case (see also Sect. 23.2.2.1) [15, 16].

Additional cardiac TF genes mutated in patients with non-syndromic VSD comprise *TBX1*, *ZIC3* (Zic family member 3), and *PITX2*. A nonsense mutation in *TBX1* (p.Gln277X) has been found in a family including relatives with isolated VSD as well as one individual with VSD and PDA (see Sect. 23.2.2.1) [19]. *ZIC3*, a zinc finger TF known for its association with laterality defects (see Chap. 38), was mutated in one sporadic heterotaxy case showing VSD in combination with ASD, pulmonary stenosis, and TGA [33]. Wei et al. identified two subjects with VSD and TGA from two affected families who carried *PITX2* missense mutations (see also Sect. 23.2.2.1) [18].

23.3.2.2 Signaling Molecules

As described before, various signaling pathways are active during cardiac development such as the NODAL signaling pathway (see Sect. 23.2.2.2). In a cohort of 362 severe CHD cases, the *NF1* gene encoding neurofibromin 1, a negative regulator of the RAS signaling pathway, was found to be mutated in one case with VSD accompanied by pulmonary atresia and multiple aorticopulmonary collaterals [34].

23.3.2.3 Sarcomere Genes

Contraction of the heart involves the shortening of sarcomeres by the ATP-dependent interaction between thin (actin) and thick (myosin) filaments (see Chap. 17). Mutations in genes encoding sarcomeric proteins have been well established as disease-causing for different forms of cardiomyopathy (see Chap. 59). For *MYH7* encoding cardiac specific β-myosin heavy chain, two mutations (p.Met362Arg and p.Glu1220del, respectively) in two families characterized by Ebstein's anomaly (EA), left ventricular noncompaction cardiomyopathy (LVNC), and VSD were reported [35, 36]. Two mutation carriers from each family showed the phenotype with the combination of EA, LVNC, and VSD [35, 36]. Troponin I (encoded by *TNNI3*) is a cardiac specific thin filament component important for calcium sensing during contraction of the heart muscle. Yang et al. detected a *de novo* missense mutation in *TNNI3* (p.Arg204His) in a patient first diagnosed with perimembranous VSD who then gradually developed restrictive cardiomyopathy [37].

23.4 Syndromic VSD

In the following section, we describe VSDs observed in patients showing additional congenital malformations in other organs. Those so-called syndromic forms include well-defined syndromes with known genetic cause (e.g., DiGeorge syndrome) as

well as so far undefined syndromic forms with unspecific symptoms such as mental retardation and dysmorphic features of unknown genetic etiology.

23.4.1 Aneuploidy Syndromes

Chromosomal aneuploidy is the presence of an abnormal number of chromosomes in the cell leading to various syndromic genetic disorders. Aneuploidy syndromes can occur with almost any cardiac malformation [38]. CHD occurs in about 45–50 % of patients with Down syndrome (trisomy 21), the most common aneuploidy syndrome [39, 40]. Källen et al. showed in a large epidemiologic study with more than 5000 patients with trisomy 21 that VSD was present in 28 % of individuals with cardiac malformation [41]. In the National Down Syndrome Project cohort, Freeman et al. found a VSD rate of 19 % regarding all registered infants (65 % were membranous and 35 % were muscular VSDs) [40]. A retrospective cohort study including about 4300 Down syndrome patients undergoing CHD surgery was performed by Fudge et al. to examine postoperative outcomes [42]. VSD closure of any type was the second most common procedure (19 %) performed for patients with Down syndrome [42].

Further, CHD occurs in about 35 % of cases of Patau syndrome (trisomy 13) and about 45 % of cases with Edwards syndrome (trisomy 18) as Pont et al. described in a large epidemiologic study of hospitalizations of live-born infants with chromosomal abnormalities [43]. VSD was the most common heart defect in trisomy 13 (18 %) and in trisomy 18 (31 %) [43]. Pallister–Killian syndrome, a sporadic multisystem developmental disorder, is typically caused by the presence of a supernumerary isochromosome composed of the short arms of chromosome 12 resulting in tetrasomy 12p [44]. Tilton et al. evaluated 30 patients with this syndrome and CHD and found a VSD in 10 % of those [44].

23.4.2 Copy Number Variation in Syndromic VSD

A number of studies have shown the importance of CNVs, mainly microdeletions and microduplications, in syndromic CHD [38, 45]. Those studies reported subjects with well-known syndromes as well as so far undefined syndromic forms.

In Table 23.5, clinically delineated microdeletion and microduplication syndromes are listed in which a specific association to VSD was described. Of note, the most frequent genomic disorder associated with CHD is DiGeorge syndrome (DGS; 22q11 deletion or velocardiofacial syndrome) (see, e.g., Chap. 38). Cardiovascular anomalies are present in about 80 % of neonates with DGS [54]. Ryan et al. evaluated a cohort of 545 DGS patients and could show that VSDs were observed in 14 % of these patients (among other heart defects) [55]. Momma described in his review of several studies of DGS cohorts (ranging from 100 up to 222 patients) quite similar VSD rates [54]. All other syndromes show lower VSD rates. For example, in Williams–Beuren syndrome (caused by a deletion of about 1.5 megabases in chromosome 7q11.23), VSDs, mainly muscular ones (75 %), are present in 4–9 % of all

Table 23.5 Copy number variation in well-defined syndromic VSD

Cytoband	Copy number	Syndrome	Candidate genes[a]	References
4p16.3	Deletion	Wolf–Hirschhorn	*WHSC1, FGFRL1*	[46]
5p15.2	Deletion	Cri-du-chat	*TERT*	[47]
5q35.2-q35.3	Deletion	Sotos	*NSD1*	[48]
7q11.23	Deletion	Williams–Beuren	*ELN*	[49]
9q34.3	Deletion	Kleefstra	*EHMT1*	[50]
11q23	Deletion	Jacobsen	not specified	[51]
17p11.2	Deletion	Smith–Magenis	*RAI1*	[52]
17p11.2	Duplication	Potocki–Lupski	*MAPK7*	[53]
22q11.2	Deletion	DiGeorge	*TBX1*	[54, 55]

Abbreviations: *WHSC1* Wolf–Hirschhorn syndrome candidate 1, *FGFRL1* fibroblast growth factor receptor-like 1, *TERT* telomerase reverse transcriptase, *NSD1* nuclear receptor binding SET domain protein 1, *ELN* elastin, *EHMT1* euchromatic histone-lysine *N*-methyltransferase 1, *RAI1* retinoic acid induced 1, *MAPK7* mitogen-activated protein kinase 7, *TBX1* T-box 1

[a]These include known disease genes (such as TBX1 and ELN) as well as genes causing heart defects when deleted in mice and/or by mutations in CHD patients

patients [49]. A similar VSD rate (8 %) was described by Jefferies et al. in patients with Potocki–Lupski syndrome whereby most individuals harbor a common 3.7 megabase duplication within chromosome 17q11.2 [53].

Besides well-known syndromes, there are a number of studies describing syndromic patients with VSD who present different unspecific symptoms (see Table 23.6). Using array-based CGH, Syrmou et al. screened a cohort of 55 syndromic CHD patients and detected CNVs in 37 of them [57]. They found five patients with VSD showing either one CNV or a combination of up to three different deletions and duplications (ranging from 0.023 to 6.6 megabases) [57]. Through a genome-wide survey of two independent cohorts of CHD subjects with extracardiac abnormalities (700 subjects in total), Lalani et al. identified 16 CNV regions, present in two or more cases and absent in about 3000 controls [60]. Interestingly, one of the most frequent CNVs they found was a *de novo* copy number loss of 16q24.3 (affecting *ANKRD11* encoding ankyrin repeat domain 11) in five subjects of whom four presented with VSD together with other CHDs (two with perimembranous and one each with muscular and conoventricular VSD) [60]. Screening a cohort of 60 syndromic CHD cases by array CGH, Thienpont et al. identified among others one patient with muscular VSD and various extracardiac manifestations showing a 3.8 megabase duplication at locus 19p13.12-13.11 [61]. Using the same detection method, a similar cohort of 90 patients was analyzed by Breckpot et al. [56]. They found two deletions at locus 1p36.33 (ranging from 3.5 to 5.9 megabases) in two patients with VSD and minor extracardiac malformations, one subject with additional hypertrophic cardiomyopathy and one with microcephaly [56]. Goldmuntz et al. analyzed 58 syndromic CHD cases and reported six different CNVs in six patients characterized by VSD and further congenital abnormalities and dysmorphic features such as cleft palate [59]. They detected copy number gains at loci 5q21.1-21.2 and 18p11.32, whereas losses were detected for the loci 9p23,

Table 23.6 Copy number variation in syndromic VSD with unspecific symptoms

Cytoband	CNV start (hg18)	CNV end (hg18)	Size	Copy number	Candidate genes	References
1p36.33	1 kb	4608–5866 kb	4.61–5.87 Mb	Deletion		[56]
1p36.33	1 kb	3514–3519 kb	3.51–3.52 Mb	Deletion		[56]
1q41 Xp11.3 Xq21.31			0.37 Mb 0.042 Mb 0.118 Mb	Duplication Deletion Deletion	*DISP1*	[57]
3p24.1-23	29,757 kb		1488 kb	Deletion		[58]
3q13.32 10q26.3 16p12.1			0.074 Mb 0.043 Mb 0.023 Mb	Duplication Deletion Duplication		[57]
4q34.1	185603346[b]	185638397[b]	35.1 kb	Deletion		[7]
5q21.1-21.2	100934417[a]	103557609[a]	2.6 Mb	Duplication		[59]
6p24.1-22.3			6.64 Mb	Deletion	*EDN1, DTNBP1, MYLIP*	[57]
6q24.2-25.1	143649–143708 kb	150253–150271 kb	6.62 Mb	Deletion		[56]
9p23	9163303[a]	9606645[a]	0.44 Mb	Deletion		[59]
10p12.1-11.21	28728683[a]	35905297[a]	7.1 Mb	Deletion	*NRP1*	[59]
15q26.1	86439405[a]	90753814[a]	4.3 Mb	Deletion	*NTRK3, MESP1*	[59]
16q24.3	86406011–86406037	87962518–87962533	1.58 Mb	Deletion	*ANKRD11*	[60]
16q24.3	86051611	88133224	2.07 Mb	Deletion	*ANKRD11*	[60]
16q24.3	87822867–87862929	88001859–88011936	139 kb	Deletion	*ANKRD11*	[60]
16q24.3			1.8 Mb	Deletion	*ANKRD11*	[60]
18p11.32	284494[a]	918189[a]	0.64 Mb	Duplication		[59]
19p13.12-13.11			3.86 Mb	Duplication		[61]
20p12.2 21q21.3			0.219 Mb 0.095 Mb	Duplication Duplication	*ADAMTS, ADAMTS5*	[57]
22q11.21	18351387[a]	20306802[a]	1.96 Mb	Deletion		[59]

Each row refers to one case
hg18 human reference genome (full sequence) version 18, *kb* kilobases, *Mb* megabases
[a]Genomic coordinates refer to hg17
[b]Genomic coordinates refer to hg19

10p12.1-11.21, 15q26.1, and 22q11.21 (ranging from 0.4 to 7.1 megabases in length) [59]. Analyzing several hundred CHD trios (non-syndromic and syndromic cases) by SNP arrays and whole exome sequencing, a paternally inherited deletion of 35 kilobases at chromosome 4q34.1 was detected in a patient with VSD, TGA, and extracardiac manifestations [7]. Fakhro et al. genotyped a cohort of more than

250 heterotaxy cases by SNP arrays: they identified a heterozygous 1.5 megabase deletion at locus 3p24.1-23 (affecting *TGFBR2* encoding TGFβ receptor II) in a patient characterized by VSD, ASD, partial anomalous pulmonary venous return, and situs inversus (see Chap. 38) [58].

23.4.3 Single Gene Defects in Syndromic VSD

In addition to structural genomic variations, syndromic CHD can be caused by point mutations influencing the dosage of genes functioning in developmental pathways that are broadly used in organogenesis and therefore affecting many organs. As patients covered in Sect. 23.4.2, the cases presented in the following section are also characterized by a variety of extracardiac manifestations. All described single gene syndromes are summarized in Table 23.7.

23.4.3.1 Transcription Factors

Mutations in the transcription factor *TBX5* (T-box 5) cause Holt–Oram syndrome (HOS), which is characterized by upper-extremity malformations and cardiac defects including VSD [106] (see Chap. 20). Septal defects (VSD and ASD) are the most common cardiac malformations observed in HOS as Al-Qattan et al. showed in their meta-analysis of 16 different studies [106]. In total, 13 different TBX5 mutations (mostly missense ones) were associated with VSDs as part of complex cardiac malformations [106]. Borozdin et al. described VSDs in five out of 23 HOS patients with *TBX5* mutations of which four were accompanied by ASD [75]. Another T-box gene, *TBX3*, is causative to ulnar–mammary syndrome characterized by limb malformations and sporadically associated with VSDs [73, 74].

Cardiac malformations, in particular VSDs, are described in about 14 % of Townes–Brocks syndrome patients, which are further characterized by hand and ear abnormalities and are caused by mutations in *SALL1* encoding spalt-like transcription factor, a zinc finger TF [70]. In contrast, mutations of *SALL4* (spalt-like transcription factor 4) cause Okihiro syndrome that is characterized by forearm and renal malformations (also known as Duane-radial ray syndrome) and rarely associated with VSD [71, 72].

VSDs are frequently seen in syndromes such as oculofaciocardiodental (OFCD), Ellis–van Creveld, Mowat–Wilson, and congenital diaphragmatic hernia with CHD. OFCD syndrome is an inherited X-linked dominant disorder caused by mutations in *BCOR* (BCL6 corepressor), a key transcriptional regulator during embryogenesis [62]. Ng et al. were the first to describe three OFCD patients who showed a VSD (one perimembranous, one subpulmonary, and one not further characterized VSD) [62]. In a later study, Hilton et al. presented six OFCD patients with VSD from 21 families showing that VSD was the second most common CHD in these patients [63].

Mutations in *EVC1* and *EVC2* (two leucine zipper transcription factors) lead to Ellis–van Creveld syndrome, which is mainly associated with atrioventricular septal defect and more sporadically with VSDs [64, 65]. Mowat–Wilson syndrome is

Table 23.7 Single gene defects in well-defined syndromic VSD

Gene	Protein function	Syndrome	References
Transcription factors			
BCOR	Transcriptional corepressor	Oculofaciocardiodental (OFCD) (X)	[62, 63]
EVC1, EVC2	Leucine zipper proteins	Ellis–van Creveld	[64, 65]
FOXL2	Forkhead TF	Blepharophimosis (AD)	[66, 67]
GATA6	GATA binding TF	Diaphragmatic hernia	[68, 69]
SALL1	Spalt-like TF	Townes–Brocks	[70]
SALL4	Spalt-like TF	Okihiro	[71, 72]
TBX3	T-box TF	Ulnar–mammary	[73, 74]
TBX5	T-box TF	Holt–Oram	[75]
TFAB2B	AP-2 TF	Char	[76]
ZEB2/ZFHX1B	Zinc finger homeobox TF	Mowat–Wilson	[77]
Signaling molecules			
FGFR3	Fibroblast growth factor	Muenke	[78]
HRAS	Ras/MAPK signaling	Costello	[79]
JAG1, NOTCH2	Notch ligand, Notch signaling	Alagille	[80]
KRAS	Ras/MAPK signaling	Noonan 3	[81]
NOTCH1	Notch signaling	Adams–Oliver 5	[82]
PTPN11	Protein tyrosine phosphatase	Noonan 1	[83, 84]
ROR2	Receptor protein tyrosine kinase	Robinow (AR)	[85]
SHOC2	Ras/MAPK signaling	Noonan-like	[86]
SOS1	Ras/MAPK signaling	Noonan 4	[87]
WNT5A, DVL1	Wnt signaling	Robinow (AD)	[88, 89]
Chromatin regulators			
CHD7	DNA helicase	CHARGE	[90]
MLL2	Methyltransferase	Kabuki	[91, 92]
NIPBL	Sister chromatid cohesin	Cornelia de Lange 1	[93, 94]
NSD1	Methyltransferase	Sotos (AD)	[95]
LBR	Chromatin binding	Pelger–Huet	[96]
Structural and cell adhesion molecules			
FBN1	Fibrillin	Marfan with hypophosphatemia (X)	[97]
GPC3	Proteoglycan	Simpson–Golabi–Behmel (X)	[98]
SH3PXD2B	Adapter protein	Frank–ter Haar (AR)	[99]
Enzymes for posttranslational modification			
B3GALTL	Glycosyltransferase	Peters plus	[100–102]
B3GAT3	Glucuronyltransferase	Larsen-like	[103]

(continued)

Table 23.7 (continued)

Gene	Protein function	Syndrome	References
Other			
DNAI1, DNAH5	Ciliary dynein arm proteins	Kartagener	[104]
MID1	RING finger ubiquitin ligase	Opitz (X)	[105]
MKRN2	E3 ubiquitin ligase	Heterotaxy	[34]

AD autosomal recessive, *AR* autosomal recessive, *X* X linked, *TF* transcription factor

caused by mutations in *ZEB2 (ZFHX1B)* encoding a zinc finger E-box binding factor and is frequently accompanied by cardiac malformations including VSD and ASD [77]. Mutations in *GATA6* have been found in familial cases of congenital diaphragmatic hernia accompanied by VSD [68, 69].

Char and blepharophimosis syndrome infrequently present with VSD. Char syndrome is caused by mutations in the gene *TFAP2B* (transcription factor AP-2 beta) and associates with patent ductus arteriosus (PDA) [76]. However, in one case Char syndrome was accompanied by VSD [76]. Blepharophimosis syndrome is an autosomal dominant disorder that is characterized by a malformation of the eyelids and is caused by loss-of-function mutations in the forkhead TF *FOXL2* (forkhead box L2) [66, 67]. VSDs are rare (about 1 %) in patients with this syndrome [66, 67].

23.4.3.2 Signaling Molecules

Inter- and intracellular communication via signaling molecules coordinates heart development, as has been shown, for example, for the Notch signaling pathway (see Chap. 11). Alagille syndrome, an autosomal dominant multisystem disorder, is associated with CHD in about 25 % of patients [80]. This syndrome is caused by mutations in either *JAG1* or *NOTCH2*, with Jagged 1 being a ligand of the membranous Notch 2 receptor. Emerick et al. showed in a cohort of 73 Alagille syndrome patients a frequent association with cardiac malformation of which 32 % showed VSDs [80]. A further NOTCH signaling driven syndrome is Adams–Oliver syndrome characterized by limb defects and less frequent cardiac malformations including VSDs [82]. A study based on 11 familiar cases identified truncating mutations in *NOTCH1* of which three cases showed VSDs [82].

Mutations of genes of the Ras/MAPK (Ras/mitogen-activated protein kinase) signaling pathway represent a frequent cause of Noonan syndrome, which is after Down syndrome the most common syndromic disorder involving cardiac malformations [83]. Noonan syndrome is genetically heterogenous and characterized by short stature, facial dysmorphism, and cardiac defects of different nature such as pulmonary stenosis, hypertrophic cardiomyopathy, ASD, and at a lower rate VSDs. *PTPN11* (protein tyrosine phosphatase non-receptor type 11) causing Noonan syndrome 1 was described being mutated in about 50 % of cases [83]. Snayer et al. reported an incidence of 7 % of VSDs in Noonan syndrome 1 patients [84]. Single case reports showed VSDs in the less frequent Noonan syndrome 3 caused by mutations in KRAS (Kirsten rat sarcoma viral oncogene homolog) [87] and Noonan

syndrome 4 caused by mutations in SOS1 (son of sevenless homolog 1) [81]. In 25 patients with a phenotype termed Noonan-like syndrome with anagen hair, the missense mutation p.Ser2Gly in *SHOC2* (Soc-2 suppressor of clear homolog), a leucine-rich repeat-containing protein, was detected by Cordeddu et al. [86]. While the majority of patients have cardiac malformations, VSDs were found only in two cases [86]. Mutations in another member of the Ras/MAPK pathway, *HRAS* (Harvey rat sarcoma viral oncogene homolog), cause Costello syndrome, a disorder phenotypically closely related to Noonan syndrome. For Costello syndrome, one case report with VSD has been published [79].

Robinow syndrome is a rare skeletal dysplasia, inherited either in an autosomal dominant or recessive manner. Autosomal dominant Robinow syndrome, either caused by pathogenic variants in *WNT5A* (wingless-type MMTV integration site family member 5A) or *DVL1* (dishevelled segment polarity protein 1), is frequently accompanied by VSDs [88, 89]. In contrast, VSDs are rarely present in autosomal recessive Robinow syndrome, caused by biallelic pathogenic variants in *ROR2* (receptor tyrosine kinase-like orphan receptor 2) [85]. A further rare case with VSD has been documented for Muenke syndrome, which is characterized by fusion of cranial bones and is caused by mutations of *FGFR3* encoding fibroblast growth factor receptor 3 [78].

23.4.3.3 Chromatin Regulators

In syndromes related to chromatin regulators, the VSD rates greatly vary ranging from about 40 % in Cornelia de Lange syndrome to about 4 % in Sotos syndrome. Cornelia de Lange syndrome 1 is caused by mutations in *NIPBL* encoding Nipped-B homolog with putative sister chromatid cohesion function [93, 94]. From a cohort of 24 typical Sotos cases, Cecconi et al. observed one individual with VSD [95]. Sotos syndrome is caused by haploinsufficiency of the *NSD1* gene encoding a histone methyltransferase [95]. Microdeletions involving this gene are the major cause of the syndrome in Japanese patients (see Sect. 23.3) [48], whereas intragenic mutations are more frequent in non-Japanese patients [95].

About two-thirds of patients with CHARGE syndrome show cardiac malformations including 12 % with VSDs [90]. Genetically CHARGE syndrome is caused by mutations in *CHD7* encoding a chromodomain helicase DNA binding protein. A far less frequent association of VSDs is given in Pelger–Huet anomaly, where one case was described in a cohort of 20 families harboring mutations in *LBR* encoding for the lamin B receptor, which mediates the interaction of chromatin and lamin B [96].

Finally, mutations in *MLL2* encoding a DNA methyltransferase have been found in cases of Kabuki syndrome [91]. Half of these patients present with VSDs often accompanied by an ASD. In a meta-analysis, Yuan examined the cardiac phenotype of 76 published Kabuki cases and showed that about 20 % presented a VSD either isolated or as part of a complex cardiac malformation [92].

23.4.3.4 Structural and Cell Molecules

The integrity of the cell is maintained by structural proteins while cell adhesion factors are crucial for cell–cell contacts in a respective tissue. There are three different syndromes associated with VSD caused by mutations in structural and cell adhesion proteins.

Mutations in *SH3PXD2B* (SH3 and PX domains 2B, involved in cell adhesion and migration of numerous cell types) cause Frank–ter Haar syndrome, an autosomal recessive skeletal dysplasia characterized among others by cardiovascular malformations [99]. VSD is commonly seen in patients with this syndrome (in 50 % of mutation carriers) as Iqbal et al. showed in their study [99]. Lin et al. reviewed 26 patients with genetically confirmed Simpson–Golabi–Behmel syndrome, a disorder characterized by high birth weight and length, which is caused by mutations in *GPC3* encoding glypican 3, a cell surface proteoglycan [98]. A VSD in association with other CHDs was present in five of them (19 %) [98]. One case with Marfan syndrome (with the typical cardiovascular feature of aortic aneurysm) combined with X-linked hypophosphatemia showing VSD and ASD was described by Sheng et al. [97]. They found a *de novo* missense mutation in *FBN1* encoding fibrillin 1, a large extracellular matrix glycoprotein, using whole exome sequencing [97].

23.4.3.5 Enzymes for Posttranslational Modification

Enzymes involved in posttranslational modifications of proteins such as glycosylation play a role in two syndromes associated with VSDs. Peters plus syndrome (named after the Peters anomaly, an eye-chamber defect) is caused by mutations in *B3GALTL* encoding a glycosyltransferase involved in addition of glycans to proteins and is associated with VSDs in about one-third of cases [100–102]. The rare Larsen-like syndrome is caused by mutations in *B3GAT3* coding for glucuronyltransferase-I and is characterized by joint dislocations, short stature, craniofacial dysmorphism, and heart defects including VSDs [103].

23.4.3.6 Other Genes

Defects in ciliary structure cause Kartagener syndrome that is characterized by primary ciliary dyskinesia leading to situs inversus, the mirror image arrangement of all internal organs. It is caused by mutations in a number of genes encoding dynein arm components (see more details in Chap. 38). Kennedy et al. examined 21 Kartagener patients and found VSDs among other CHDs in three individuals (14 %) [104]. Zaidi et al. searched for *de novo* mutations in 362 severe CHD cases [34]. In one heterotaxy patient with abdominal situs inversus and CHD including VSD, they identified a missense mutation (p.Ala251Val) in *MKRN2* (encoding makorin ring finger protein 2, a putative E3 ubiquitin ligase) [34]. The gene implicated in the X-linked form of Opitz syndrome, *MID1* (midline 1), encodes a RING finger protein and is involved in the formation of multiprotein structures acting as anchor points to microtubules [105]. In a meta-analysis of several studies, Fontanella et al. showed that about 22 % of cases have heart defects with a high incidence of VSDs and ASDs [105].

23.5 Associations with Common Variations

Genome-wide associations studies (GWAS) look for associations between DNA sequence variants and phenotypes of interest [107]. They do so by studying many hundreds of individuals (affecteds and non-affecteds) and determining their

genotype at the positions of hundreds of thousands of single nucleotide polymorphisms (SNPs) followed by statistical analysis and subsequent confirmation experiments in replication cohorts [107].

A small case–control association study genotyped 58 SNPs in the TBX5 region in 192 VSD cases and matched controls of Han Chinese origin and identified a significant association of SNP rs11067075 within intron eight of the TBX5 gene with VSD [108]. Hu et al. performed a large GWAS in 945 cases with septation defects (ASD, VSD, and ASD/VSD) and 1246 controls of Han Chinese ancestry followed by two-stage validation with further 2160 cases and 3866 controls [109]. They identified a highly significant association of two SNPs at chromosome 1p12 (SNP rs2474937 near *TBX15*) and 4q31.1 (SNP rs1531070 near *MAML3* encoding mastermind like three involved in Notch signaling) [109]. A GWAS with 1995 CHD cases (including VSD) and 5159 controls of European Caucasian origin was carried out by Cordell et al. and failed to identify any SNPs with genome-wide significance [110]. A further replication study focusing on a subgroup analysis of about 200 VSD cases also did not find any significant association [110].

Conclusion

VSDs, one of the most common cardiac malformations in the general population, are commonly associated with a high number of well-known genetic syndromes caused by chromosomal aberrations or by smaller deletions and duplications as well as by single point mutations. Moreover, so far undefined syndromic disorders are often accompanied by VSDs.

As shown by a number of studies, mutations in genes encoding cardiac transcription factors (e.g., *NKX2-5*, *GATA4*, and *TBX5*) and signaling molecules (e.g., *CFC1*) have been most frequently found in VSD cases and families. This holds true for isolated VSD as well as for syndromic and non-syndromic forms. Of note, there is a great overlap of genes associated with other CHDs such as ASD (see Chap. 20), atrioventricular septal defect (see Chap. 26), tetralogy of Fallot, and double outlet right ventricle (see Chap. 32), as well as situs defects (see Chap. 38).

Over the last decade, tremendous effort has been made in the area of genetic technologies such as calling of CNVs and next-generation sequencing of whole exomes and genomes [111]. As a consequence, we better understand the genetic basis of VSDs and other CHDs. However, future studies are still required to unravel the effects of altered gene dosage or loss of function on formation of VSD. In addition, the interpretation of complex patterns of inheritance and phenotypic heterogeneity remains a difficult obstacle in individual VSD cases and families [45]. Nevertheless, these new technologies hold the potential to improve patient care. Especially in VSD cases with a growing number of surviving adults after successful cardiac surgery or intervention, the urgency for a better characterization of the CHD-related morbidities and mortality occurring late after surgery is evident [112]. Recently, Menting et al. reported on the outcomes in about hundred late survivors up to 40 years after VSD closure and demonstrated the ongoing clinical problems in this patient cohort such as arrhythmias and heart

failure [113]. In fact, genetics may open new ways for identifying novel risk-stratifying factors in those patients and offer them earlier and better-tailored treatment.

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Molecular Pathways and Animal Models of Ventricular Septal Defect

24

Lucile Houyel

Contents

Abstract

Ventricular septation is a complex process which involves the major genes of cardiac development, acting on myocardial cells from first and second heart fields, and on mesenchymal cells from endocardial cushions. These genes, coding for transcription factors, interact with each other, and their differential expression conditions the severity of the phenotype. In this chapter, we will describe the formation of the ventricular septum in the normal heart, as well as the molecular mechanisms leading to the four main anatomic types of ventricular septal defects: outlet, inlet, muscular, and central perimembranous, resulting from failure of development of

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the different parts of the ventricular septum. Experiments on animal models, particularly transgenic mouse lines, have helped us to decipher the molecular determinants of ventricular septation. However, a precise description of the anatomic phenotypes found in these models is mandatory to achieve a better comprehension of the complex mechanisms responsible for the various types of VSDs.

24.1 Introduction

Isolated ventricular septal defects are the most frequent congenital heart defects in children [1] and are also an integral part of a large number of congenital heart malformations. The term "ventricular septal defect" is widely used to describe any type of hole, or channel, or communication, between the two ventricles [2, 3]. However, it is possible to make a distinction between the "true" ventricular septal defects (VSD) which represent holes, or channels, within the ventricular septum itself, and the interventricular communications, which are the space between two components of the ventricular septum which did not fuse properly during embryonic life [2, 3].

Many classifications have been used to describe the communications between the ventricular chambers. Recently, some decisive steps have been taken toward a consensus between anatomists and clinicians, based on geographic approach and phenotypic features [3], allowing us to distinguish four types of interventricular defects (Fig. 24.1):

- Muscular defects
- Defects opening into the outlet of the ventricles (outlet defects)
- Defects opening into the inlet of the ventricles (inlet defects)
- Central membranous defects

The anatomic characteristics of these defects and their assumed embryologic origin are depicted in Table 24.1.

Ventricular septation, as a crucial step in heart development, was studied first in human embryos from a purely morphologic point of view, then on chick and mouse. Recent techniques such as high-resolution episcopic microscopy and high-resolution magnetic resonance imaging (MRI) have been used in mouse and human embryos to better analyze the development of the atrial and ventricular septa [4, 5]. Despite these recent advances, the precise mechanisms leading to normal ventricular septation are not fully understood and controversies still exist. Some of them are due to some developmental differences in the chick and in the mouse. On the other hand, despite some minor differences, the steps of cardiac development are very similar in mouse and human [6].

24.2 Normal Ventricular Septation

Ventricular septation is a complex process starting from the onset of cardiac looping to its final steps which are the formation of the membranous septum and later on, during fetal life, compaction of the ventricular myocardium.

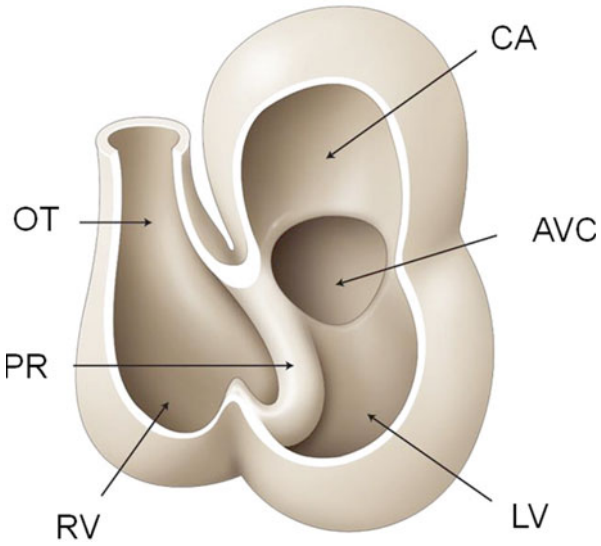

Fig. 24.1 The embryonic heart just after cardiac looping. The common atrium (*CA*) communicates with the left ventricle (*LV*) only, via the atrioventricular canal (*AVC*). The primary ring (*PR*) marks the boundary between the left ventricle, derived from the first heart field (straight heart tube), and the developing right ventricle (*RV*), derived from the anterior part of the second heart field. The outflow tract (*OT*) is entirely above the developing right ventricle

Immediately after cardiac looping (early looping stage), the inlet segment (atria and atrioventricular canal) is located entirely above the future left ventricle, and the outlet segment (the conotruncus or outflow tract) is located entirely above the future right ventricle, leading to both double-inlet left ventricle and double-outlet right ventricle types of atrioventricular (AV) and ventriculo-arterial connection (Fig. 24.2). From this stage on, the heart continues to grow by addition of myocardial cells from two cellular populations: the first heart field or primitive linear heart tube will form the left ventricle and a small part of the atria above the AV valves; the second heart field (SHF) is essential for the development of the right ventricle and the outflow tract (OFT) (anterior SHF) and also the venous pole, including the major part of the atria (posterior SHF or dorsal mesocardium).

Ventricular septation involves not only myocardial cells of the two heart fields, but also mesenchymal cells of the endocardial cushions; most of those will be secondarily muscularized. Proper ventricular septation can be achieved normally only if all elements of the definitive four-chambered heart are correctly aligned.

The interventricular septum first appears immediately after the cardiac loop, as a myocardial ridge developed at the posterior part and at the apex of the primitive heart, the primary ring (Fig. 24.2). Cardiac looping creates an outer curvature, formed by the ventral surface of the straight heart tube, and an inner curvature, between the outflow and the inflow parts of the heart. The outer curvature will take part to the growth of the ventricles, by ballooning of the cavities with formation of

Table 24.1 The four main anatomic types of ventricular septal defect with their anatomic characteristics and their presumably embryologic origin

Type of defect			Fibrous continuity	Embryologic origin	Time in cardiac development
Muscular	Midmuscular		NO	Compaction of myocardium, trabeculations	T
	Apical				
	Inlet muscular				
Central membranous without aortic overriding			YES (*septal* tricuspid leaflet/aortic leaflets)	Formation of membranous septum (AV cushions)	T-1
Outflow tract (outlet) defects	Malalignment defects (aortic overriding)	With aortic-tricuspid fibrous continuity (perimembranous extension)	YES (*anterior* tricuspid leaflet/aortic leaflets)	Cardiac neural crest and anterior second heart field. Outflow tract cushions	T-2
		Muscular borders	NO (muscular borders)		
	Juxta-arterial defects (fibrous continuity Ao/pulm valves)	With aortic-tricuspid fibrous continuity (perimembranous extension)	YES (*anterior* tricuspid leaflet/aortic leaflets)		
		Muscular borders	NO (muscular borders)		
Inflow tract (inlet) defects	Common atrioventricular junction (isolated ventricular component)		YES (tricuspid/mitral)	Posterior second heart field. AV cushions	T-3
	Straddling tricuspid valve (malalignment atrial/ventricular septum)			Convergence. Formation of AV junction	T-4

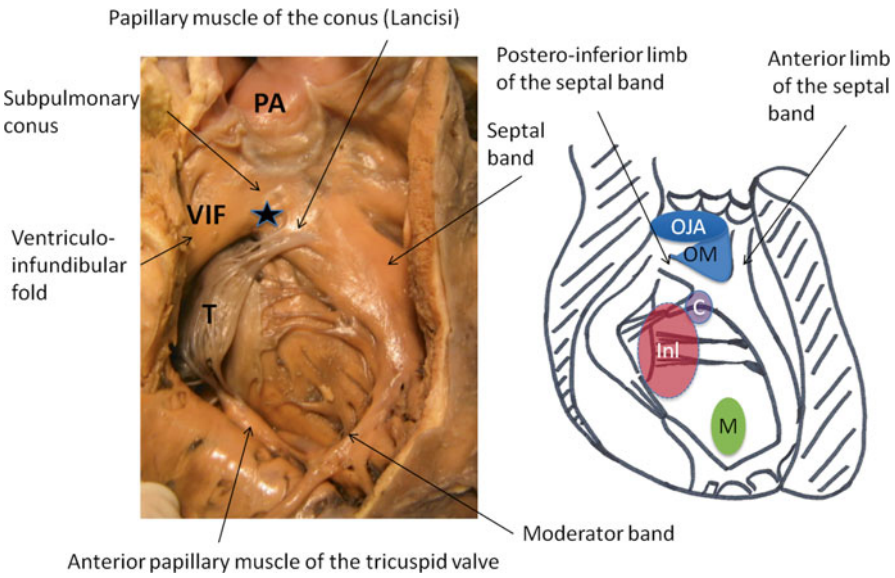

Fig. 24.2 The right ventricle in the normal heart, and the four main anatomic types of ventricular septal defect, viewed from the right ventricle. *Asterisk* outlet septum, *C* central membranous, *Inl* inlet, *OJA* outlet, juxta-arterial, *OM* outlet, malalignment, *PA* pulmonary artery, *M* muscular, *T* tricuspid valve, *VIF* ventriculo-infundibular fold

multiple trabeculations [7], while the remodeling of the inner curvature controls the alignment between the inlet and outlet segments [8]. The right ventricle develops later than the left ventricle [9], by addition of the cardiomyocytes of the anterior SHF [10]. This differential growth contributes to the alignment of the developing atrial and ventricular septa. The formation of the interventricular septum would thus be both passive (ballooning) and active [11–13].

The ventricular septum can be divided in several components, each one having a different embryologic origin. The muscular trabeculated septum is composed of the myocytes of both left and right ventricles [11] and thus originates from both first and second heart fields.

The origin of the inlet septum is still controversial. What is commonly called the inlet septum is the posteroinferior, or caudal, part of the ventricular septum [12, 14], but for some authors it does not exist in the normal heart [4, 15]. Experimental studies in the mouse embryo demonstrate that the larger part of the central mesenchymal mass created by the fusion of the mesenchymal tissues of the vestibular spine, the mesenchymal cap of the primitive atrial septum, and the AV cushions, becomes muscularized to form the inferior margin of the oval fossa [16]. Whether muscularization occurs or not in the posteroinferior part of the mass (the part which is lacking in hearts with a common AV junction, forming the inlet VSD) has been demonstrated in the chick embryo [17] but not in mammals, in which the posterior-inferior part of the ventricular septum would be part of the developing trabeculated muscular septum [18].

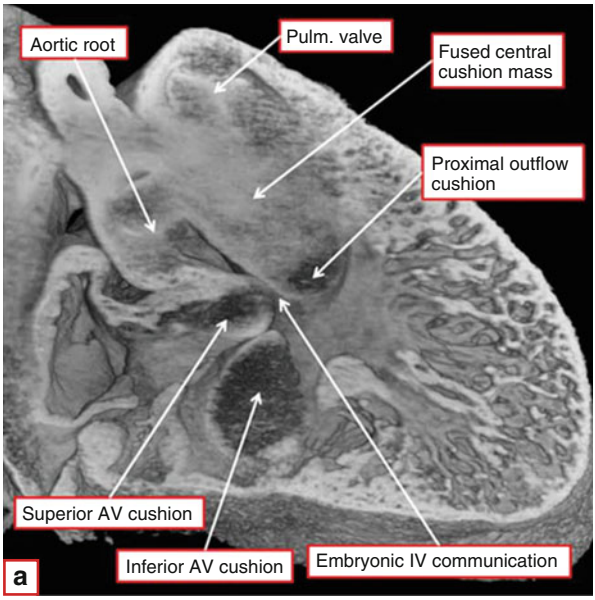

Fig. 24.3 Episcopic section from a developing mouse heart at E13.5. The aortic root is still above the right ventricle. The proximal outflow cushions have fused but have not yet muscularized. The embryonic interventricular communication lies at the confluence of the outflow tract cushions and the superior and inferior atrioventricular cushions (Courtesy of R.H. Anderson)

The outlet septum develops by fusion and muscularization of the proximal part of the endocardial cushions of the OFT [19] and is taken along leftward by the rotation of the developing aortic valve, to fuse with the upper primitive ventricular septum between the two limbs of the septal band or septomarginal trabeculation (Fig. 24.3), itself derived from the primary fold [20]. This rotation depends on the proper growth of the OFT by addition of myocardial cells from the anterior heart field, in response to the migration of cardiac neural crest cells toward the OFT [21]. The rotation of the aortic valve permits its transfer toward the left ventricle and its wedging between the tricuspid and mitral valves, establishing fibrous mitral-aortic continuity [22–24].

The membranous septum (Fig. 24.4) completes cardiac septation by closing the residual central defect, by fusion of the AV canal cushions with the left outflow tract cushion [2]. Any failure of development of these different parts (the limits of which are barely recognizable in the normal heart) leads to a corresponding phenotype of VSD.

24.3 Pathways to the Different Anatomic Types of Ventricular Septal Defects

The molecular determinants of the complex events leading to ventricular septation are not fully understood yet [25]. This is due in part to the uncertainties relative to the normal development of the ventricular septum and also to the difficulties in assessing the precise anatomic phenotype in animal models. In addition, few animal models exhibit isolated VSDs.

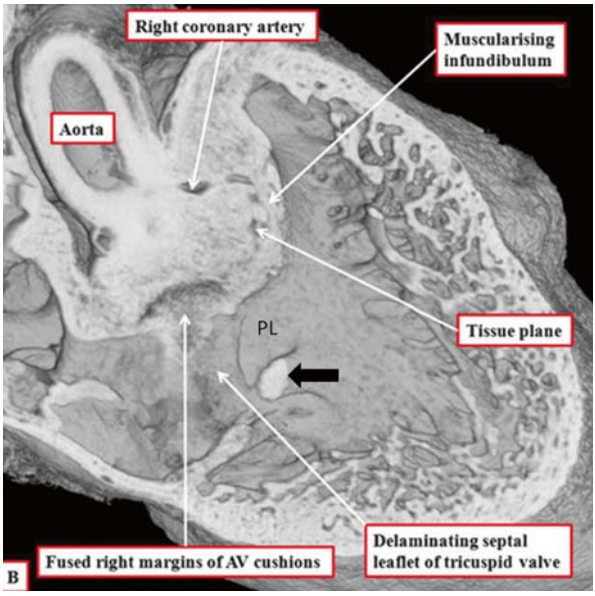

Fig. 24.4 Episcopic section from a developing mouse heart at E14.5. The fused central cushion mass is inserted between the two limbs of the septomarginal trabeculation (septal band) and has begun to muscularize. It will be transformed into the outlet septum and into the free-standing muscular subpulmonary infundibulum. The aorta has achieved its transfer toward the left ventricle. The rightward margins of the atrioventricular endocardial cushions fuse to close the right ventricular entrance to the subaortic vestibule, completing the process of ventricular septation. The membranous septum which results from this fusion is visible under the posterior limb (*PL*) of the septal band (*black arrow*) (Courtesy of R.H. Anderson)

A lot of genes coding for transcription factors have been recognized as playing a role in ventricular septation [26, 27]. However, the major ones, T-box 5 (Tbx5), T-box 20 (Tbx20), GATA binding protein 4 (Gata4), GATA binding protein 6 (Gata6), and NK2 homeobox 5 (Nkx2-5), when deleted in mouse or mutated in human, produce VSDs but also atrial septal defects (ASDs) and atrioventricular septal defects (AVSD) (see Chaps. 20 and 26). An explanation for this could be that several genes act on cells from both parts of SHF, anterior and posterior. For example, sonic hedgehog (Shh) signaling has been found essential for the contribution of the anterior heart field to the developing outflow tract in mice [28] and regulates the expression of Tbx1 and the semaphorin receptor neuropilin 2 in the SHF [29]. Shh signaling has also been demonstrated to play a crucial role in the formation of the vestibular spine, which originates from the posterior SHF, loss of Shh from the pharyngeal endoderm resulting in AVSD in mice [30].

24.3.1 Pathways to the Outlet VSDs

All outflow tract defects share the same anatomic type of VSD, opening to the outlet of the right ventricle and located between the two limbs of the septal band (except

transposition of the great arteries, in which this type of VSD is not constant, and double-outlet right ventricle (DORV) with noncommitted VSD) [31]. The phenotypic differences observed, especially regarding the posteroinferior rim of the VSD (fibrous, with fibrous continuity between the anterior leaflet of the tricuspid valve and the aortic valve, or muscular, due to the fusion of the posterior limb of the septal band with the ventriculo-infundibular fold), are related to the degree of rotation of the aortic valve during heart development [31]. Separation of the posterior limb of the septal band from the ventriculo-infundibular fold (so-called outlet VSD with perimembranous extension) would indicate a higher degree of rotation of the aortic valve, by analogy with the normal heart [31]. Therefore, the molecular pathways that lead to an outlet VSD have to be the same than those leading to so-called conotruncal, or cardiac neural crest, defects, which include tetralogy of Fallot and its anatomic variants, common arterial trunk, interrupted aortic arch type B, isolated outlet VSDs, and DORV with outlet VSD. The genetic background of these anomalies is well known, because of their frequent association with 22q11 microdeletion or DiGeorge syndrome (see Chaps. 23 and 32). The major candidate gene for DiGeorge syndrome is Tbx1, which is expressed in the anterior part of the SHF [29] and is a key regulator of SHF development. Tbx1+/– mice have defects of the aortic arches (especially the 4th aortic arch) and great arteries, while Tbx1–/– mice have outflow tract defects, with variable severity according to the dosage of the gene [32]. This dosage effect, interacting with the complex molecular pathways affecting the anterior SHF, could explain the phenotypic differences observed between the various outflow tract defects and within the same malformation. Other animal experiments have shown that Tbx1 is expressed in a particular SHF subdomain, which contributes to the subpulmonary myocardium [29]. In Tbx1 mutant mice, the outflow tract is shorter and narrower, due to reduction in size of the future subpulmonary myocardial domain [33].

24.3.1.1 Malalignment-Type Outlet VSDs

These defects are due to a malalignment between the developing outflow tract and the ventricles, due to lack of rotation of the aortic valve, and result in a failure of fusion of the outlet septum with the primitive ventricular septum between the two limbs of the septal band. This is due to a lack of addition of myocardial cells by the anterior SHF, in interaction with neural crest cells, to the developing outflow tract [34], resulting in an underdevelopment of the subpulmonary myocardium [29]. All the genes involved in regulation of anterior SHF, when mutated in animals and in humans, can determine, in addition to so-called conotruncal defects, isolated malalignment outlet VSDs.

24.3.1.2 Doubly Committed and Juxta-arterial Outlet VSDs

This particular type of outlet VSD is due to a failure of fusion and of muscularization of the proximal part of the endocardial cushions of the outflow tract, resulting in absent or fibrous outlet septum [4]. The genetic background is different whether this type of VSD is found in isolation (with a particular frequency in populations of Asian origin) or in the setting of a common arterial trunk. Common arterial trunk results from a complete failure of septation of the outflow tract, involving a defect of migration of the neural crest cells [35]. Several animal models exhibit common

arterial trunk, Tbx1, Sema3c (semaphorin 3C), Splotch (also known as Pax3 encoding paired box 3), and Raldh2 (also known as Aldh1a2 encoding aldehyde dehydrogenase 1 family member A2), with various phenotypes according to the position of the pulmonary branches on the common arterial trunk.

24.3.2 Pathways to the Inlet VSDs

The majority of the defects in the posterior, or caudal, part of the ventricular septum are part of an AVSD, in the setting of a common AV junction.

24.3.2.1 Inlet VSD in the Setting of a Common Atrioventricular Junction

Many experiments have recognized as the major mechanism leading to abnormal AV septation a deficient growth of the vestibular spine, or dorsal mesenchymal protrusion [16, 30, 36]. This mechanism was confirmed in human T21 embryos with AVSD [37]. Thus the vestibular spine would participate in the normal septation of the AV canal and presumably to the posteroinferior part of the ventricular septum. Mutant mice heterozygous for Gata4 exhibit a common AV junction phenotype, including hearts with an isolated inlet VSD [38]. Indeed, a defect of the posteroinferior part of the ventricular septum is found anatomically in all hearts with a common atrioventricular junction, even if the shunt through this defect may disappear because of fusion of the valvar leaflets with the crest of the ventricular septum. Other experiments on mice have found a dose effect for various genes, which could explain the occurrence of the different forms of common AV junction [38, 39] and also the frequent association with outlet defects such as DORV.

24.3.2.2 Inlet VSD Associated with Straddling Tricuspid Valve

Tgf-β2 (tissue growth factor beta 2) knockout mice exhibit as part of their phenotype an inlet VSD, associated in 2/3 of cases with a straddling tricuspid valve. This anomaly was associated in most embryos with a DORV [40]. These defects could be due to a lack of myocardialization of the endocardial cushions, with altered neural crest cell migration and excessive apoptosis [40]. This would alter the final phase of wedging, resulting in a malalignment between atrial and ventricular septa which precludes normal closure of the posterior part of the primitive interventricular foramen and impairs the normal rotation of the outflow tract.

24.3.3 Pathways to the Muscular VSD

In an experimental study using two different Mlc (myosin light chain) transgenic mouse lines (Mlc1v expressed in the outflow tract and right ventricular myocardium and Mlc3f expressed in left ventricular myocardium and the atrial appendages), Franco et al. demonstrated that cells contributing to the trabeculated ventricular septum originate from both first and second heart fields [11]. During the early stages

of development, the expression of the two transgenes is symmetrical in the developing interventricular septum in the mouse. Then the left ventricular cardiomyocytes become progressively predominant, particularly in the dorsal part of the septum. A third population of cardiomyocytes could be added by the inner curvature of the heart at the upper part of the muscular trabeculated septum. Some muscular VSDs could occur at the zones of junction of these different cell populations.

Several transcriptions factors, like Tbx5, Gata4, and Nkx2-5, are involved in the development of the primitive ventricular septum and seem to interact at some stages of cardiac development [41]. Some of these genes exhibit gradients of expression along the ventricular septum, which are critical for ventricular septation [42]. Tbx18 was expressed on both sides of the dorsal aspect of the developing ventricular septum in mice, but was restricted to the left side in its ventral aspect [11]. In the mouse, Tbx5 expression is stronger in the left ventricle, weaker in the right ventricular inlet, and almost negative in the right ventricular outflow tract [43]. When Tbx5 is deleted from all or part of the ventricular myocardium, ventricular septation is deficient [44]. Hand1/eHand (heart and neural crest derivatives expressed transcript 1) knockout mouse embryos exhibited normal morphology of the inner curvature, the AV junction, and the outflow tract, but the interventricular septum failed to form and the compact myocardium was thinner than normal, leading to embryonic lethality [45]. Takeuchi et al. demonstrated in chicken and mouse embryo that Tbx5 may control Hand1/eHand expression and that the boundary of Tbx5 and Tbx20 expression may determine the position of the ventricular septum [46]. The absence of Tbx5 and Hand1/eHand expression in the region between the two developing ventricles may be necessary for normal development of the ventricular septum [45].

Compaction of the primary trabeculated myocardium may play a major role in the formation of the ventricular septum and could be mediated by epicardial-derived cells [43]. Pdpn (podoplanin)-mutant mice exhibit an underdeveloped epicardium, a thin muscular septum with few epicardial cells, spongy inlet septum, and septal defects including AVSDs [47]. Multiple muscular VSDs could be related to a certain degree of noncompaction of the trabeculated ventricular septum [47].

24.3.4 Pathways to the Central Membranous VSD

Central membranous (or perimembranous) VSD must be differentiated from malalignment outlet defects with perimembranous extension [31]. The latter also exhibit a fibrous continuity between the tricuspid and the aortic valve, but this continuity involves the anterior leaflet of the tricuspid valve and not the septal leaflet like in central membranous VSD [31]. The central membranous defect is located below the posterior limb of the septal band and under the ventriculo-infundibular fold, while the malalignment outlet defect is located between the two limbs of the septal band. This is extremely important because the genetic and molecular pathways are different: central membranous defects depend on the AV cushions, while

outlet defects depend on anterior SHF and cardiac neural crest [43]. The main phenotype associated with trisomy 18 is a central membranous VSD. However, the phenotypic distinction is rarely made on animal models such as mouse and chick.

Likewise malalignment outlet defects can extend posteriorly and have a perimembranous extension, central perimembranous defects can extend posteriorly towards the inlet. This underlines the common origin of central perimembranous and inlet defects, namely, the mesenchyme of the AV endocardial cushions.

Conclusion

Ventricular septation is a complex phenomenon which involves the major genes of cardiac development, acting on myocardial cells from first and second heart fields, and mesenchymal cells from endocardial cushions. These genes, coding for transcription factors, interact with each other, and their differential expression conditions the severity of the phenotype. Gene deletion experiments in animal models have helped to decipher their role in heart development, although heterozygous models are probably closer to human phenotypes. The complexity of the molecular determinants of ventricular septation and the distinct origin of each part of the ventricular septum underline the extreme importance for developmental biologists of being able to determine the exact anatomic phenotype in their animal models and not to refer only to "VSDs."

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Part VI

Atrioventricular Septal Defect

Clinical Presentation and Therapy of Atrioventricular Septal Defect

25

David J. Driscoll

Contents

Abstract

Atrioventricular septal defects (AVSDs) consist of a number of cardiac malformations that result from abnormal development of the endocardial cushions. AVSDs occur in 0.19 of 1000 live births and constitute 4–5 % of congenital heart defects. AVSDs can be categorized as (1) incomplete (or partial) or (2) complete (Fig. 25.1) and (3) intermediate or transitional.

25.1 Introduction

Atrioventricular septal defects (AVSDs) consist of a number of cardiac malformations that result from abnormal development of the endocardial cushions. AVSDs occur in 0.19 of 1000 live births and constitute 4–5 % of congenital heart defects.

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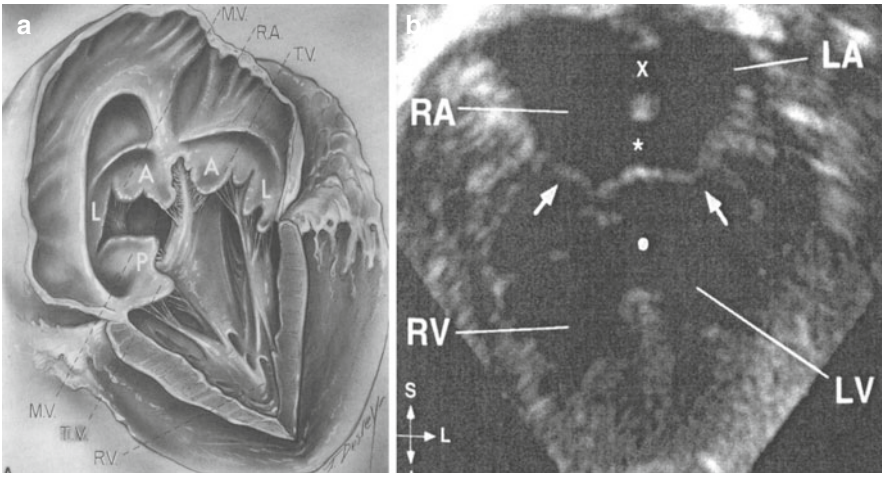

Fig. 25.1 Diagrammatic representation of a complete atrioventricular septal defect on the *left*. Note that instead of a mitral and a tricuspid valve, there is only one large atrioventricular valve. There are cordal attachments to the crest of the ventricular septum. The ostium primum ASD and an inlet VSD are readily apparent. On the *right* is an echocardiographic four-chamber view of a complete atrioventricular canal defect. The arrows represent the annulus of the common AV valve. The "X" indicates an associated ostium secundum ASD and the "*" shows the location of the ostium primum ASD. The "o" shows the location of the VSD. Abbreviations: *MV* mitral valve remnant, *TV* tricuspid valve remnant, *RA* right atrium, *RV* right ventricle, *L* lateral valve leaflet, *A* anterior leaflet, *P* posterior leaflet, *LV* left ventricle (Adapted from Feldt et al. (1976) Atrioventricular Canal Defect, WB Saunders, Philadelphia and reproduced or adapted from Driscoll, David (2006) Fundamentals of Pediatric Cardiology. Lippincott Williams & Wilkins, Baltimore with permission of the author and publisher)

AVSDs can be categorized as (1) incomplete (or partial) or (2) complete (Fig. 25.1) and (3) intermediate or transitional.

Incomplete AVSDs include ostium primum ASD, common atrium, cleft mitral valve, and defects of the atrioventricular septum producing a left ventricular to right atrial shunt (Gerbode defect). Ostium primum ASD results from lack of closure of the ostium primum by the endocardial cushions. Since the endocardial cushions also form major portions of the mitral and tricuspid valves, abnormalities of the atrioventricular valves are associated with ostium primum ASDs. A cleft in the septal leaflet of the mitral valve invariably is associated with ostium primum ASDs.

Complete AVSD is a condition in which the inferior portion of the atrial septum and the posterior portion of the ventricular septum are absent. These two defects are contiguous. In addition, instead of two separate atrioventricular valves, there is one large common atrioventricular valve. Hence, the entire central portion of the heart is missing (Fig. 25.1).

Intermediate or transitional AVSDs consist of an ostium primum ASD and a restrictive posterior VSD but two complete atrioventricular valve rings. Thus, the ASD and VSD are not contiguous.

25.2 Pathologic Physiology

The pathologic physiology of atrioventricular septal defects that result in defects in the atrial septum is the same as that for atrial septal defects as discussed previously (Chap. 19). Also the pathologic physiology for AVSD involving the ventricular septum is the same as for ventricular septal defects as discussed previously (Chap. 22).

25.3 Clinical Presentation and Physical Examination

The presentation and clinical examination of patients with ostium primum ASD and common atrium are the same as ASD as discussed in Chap. 19. However, because of the associated cleft of the septal leaflet of the mitral valve associated with ostium primum ASD, there can be a systolic murmur of mitral insufficiency and, if the leakage is severe, there can be a diastolic mitral flow murmur. The presentation and clinical examination of patients with complete AVSDs are similar to patients with a large VSD and are discussed in Chap. 22.

25.4 Echocardiographic and Cardiac Catheterization Issues

The diagnosis and type of AVSD as well as associated anomalies can be determined using echocardiography (Fig. 25.1). Using Doppler techniques, right ventricular and pulmonary artery pressure can be estimated. For patients with complete AVSDs who may have pulmonary vascular obstructive disease, cardiac catheterization is essential to determine if the patient is a candidate for complete repair.

25.5 Treatment

The same guidelines for treatment of ASD can be applied to patients with incomplete or partial AVSDs. The same treatment guidelines for VSD can be applied to patients with complete AVSDs.

25.6 Outcome

For partial atrioventricular septal defects of the ostium primum type, the surgical mortality is low and the long-term outlook is good. There is, however, an 8 % chance that a second operation will be necessary to deal with subaortic stenosis or mitral valve abnormalities.

The surgical mortality is 5 % or less for the complete forms of atrioventricular septal defect, and there is a late hazard for reoperation either for mitral valve or tricuspid valve insufficiency or left ventricular outflow tract obstruction.

Human Genetics of Atrioventricular Septal Defect

26

Cheryl L. Maslen

Contents

Abstract

Atrioventricular septal defects (AVSD), also known as a common atrioventricular canal (CAVC), are clinically severe heart malformations that affect about 1 out of every 2,100 live births. AVSD is associated with cytogenetic disorders, such as Down syndrome and numerous rare genetic syndromes, but also occurs as a simplex trait. Studies in mouse models have identified over 100 genetic mutations that have the potential to cause an AVSD. However, studies in humans indicate that AVSD is genetically heterogeneous and that the cause in humans is very rarely a single-gene defect. Familial cases do occur, usually with autosomal dominant inheritance, and the mutations identified in those families suggest biochemical pathways of interest. In addition, the frequent occurrence of AVSD in some syndromes, such as heterotaxy syndrome, points to additional genes/pathways that increase AVSD risk. Accordingly, while the genetic underpinnings for

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most AVSD remain unknown, there have been advances in identifying genetic risk factors for AVSD in both syndromic and nonsyndromic cases. This chapter summarizes the current knowledge of the genetic basis for AVSD.

26.1 Introduction

Atrioventricular septal defects (AVSD) are a group of phenotypically and genetically heterogeneous heart malformations (see Chap. 25). The most commonly occurring genetic association for AVSD is with trisomy 21, which causes Down syndrome. An estimated 65 % of all cases of complete AVSD occur in individuals with Down syndrome. However, only about 25 % of trisomy 21 cases have a complete AVSD, indicating that an extra copy of chromosome 21 is not sufficient to cause the heart defect. In addition, individuals with heterotaxy syndrome also frequently have a complete AVSD or related heart malformation. Heterotaxy syndrome is caused by mutations in genes that control left-right patterning of the body during development (see Chap. 38). Other rare syndromes that sometimes include AVSD as part of the phenotypic spectrum include Ellis-van Creveld syndrome, CHARGE syndrome, Ivemark syndrome, Kaufman-McKusick syndrome, Noonan syndrome, Holt-Oram syndrome, and 3p-syndrome. Families with isolated AVSD inherited in an autosomal dominant pattern with incomplete penetrance have also been described [1, 2]. However, with the exception of Down syndrome, the majority of cases of AVSD occur sporadically without a clear familial component.

26.2 Disease Model

AVSD is a complex trait that can be described using a threshold model, where otherwise benign factors occur in combination to breach the disease threshold resulting in a heart defect. The number of required risk factors likely varies depending on an individual's genetic background and could include multiple factors each of small effect size, a combination of large and small effect size variants, or a single variant of very large effect size sufficient to cause the defect (dominant inheritance). Risk factors may be genetic, epigenetic, environmental, or a combination of factors. In the case of Down syndrome, there is a clear but poorly defined contribution of trisomy 21 that greatly increases risk, but alone is not sufficient to cause the defect. This is likely true in general for other syndromes, where the primary cause of the syndrome increases risk but additional risk factors push the risk over the threshold resulting in a heart defect.

26.3 AVSD Genes and Pathways

26.3.1 *CRELD1* Mutations

Cysteine-rich protein with EGF-like domain 1 (*CRELD1*) was first identified through a study of infants with the cytogenetic disorder, 3p-syndrome. These children are missing the distal portion of the short arm of chromosome 3 and have a

variable phenotype with AVSD found in those with a more proximal breakpoint. This defined a "critical region" of 3p that encompassed the *AVSD2* locus, which presumably included a gene, or genes, which when deleted caused the heart defect [3]. Molecular mapping identified a previously unrecognized gene, *CRELD1*, in that interval [4]. Expressed in the developing heart, *CRELD1* became a candidate for the *AVSD2* locus. Further studies determined that missense mutations in *CRELD1* were associated with simplex AVSD [5], which were confirmed in multiple populations around the world, occurring in about 5 % of simplex cases [6–10]. This includes a recurrent mutation, p.Arg329Cys, which has been identified in numerous unrelated individuals. Family studies showed that *CRELD1* missense mutations are incompletely penetrant, which is consistent with the proposed disease threshold model for AVSD [5]. Additional studies demonstrated that missense mutations in *CRELD1* were also associated with AVSD in Down syndrome [11] and heterotaxy syndrome [12], demonstrating a specificity of the mutations for AVSD regardless of genetic background.

The contribution of CRELD1 defects to the risk of heart malformations was confirmed in an innovative study using the Ts65Dn mouse model as a sensitized genetic background for heart defects [13]. This mouse model of Down syndrome has an incidence of cardiac septal defects at birth of approximately 4.7 %. However, when crossed with a *Creld1* heterozygous null mouse, the incidence of heart defects at birth increased to 33.3 %, indicating that deficiency of Creld1 does indeed contribute to the risk of heart defects.

Mouse models also have helped define CRELD1 function and the mechanism of disease when there are mutations. The *Creld1* knockout mouse model demonstrated that Creld1 function is essential for heart development and that absence of Creld1 interferes with VEGFA-dependent proliferation of endocardial cells [14]. CRELD1 activates calcineurin/nuclear factor of activated T-cell-1 (NFATc1) signaling under the control of VEGFA thereby promoting the expression of NFATc1 targets, which is required for the endocardial cushions to remodel into valves. Two *CRELD1* missense mutations associated with AVSD in humans, the recurrent p.Arg329Cys mutation and another mutation, p.Arg107His, have been shown to directly interfere with activation of the calcineurin/NFATc1 signaling cascade thereby impairing valve formation [15]. The mechanistic action for other *CRELD1* mutations is unknown at this point.

26.3.2 VEGFA Pathway Gene Mutations

Vascular endothelial growth factor A (*VEGFA*) has long been known to play a key role in heart valve development, and the interaction between CRELD1 and VEGFA suggested that mutations in other VEGFA pathway genes might be additional risk factors for AVSD. Using a candidate gene approach, it was demonstrated that there is an excess of damaging missense mutations in VEGFA pathway genes in individuals with Down syndrome-associated AVSD, including several recurrent mutations in GATA binding protein (*GATA)* 5 that altered transcriptional activity [16]. Although individually rare, recurrent missense mutations in these genes were identified in 10 % of cases studied, indicating a significant contribution to the risk of

AVSD in Down syndrome. Further study is needed to determine if this phenomenon is specific to Down syndrome or if it extends to simplex AVSD.

26.3.3 *NR2F2* Mutations

Another transcription factor gene, nuclear receptor subfamily 2/group F (*NR2F2*), has been shown to have AVSD-associated mutations in simplex AVSD cases and familial cases of mixed heart malformations including AVSD. *N2RF2* was identified as an AVSD candidate gene based on a finding of enrichment of *de novo* mutations in cases compared to controls in a whole-exome sequencing study [17]. The premise of the study was that *de novo* variants are likely to be the most damaging variants, thereby providing a strategy for filtering the exome data. Targeted gene sequencing identified additional *N2RF2* mutations in more simplex cases and also identified inherited mutations in families with multigenerational congenital heart disease. Co-segregation of those mutations with the heart defects indicates that *N2RF2* mutations can be causal. There appeared to be a possible genotype-phenotype correlation, with loss-of-function variants biased toward left ventricular outflow tract obstruction and functional missense mutations most often associated with septal defects.

26.4 Additional Risk Factors

There are several isolated reports of single-gene mutations associated with AVSD (Table 26.1), including protein tyrosine phosphatase, non-receptor type 11 (*PTPN11)*, *GATA4*, *GATA6*, activin A receptor, type I1 (*ACVR1/ALK2)*, and forkhead box P1 (*FOXP1)* that likely represent rare or family-specific events that may be important in understanding AVSD biology but do not account for the majority of cases [18–23]. This underscores the substantial genetic heterogeneity in AVSD and may point to biochemical pathways or biological functions that are central to the cause of this defect. For instance, the greatly increased incidence of AVSD in heterotaxy syndrome (60–70 %) implicates genes involved in left-right patterning of the embryo in the pathogenesis of AVSD. This includes genes such as activin A receptor, type IIB (*ACVR2B)*, cryptic family protein 1 (*CFC1)*, *FOXP1*, left-right determination factor 2 (*LEFTY2)*, *NODAL*, and Zic family member 3 (*ZIC3)* [21, 24–27] (see Chap. 38). Mutations in these genes clearly cause heterotaxy syndrome and significantly increase risk for AVSD and related heart malformations. Heterotaxy syndrome is one of the many disorders that are classified as ciliopathies, or diseases that occur due to dysfunction of primary and/or motile cilia (see also Chap. 7). AVSD also occurs in association with other ciliopathies such as Bardet-Biedl and Meckel-Gruber syndromes. It is notable that defects in cilium-related genes disrupt second heart field development in mice, leading to AVSD. In addition, a study of children with Down syndrome showed that rare large genetic deletions involving genes encoding components of the cilia were enriched in the children with an AVSD [28]. Collectively these

Table 26.1 Genetic variants associated with AVSD

Gene	Protein variant	Function	Phenotypes	Reference
ACVR2B	p.Arg40His	ND	ubAVSD, CVM, HTX, AVSD, TGA	[25]
ALK2	p.His286Asp	Reduced BMP signaling	pAVSD, DS	[18]
CFC1	p.Ala145Thr	ND	AVSD, HPE	[27]
CRELD1	p.Arg107His	Reduced calcineurin/NFATc1 signaling	pAVSD, HTX	[5]
	p.Pro162Ala	ND	AVSD	[10]
	p.Pro286Arg	ND	pAVSD	[6]
	p.Glu325Lys	ND	pAVSD, DS	[6]
	p.Thr311Ile	ND	pAVSD	[5]
	p.Arg329Cys	Misfolding, reduced calcineurin/NFATc1 signaling	pAVSD	[5, 7, 15]
	p.Arg329Cys	ND	cAVSD, DS	[32]
	p.Arg329Cys	ND	cAVSD, HTX	[12]
	p.Glu414Lys	ND	cAVSD, DS	[32]
FOXP1	p. Pro568Ser	Decreased transcriptional activity	ubAVSD, PA, single ventricle, HTX	[21]
GATA4	p.Gly296Ser	Decreased transcriptional activity	Familial CHD, incl. AVSD	[22]
GATA5	p.Gln3Arg	Increased transcriptional activity	cAVSD, DS	[16]
	p.Tyr142His	Increased transcriptional activity	cAVSD, DS	[16]
	p.Phe159Leu	Decreased transcriptional activity	cAVSD, DS	[16]
GATA6	p.Arg178Val	Increased transcriptional activity	AVSD, HLH, VSD	[11]
LEFTY2	p.Ser342Lys	Disruption of L/R axis determination	AVSD, HLH, AV shunt, HTX	[24]
	p.Arg314X[a]	Disruption of L/R axis determination	AVSD, HLV, AV shunt, HTX	[24]
NODAL	p.Arg275Cys	Decreased transactivation of NODAL targets	cAVSD, d-TGA, HTX	[26]
NR2F2	p.Gln75dup	Increased transcriptional activity	cAVSD	[17]
	p.Asp179Val	No change in transcriptional activity	pAVSD	[17]
	p.Asn205Ile	Increased transcriptional activity	iAVSD	[17]
	p.Glu251Asp	Decreased transcriptional activity	ubAVSD	[17]
	p.Ser341Tyr	Decreased transcriptional activity	cAVSD	[17]
	p.Ala412Ser	No change in transcriptional activity	cAVSD	[17]
PTPN11	p.Leu43Phe	ND	cAVSD	[20]
ZIC3	p.Ser402Pro	Disruption of L/R axis determination	AVSD, HTX	[25]

Abbreviations: *AVSD* atrioventricular septal defects, *cAVSD* complete AVDS, *iAVSD* intermediate AVSD, *pAVSD* partial AVSD (ostium primum ASD), *ubAVSD* unbalanced AVSD, *CVM* complex cardiac vascular abnormalities, *DS* Down syndrome, *HLH* hypoplastic left heart, *HLV* hypoplastic left ventricle, *HPE* holoprosencephaly, *HTX* heterotaxy syndrome, *ND* not determined, *PA* pulmonary atresia, *TGA* transposition of the great arteries, *dup* duplication

[a]Translation termination (stop) codon

data suggest that defects in cilia may be a primary pathogenic event in AVSD, although this is yet to be shown for AVSD not associated with heterotaxy.

In addition to gene mutations, other potential contributing factors include rare copy number variations [29] and other chromosome abnormalities [30]. However, like most of the single-gene defects, these are rare and unlikely to contribute significantly to the incidence of AVSD. Environmental factors may also contribute, including gene x environment interactions such as genetic variants that affect folate metabolism [31] and epigenetics [32]. However, these areas are largely unexplored at this point and deserve further attention.

Conclusion

AVSD is a phenotypically and genetically heterogeneous heart defect. Numerous genes have been associated with AVSD, although few have been confirmed by additional studies and are either "private" mutations or spurious associations that may not contribute to the pathogenesis of the heart defect. However, there is substantial evidence that there are causal mutations in two genes, *CRELD1* and *N2RF2*, which have been identified in numerous unrelated affected individuals with confirmed pathogenicity in animal models. Mutations in these genes contribute to only a small percentage of AVSD, although they may define pathways or gene networks that will yield additional causative variants. Even so, combining all of the reported known and potential risk factors still falls short of accounting for the incidence of AVSD indicating that additional discovery is still needed.

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Molecular Pathways and Animal Models of Atrioventricular Septal Defect

27

Andy Wessels

Contents

Abstract

The development of a properly functioning 4-chambered heart relies on the correct formation of the septal structures that separate the atrial and ventricular chambers. Perturbation of this septation process results in a spectrum of cardiac malformations involving the atrial and ventricular septal structures. Atrioventricular septal defects (AVSDs) form a class of congenital heart defects that are characterized by the presence of a primary atrial septal defect, a common

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atrioventricular junction, and frequently also a ventricular septal defect. While AVSD were historically considered to result from failure of the endocardial atrioventricular cushions to properly develop and fuse, more recent studies have determined that inhibition of the development of the dorsal mesenchymal protrusion (DMP), a derivative of the second heart field, can lead to AVSDs as well. In this chapter, we review what is currently known about the molecular mechanisms and pathways that are involved in DMP development and the pathogenesis of AVSD.

27.1 Introduction

Atrioventricular septal defects (AVSDs) are found in approximately 5 % of individuals with congenital heart disease (CHD). Two major AVSD subtypes can be distinguished based on the potential for shunting between the left and right side of the heart [1]. In "incomplete/partial" AVSDs, shunting is restricted to the atrial level via a primary atrial septal defect (pASD), also known as the *ostium primum defect*. In "complete" AVSDs, we find, in addition to a pASD, an inlet-type ventricular septal defect (VSD). In complete AVSDs, shunting can occur at both atrial and ventricular level. For many years, it was believed that perturbation of the development of the endocardial AV cushions (or failure of their fusion) was the only mechanism leading to AVSD (hence the term "endocardial cushion defect" as a synonym for AVSD). However, a number of observations in the human heart [2, 3] as well as a growing number of experimental studies have revealed that abnormal development of the posterior second heart field (pSHF)-derived dorsal mesenchymal protrusion (DMP) plays a critical role in the pathogenesis of AVSDs [4–6]. In this chapter, we will discuss several conditions and models in which AVSDs are observed. Unless we specifically mention "complete" or "incomplete", the term AVSD refers to either form.

27.2 AVSDs and Genetic Syndromes

Down syndrome (trisomy 21), the most common genetic syndrome in humans, is strongly associated with AVSDs: approximately 1/4 of children with Down syndrome have an AVSD, and roughly 2/3 of cases of complete AVSD are found in patients with trisomy 21 [7]. The fact that not all individuals with Down syndrome have an AVSD indicates that the pathogenesis of AVSD in Down syndrome may involve multiple genetic modifiers and/or an environmental component. Other syndromes in which AVSDs are frequently observed include Smith-Lemli-Opitz syndrome (SLOS), Ellis-van Creveld syndrome, Noonan syndrome, and heterotaxy syndrome.

27.3 The Role of the Dorsal Mesenchymal Protrusion (DMP) in the Pathogenesis of AVSDs

27.3.1 The Contribution of the DMP to the AV Mesenchymal Complex

The DMP, sometimes referred to as the "vestibular spine" [2, 3, 8, 9] or "spina vestibuli" [10, 11], plays an essential role in atrial and atrioventricular septation [12–14]. The window in which the DMP develops is relatively narrow. The high proliferative activity of the pSHF cells located ventral to the foregut at around E9-9.5 (murine embryonic development) is responsible for a rapid expansion of this cell population. Around E10-10.5 the expanded pSHF-derived cell population protrudes into the common atrium as the DMP, using the dorsal mesocardium as its portal of entry [11, 13]. Here the DMP becomes a critical player in the septation process. As described in Chap. 5, an important step in atrial septation is the growth and descend of the myocardial primary atrial septum (or *septum primum*) from the atrial roof into the common atrium [15]. On the leading edge of this septum is located an endocardially derived mesenchymal ridge (also known as the mesenchymal cap). Although this ridge and the DMP have different origins, the two mesenchymal tissues are contiguous with each other. The space located under the primary atrial septum, through which the left and right atria are in open communication with each other, is called the primary foramen (or *ostium primum*). During normal development, the mesenchymal ridge, the DMP, and the major AV cushions will eventually fuse and close the primary foramen. The body of mesenchyme formed after the fusion of the mesenchymal ridge, DMP, and AV cushions is known as the AV mesenchymal complex [14]. Eventually, the mesenchyme of the DMP, but not the mesenchyme of the ridge and cushions, undergoes a myocardial differentiation [14]. In the formed heart, the muscularized DMP can be recognized as the inferior muscular rim at the base of the atrial septum [14–16].

27.3.2 Failure of the DMP to Properly Develop Results in AVSD

As described above, an important event in the development of the DMP is the proliferation-driven expansion of the pSHF cell population. When the pSHF cell proliferation is inhibited/impaired, the pSHF precursor population does not expand and the DMP fails to develop (or becomes severely truncated). In this case, the DMP will not be able to participate in atrioventricular septation; the AV mesenchymal complex will not form properly and the primary foramen will not be closed. This results in a common AV valve (cAVV) and a primary atrial septal defect (pASD). In addition to cell proliferation, the development of the DMP also involves cell migration [5]. Cell migration is important for organogenesis as well as numerous other developmental processes. Extracellular components, including growth factors,

cytokines, and/or extracellular matrix (ECM) proteins, are typically required for the initiation and progression of cell migration [17]. These components may be produced by neighboring cells and/or are present in the extracellular matrix and can be involved in the regulation of intracellular signaling pathway factors, including *Rho*, *Rac*, and other members of the small GTPase family that facilitate migration by stimulating extension of lamellipodia, turnover of cell-substrate adhesion, and cell body contraction [18–20]. Perturbation of SHF cell migration has also been proposed to be a mechanism that can lead to inhibition of DMP formation, failure of formation of the AV mesenchymal complex, and eventually resulting in cAVV and pASD which are the common components of most AVSDs [5].

27.4 Emerging Insights into the Molecular Pathways That Are Involved in DMP Development

Insights into the cellular and molecular mechanisms that govern proper DMP development and its maturation are slowly emerging. Below we will discuss a number of studies, performed in recent years on mouse models with AVSDs, which have increased our understanding of how the development and growth of the pSHF and DMP are regulated and have provided insights into the etiology of AVSDs.

27.4.1 The BMP Signaling Pathway and AVSDs

Bone morphogenetic proteins (Bmps) play an important role in cardiovascular development. At least 6 *Bmp* isoforms are expressed in the heart (*Bmp2,4,5,6,7,10*). Of these isoforms, *Bmp2* has been most frequently studied. *Bmp2* is highly expressed in the myocardium of the AV junction at early stages of development [21] where it plays a crucial role in regulating the endocardial-to-mesenchymal transformation necessary for formation of the AV cushions [22]. Mice that do not express *Bmp2* (*Bmp2* knockout mice) die before E12.5. Embryos isolated at this stage show severe defects in AV cushion development [22, 23]. *Bmp4* is also very important for heart formation [24–26]. Mice deficient for this isoform also die early in development [27]. Transgenic *Bmp4* hypomorphic mice (i.e., mice that express a severely reduced amount of *Bmp4*) survive until after birth but die within 1 week. Histological examination of *Bmp4* hypomorphic mice shows a 100 % penetrance of AVSD [28]. Furthermore, when *Bmp4* is specifically deleted from the myocardium, the offspring also have AVSDs [28]. In this context, it is important to note that *Bmp4* is abundantly expressed in the myocardial walls of the dorsal mesocardium (a.k.a. the mesocardial reflections) but that *Bmp4* is not expressed in the myocardial AV junction [4]. The mesocardial reflections flank the space through which the expanding pSHF protrudes into the common atrial cavity [1, 12]. In a recent study, it was shown that the pSHF population that eventually will form the DMP expresses *BmpR1A/Alk3*, a *Bmp* receptor known to interact with *BMP4*. Furthermore, positive staining for activated *pSmad1,5,8* in this cell population indicates ongoing *BMP*

signaling. The importance of *BmpR1A/Alk3*-dependent *Bmp* signaling for the formation of the DMP and atrioventricular septation was demonstrated in experiments in which *BmpR1A/Alk3* was conditionally deleted from the SHF using the myocyte enhancer factor 2C (*Mef2C)-AHF-Cre* mouse line. SHF-specific deletion of Alk3 resulted in impaired formation of the DMP and a completely penetrant incomplete AVSD (*ostium primum defect*) phenotype at stages ED13.5–15.5. Analysis of *Mef2C-AHF-Cre;Alk3fl/fl* mutants at E10-10.5 revealed decreased proliferative index of SHF cells and, consequently, a reduced number of pSHF cells at the cardiac venous pole [4].

27.4.2 The (Sonic) Hedgehog Signaling Pathway and AVSDs

Sonic hedgehog (*Shh*) is a secreted morphogen that plays an important role in the development of the heart. Specifically, *Shh* is crucial for regulating SHF contributions to the cardiac inflow tract, to the cardiac outflow tract, and to cardiac septation [5, 29, 30]. Interestingly, while *Shh* knockout mice have severe cardiac defects [31, 32], *Shh* is not expressed within the heart itself. This indicates that extracardiac sources are involved in *Shh*-dependent regulation of heart development. One of the tissues secreting *Shh* is the ventral pharyngeal endoderm [30]. Conditional deletion of *Shh* with an NK2 homeobox 5 (*Nkx2-5)-Cre* mouse, driving expression in a number of tissues including the pharyngeal endoderm, leads to a fully penetrant AVSD phenotype [30]. *Shh* signaling involves two co-receptors. *Smoothened* (*Smo*) is a G-protein-coupled-like receptor acting downstream of *Patched (Ptc1)* to confer *Shh* signaling; when secreted *Shh* binds to *Ptc1,* it abolishes an inhibitory effect on *Smo*, allowing *Smo* to transduce signals via *Gli* transcription factors. *Shh* expressing ventral pharyngeal endoderm is juxtaposed to the SHF/DMP-precursor cell population located between the foregut and the heart. Conditionally deleting Smo from the SHF/DMP-precursor cell with the *Mef2c-AHF-Cre* mouse (*Mef2c-AHF-Cre;Smo*$^{fl/-}$) inhibits *Shh* signaling in these cells, perturbs DMP formation, and results in a fully penetrant AVSD phenotype [5]. Thus, interaction between *Shh* (expressed in and secreted from pharyngeal endoderm) and the *Shh* receptor *Smo* (expressed in the SHF/DMP-precursor cell population) is essential for the development of the DMP and, hence, for proper AV septation.

27.4.3 The Wnt/β-Catenin Signaling Pathway and AVSDs

The wingless-type MMTV integration site family *Wnt/β-catenin* signaling pathway is involved in many developmental events. In the heart, *Wnt/β-catenin* signaling has been shown to regulate the development of the outflow tract and right ventricle, components of the heart that are derived from the SHF [33, 34]. In addition, the *Wnt/β-catenin* signaling pathway is also import for the development of the venous pole of the heart. Deletion of β-catenin with the islet 1 (*Isl1)-Cre* mouse (like the *Mef2c-AHF-Cre* mouse driving Cre-expression in the SHF) results in embryonic

lethality, pharyngeal arch defects, and cardiac malformations including AVSD (pASD) [35]. Conditional deletion of β-catenin results in reduction of proliferation of SHF-derived cardiac progenitor cells and significant downregulation of a number of downstream genes including *Isl1, T-box (Tbx) 3, Wnt11,* and *Shh* [35]. One specific *Wnt* variant found to be directly associated with the development of the posterior SHF and the DMP, and therefore with atrioventricular septation, is *Wnt2* [6]. At the critical stages of DMP development (ED9.5–10.5), *Wnt2* and lymphoid enhancer-binding factor 1 (*Lef-1*), a central transcription mediator of *Wnt/β-catenin* signaling, are expressed in the SHF mesodermal cell population and the DMP. *Wnt2* and *Lef-1* are, however, not expressed in the ventricles, AV canal, or AV cushions [6, 36]. Mice that do not express *Wnt2* (*Wnt2* null mutants) show reduced expression of *axin2, Isl1, Lef1*, and fibroblast growth factor 10 (*Fgf10*), a direct target of *Wnt/β--catenin* signaling [6, 34]. Staining for Ki67 shows a decrease in proliferative activity of the SHF/DMP-precursor population, and histological analysis reveals severe inhibition of DMP development. The proliferation defect and associated failure of the DMP to properly develop resemble what is observed in the conditional SHF-Alk3 knockout mouse [4] (see also above). Approximately 85 % of *Wnt2* null mutants die at birth, and histological analysis of *Wnt2* null specimens reveals the presence of AVSDs. LiCl is a pharmacological inhibitor of *Gsk-3b* and a strong activator of *Wnt/β-catenin* signaling. Administration of LiCl to pregnant female mice carrying ED8-10 mutant Wnt2 embryos leads to an elevation of *Lef1* expression in the posterior SHF, restores SHF cell proliferation, rescues the AVSD phenotype, and increases the survival rate of *Wnt2* null mutant offspring.

27.4.4 Pdgf and Pdgf Receptors and AVSDs

Platelet-derived growth factors (*Pdgfs*) play major roles in cardiac development. Knockout mice for *Pdgf* ligands and their receptors (*Pdgfrs*) present with a spectrum of cardiac malformations, including abnormalities of the outflow tract [37, 38]. AVSDs are found in *Pdgfrα* and *Pdgfrβ* mutant mice. While *Pdgfrβ* knockout mice only show sporadic AVSDs, the malformations are more common in *Pdgfrα* knockout mice, where the presence of an AVSD has been associated with hypoplasia of the DMP [39]. *Pdgfrα* is expressed in the pSHF and in the pSHF-derived DMP. Analysis of *Pdgfrα* knockout mice revealed significantly elevated levels of the transcription factor NK2 homeobox 5 (*Nkx2-5*). *Nkx2-5* is a transcription factor expressed in embryonic and differentiated cardiomyocytes, and during normal development, *Nkx2-5* is switched on in cells of the DMP as they undergo mesenchymal-to-myocardial differentiation around E14 [13]. If the gradual increase in *Nkx2-5* expression in the DMP is the driving force behind the muscular differentiation of the DMP mesenchyme after the formation of the AV mesenchymal complex and if *Nkx2-5* expression is indeed elevated in the pSHF of the *Pdgfrα* knockout mouse, then premature and ectopic myocardial differentiation of the pSHF cell population should be considered as a possible mechanism involved in inhibiting normal DMP formation and causing AVSD. In this context, it is interesting to note

that premature myocardial differentiation of pSHF has also been suggested to be involved in the pathogenesis of AVSDs in *Mef2c-AHF-cre;Smo$^{fl/-}$* embryos [5].

27.4.5 Tbx5 and AVSD

Haploinsufficiency of *TBX5* causes Holt-Oram syndrome [40, 41]. Patients with Holt-Oram syndrome have a spectrum of congenital malformations, including limb and cardiac defects. In the *Tbx5* mutant mouse, ASD and AVSDs are found in approximately 40 % of *Tbx5* haploinsufficient offspring [42]. Within the pSHF, *Tbx5* is co-expressed with *Gli1*, a member of a transcription factor family responsible for *Shh* signal transduction [42]. That *Tbx5* expression in the pSHF is critical to the development of the DMP was demonstrated by deleting *Tbx5* from the SHF with two SHF *Cre* mouse models: *Mef2c-AHF-Cre* and *Gli1-Cre-ERT2* mice [42]. Both approaches resulted in fully penetrant incomplete AVSD (*ostium primum defect*). Quantitative analysis of markers for *Shh* signaling in the *Mef2c-AHF-Cre;Tbx5* embryos revealed a significant reduction in the expression of the *Shh* receptor *Ptc1* and *Gli1*, suggesting that *Shh* signaling is downstream of *Tbx5* in the regulation of pSHF and DMP development. Furthermore, the gene encoding *Odd-skipped related-1* (*Osr1*), a zinc finger transcription factor, was identified as a direct downstream target of *Tbx5* in the SHF. Given the fact that *Osr1* knockout mice also develop AVSDs [43], and in combination with additional data, implicating *Tbx5* in promoting pSHF proliferation by directly regulating cell-cycle gene transcription through the cyclin-dependent kinase 6 (*Cdk6)*, these results show that *Tbx5* plays a critical role within the pSHF.

27.4.6 Cilia and AVSDs

Primary cilia are organelles that protrude from the cell surface. The core structure of primary cilia is composed of microtubule bundles (the ciliary axoneme), which extends, via a transition zone, from a microtubule-based basal body. The formation, maintenance, and function of cilia are dependent largely on trafficking of particles along the axoneme by the intraflagellar transport (IFT) machinery. Proteins of the IFT-B complex are required for anterograde trafficking from the base to the tip of the cilium, whereas the IFT-A complex is believed to be involved in controlling retrograde ciliary trafficking. Primary cilia are found on almost all mammalian cell types, and a growing body of evidence shows their significance in the development of a variety of organs. Cilia "dysfunction" has been associated with a spectrum of human congenital malformations, including cardiac defects and heterotaxy (see Chaps. 7 and 38). For instance, a recent publication reports atrial and ventricular septal defects in humans with mutations in nimA-related kinase 8 (NEK8), a primary cilium-associated protein [44], while another recent study suggests that AVSDs and other congenital heart malformations found in Down syndrome patients may be associated with an altered ciliome [45]. A comprehensive study of the avc1

mouse mutant, a mouse carrying a hypomorphic mutation of intraflagellar transport protein *Ift172* (*Ift172*^{avc1}/*Ift172*^{avc1}), showed AVSDs in 100 % of avc1 mutants analyzed [46]. Another mouse model with impaired cilia formation is the cobblestone (*cbs*) mutant, carrying a hypomorphic allele for *Ift88* (a.k.a. *Polaris*). Analysis of *cbs/cbs* mutants also showed a 100 % penetrance of complete AVSD [47]. Cilia are important in a number of signal transduction and molecular pathways associated with the development of the pSHF/DMP, in particular the *Shh* pathway. *Shh* signaling is initiated when secreted *Shh* binds to the membrane receptor *Patched* (*Ptc1*) localized on the distal end of the axoneme. This interaction alleviates inhibition of the transmembrane protein *Smoothened* (*Smo*; see 27.4.2) by *Ptc1*. *Smo* then activates the transcriptional activator *Gli2* thereby transmitting the *Shh* signal to the nucleus. The *Shh* signaling pathway is critically dependent on intraflagellar transport which acts downstream of *Ptc1* and *Smo* but upstream of the *Gli* transcriptional activity. Thus, primary cilia present on the cells of the posterior SHF may play a pivotal role in atrioventricular septation through the regulation of the *Shh* pathway and associated molecular and cellular mechanisms.

Conclusion

The studies described in this chapter show that compromised proliferation of the pSHF/DMP precursor cell population is the most common mechanism underlying the pathogenesis of AVSD [4, 6, 42]. In addition, some observations suggest that premature myocardial differentiation of pSHF cells and/or altered migration characteristics of these cells also contribute to the pathogenesis of this cardiac defect [5, 39]. The LiCl embryonic rescue experiment in the *Wnt2* knockout mouse [6] that resulted in lowering the penetrance of the AVSD phenotype in the *Wnt2* knockout offspring provides hope that in the foreseeable future pharmacological (or other) approaches may be developed which, in specific situations, can lower the risk of developing this congenital heart defect.

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Part VII

Total Anomalous Pulmonary Venous Return

Clinical Presentation and Therapy of Total Anomalous Pulmonary Venous Return

28

David J. Driscoll

Contents

Abstract

Total anomalous pulmonary venous return (TAPVR) can be divided into four anatomic groups: (1) supracardiac (Fig. 28.1), (2) cardiac, (3) infracardiac, and (4) mixed. Also, it can be divided into two physiologic types: nonobstructed and obstructed.

28.1 Introduction

Total anomalous pulmonary venous return (TAPVR) can be divided into four anatomic groups: (1) supracardiac (Fig. 28.1), (2) cardiac, (3) infracardiac, and (4) mixed. Also, it can be divided into two physiologic types: nonobstructed and obstructed.

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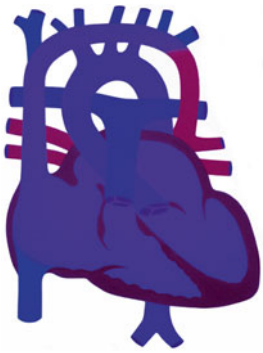

Fig. 28.1 Diagrammatic representation of supracardiac totally anomalous pulmonary venous return. The pulmonary veins drain to a left vertical vein that then connects to the innominate vein. The color scheme demonstrates the level of blood oxygenation in various parts of the heart. The blood is red (normal oxygen saturation) in the pulmonary veins and in the vertical vein. As it mixes with the systemic venous return blood, it becomes blue (low oxygen saturation) (Reproduced or adapted from Driscoll, David *Fundamentals of Pediatric Cardiology*, Lippincott Williams & Wilkins, 2006, with permission of the author and publisher)

28.2 Pathologic Physiology

Instead of connecting to the left atrium, the pulmonary veins connect to the systemic venous system. Consequently, pulmonary venous blood returns to the right atrium instead of to the left atrium. The locations of the connection of the pulmonary veins to the systemic veins (in order of frequency) are: the left vertical vein, innominate vein, coronary sinus, right atrium, umbilicovitelline system (portal vein, ductus venosus, inferior vena cava, hepatic vein), and superior vena cava. In "mixed" total anomalous pulmonary venous return, a combination of the above might occur. For example, the left pulmonary veins may connect to the left vertical vein and the right pulmonary veins directly to the right atrium. Because there is no direct communication between the pulmonary veins and the left atrium, a communication must exist between the right atrium and left atrium to allow blood to reach the left atrium and the left ventricle. Relatively complete mixing of the systemic and pulmonary venous returns occurs in the right atrium such that blood oxygen saturations in the right atrium, left atrium, right ventricle, pulmonary artery, left ventricle, and aorta are similar.

28.3 Clinical Presentation

The clinical presentation depends upon whether there is partial or total anomalous venous return and whether or not there is obstruction to the venous return. In unobstructed forms with an interatrial communication, the physiology is similar to a

large atrial septal defect. These patients may present with a murmur or signs of pulmonary overcirculation. Patients with obstructed forms will present with cyanosis and pulmonary edema.

28.4 Physical Examination

If there is an adequate interatrial communication and no significant obstruction to pulmonary venous return, the infant has mild cyanosis and evidence of increased pulmonary blood flow. There will be a systolic murmur. The clinical picture is completely different if there is an obstruction to pulmonary venous return. Characteristically, obstruction to pulmonary venous return occurs with infradiaphragmatic forms of total anomalous pulmonary venous return. In this case, there is pulmonary edema and respiratory distress.

28.5 Echocardiographic and Cardiac Catheterization Issues

The diagnosis can be established by echocardiography but may need to be confirmed by cardiac catheterization and angiography.

28.6 Treatment

Treatment is surgical correction with anastomosis of the pulmonary veins to the left atrium. Infants with obstructed totally anomalous pulmonary venous return represent a surgical emergency, and immediate repair is indicated upon diagnosis.

28.7 Outcome

Outcome depends primarily on whether or not the pulmonary veins are obstructive and how sick the infant is prior to operation. Mortality is highest in the sickest babies with obstructed pulmonary venous return. In all other cases, the operative mortality is very low. Long-term outcome is excellent although some patients will require reoperation because of stenosis of the anastomosis of the common pulmonary venous chamber to the left atrium.

Human Genetics of Total Anomalous Pulmonary Venous Return

29

Robert E. Poelmann, Monique R.M. Jongbloed, Marco C. DeRuiter, and Adriana C. Gittenberger-de Groot

Contents

Abstract

Partial anomalous pulmonary venous connections (PAPVC) have been found after abnormal gene expressions involving several syndromes. Total anomalous pulmonary venous connection (TAPVC) is found in conjunction with heterotaxia syndrome as well as several other syndromes. It has been reported with an autosomal dominance with variable expression and incomplete penetrance. The

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occurrence is also related to environmental factors which may superimpose on a familial susceptibility for TAPVC. Many pathways are involved in the normal development of the pulmonary venous connections and as a consequence disturbance of many genetic and epigenetic pathways lead to partial or total pulmonary venous misconnections. In this chapter, an overview of current knowledge regarding human genetics of anomalous venous connections is provided.

29.1 Introduction

As total anomalous pulmonary venous connections have a developmental background [1], for an overview of events during development, we refer to Chap. 30, introducing the cellular players and the time-windows for their interactions to provide a scaffold for the genetic and inheritance aspects of the anomalies. Of note, pulmonary-to-systemic connections initially exist during normal embryology that will regress during further development. In embryos with abnormal pulmonary venous connections, an abnormal extent of pulmonary-to-systemic connections may persist (Fig. 29.1; see Chap. 30).

Clinically it is important to know that the myocardial sleeve surrounding the pulmonary vein is missing in hearts with TAPVC, while the left atrial dorsal wall is hypoplastic [2]. The accompanying sinus node dysfunction in some of these patients [3, 4] points to an abnormal contribution of the so-called posterior heart field (part of the second heart field, see Chap. 30), which is also evident in the patient group with total absence of the myocardial sleeve presenting with a low incidence of atrial arrhythmias [4]. We have to keep in mind that many other patterns of pulmonary venous return are encountered such as partial anomalous pulmonary venous return including scimitar vein [5], cor triatriatum, primary and acquired stenosis, sinus venosus defects, atrial septal defect II (ASDII), and variant numbers of pulmonary veins which may complicate an adequate diagnosis [6].

29.2 TAPVC and Genetics

Partial anomalous pulmonary venous connections have been found after abnormal gene expressions involving several syndromes, including mutations in GATA4 (GATA binding protein 4) [7] and MEK1/MEK2 (MAPK/ERK kinase 1/2; also known as MAP2K1/2, mitogen-activated protein kinase kinase 1/2) [8]. TAPVC is found in conjunction with heterotaxia syndrome involving the nodal pathway [9] and Zic3 (Zic family member 3) mutations [10], Holt-Oram syndrome, cat eye syndrome [11–13], craniofacial and skeletal dysmorphia [13], cardio-facio-cutaneous syndrome [8], and Williams syndrome [14]. It has been reported with an autosomal dominance with variable expression and incomplete penetrance [15]. Genetic screens reveal rare copy number variation [16], submicroscopic genomic aberrations [17], and mutations in histone-modifying genes [18] related to TAPVC. The

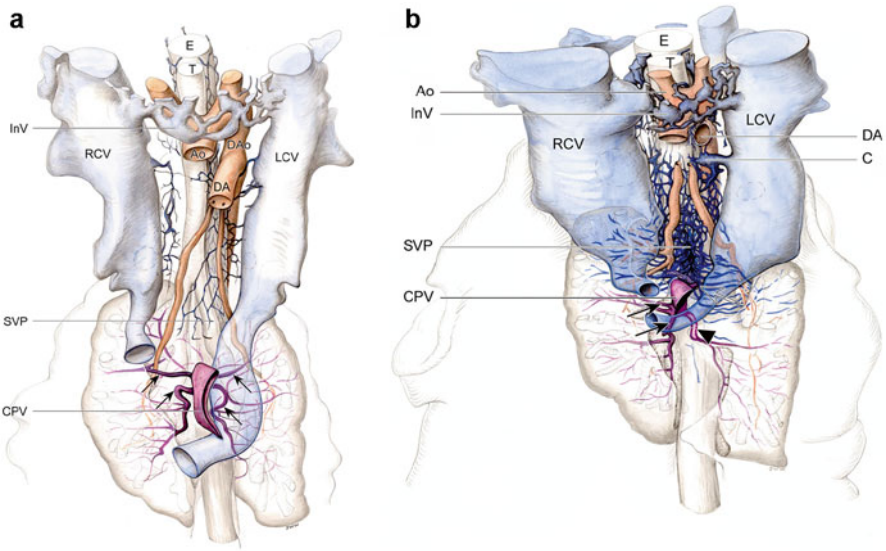

Fig. 29.1 3-D reconstructions of human embryos. Approximate gestational age 8 weeks (crown-rump length 18 m, Carnegie stage 14). Frontal views. The heart and part of the left atrial dorsal walls have been resected. (**a**) Normal embryo. *Arrows* indicate 4 pulmonary veins that drain the lung towards the left atrium (*LA*). There are no pulmonary-to-systemic connections anymore via the splanchnic venous plexus (*SVP*), although small communications of this plexus into the right and left cardinal veins (*RCV* and *LCV*) can still be observed. (**b**) Embryo with abnormal pulmonary venous connection. The *black arrows* point at 2 right-sided pulmonary veins normally draining towards the LA. The left superior pulmonary vein is lacking (*open arrow*), and the left inferior pulmonary vein is devoid of blood (*black arrowhead*). Note the extensive SVP, with numerous connections to both lungs. Also, a large connection (*C*) is present between the SVP and the LCV. Abbreviations: *AO* aorta, *DA* ductus arteriosus, *DAO* descending aorta, *E* esophagus, *INV* innominate vein, *T* trachea (Source: Modified after Rammos et al. [30])

occurrence is also related to environmental factors such as exposure to lead, solvents, and pesticides, which may superimpose on a familial susceptibility for TAPVC [19]. It may be concluded that many pathways are involved in the normal development of the pulmonary venous connections and as a consequence that disturbance of many genetic and epigenetic pathways lead to partial or total pulmonary venous misconnections. Analysis of families with high frequency of TAPVC revealed three candidate genes: TAPVR1, ANKRD1/CARP (ankyrin repeat domain 1, cardiac muscle), and SEMA3D (semaphorin 3d).

TAPVR1 has been mapped to chromosome 4q12, the centromeric region containing the kinase domain receptor (KDR) gene, that plays a role in vasculogenesis [20]. Disturbed vaculogenesis may potentially lead to deficient development of the mid-pharyngeal endothelial strand, causing PAPVC with persisting pulmonary-to-systemic connections, although the exact developmental mechanism has not been elucidated. More recently, the TAPVR1 region has been narrowed down to an intergenic region between PDGFRA and KIT [21]. Mutation analysis of the *PDGFRA*

(platelet-derived growth factor receptor, alpha polypeptide) gene in TAPVC patients revealed a rare sequence variant pointing towards a presumptive TAPVC gene. Mouse models with deficient PDGFr alpha show a small DMP (see Chap. 30) and abnormal drainage of the systemic and pulmonary veins, although a phenotype of TAPVC was not observed [22].

Furthermore, the ANKRD1/CARP gene encoding a muscle specific protein has been implicated in cardiac transcriptional regulation and myofibrillar assay [23]. It was localized to chromosome 10, proximal to a previously found translocation point [24, 25]. The ANKRD1/CARP gene is regulated by GATA4, and disruption of this signalling axis *in vitro* leads to sarcomere disarray [26]. Ankyrin repeat proteins are stress inducible that are thought to be involved in mechanosensing in response to pressure overload [27]. Therefore, the mechanism of the occurrence of TAPVC may relate to atresia caused by dysregulated flow through the embryonic mid-pharyngeal endothelial strand (MPES), although this remains speculative at this point.

As described above, pulmonary vein development starts with the early embryonic connection of the MPES to the heart [28]. The development of the early pulmonary veins depends heavily on the MPES and the associated semaphoring signalling (Chap. 30). Sequencing of *SEMA3D* in patients with TAPVC and PAPVC revealed two variants. One variant, a c.193 T >C missense, resulted in a serine-to-proline substitution, whereas a second variant, a c.1806 T >A missense mutation (phenylalanine-to-leucine), was present in an individual with PAPVC with an associated ventricular septal defect but no signs of heterotaxia. The amino acid alteration is located in the immunoglobulin-like domain of SEMA3d probably important for both endothelial neuropilin receptor binding and functional activity [29].

Conclusion

In conclusion, several mutations have been described in the human population that affect the formation of the pulmonary venous drainage. The perturbed genes are involved in PDGF signalling, sarcomere formation, and endothelial migration, thereby regulating various phases of differentiation. In view of the complexity of the interactions, it will be no surprise to find in the near future that more signalling pathways are involved.

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Molecular Pathways and Animal Models of Total Anomalous Pulmonary Venous Return

30

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Abstract

The venous pole of the heart where the pulmonary veins will develop encompasses the sinus venosus and the atrium. In the 4th week of development, the sinus venosus consists of a left and a right part receiving blood from the common cardinal vein, the omphalomesenteric and umbilical veins. Asymmetrical expansion of the common atrium corresponds with a rightward shift of the connection of the sinus to the atrium. The right-sided part of the sinus venosus including its tributing cardinal veins enlarge to form the caval veins that will incorporate into the right atrium. The left-sided part in human development largely obliterates and remodels to form the coronary sinus in adults. In approximately the same time-window (4th–5th week), a splanchnic vascular plexus surrounds the developing lung buds (putative lungs) with a twofold connection. Of note, during early developmental stages, the primary route of drainage from the pulmonary plexus is towards the systemic veins and not to the heart. After lumenization of the so-called mid-pharyngeal endothelial strand (MPES), the first Anlage of the pulmonary vein, the common pulmonary vein can be observed in the dorsal mesocardium, and the primary route of drainage will gradually change towards a cardiac drainage. The splanchnic pulmonary venous connections with the systemic cardinal veins will gradually disappear during normal development. In case of absence or atresia of the MPES, the pulmonary-to-systemic connections will persist, clinically resulting in total anomalous pulmonary venous return (TAPVR). This chapter describes the developmental processes and molecular pathways underlying anomalous pulmonary venous connections.

30.1 Introduction to Cardiac Development

The primary heart tube develops from the bilateral cardiogenic plates as part of the splanchnic mesoderm. After midline fusion of the plates, together comprising the first heart field, the primary heart tube is formed. It is connected anteriorly to the aortic sac that feeds the pharyngeal arch arteries and caudally to the omphalomesenteric veins collecting the returning blood.

The venous pole of the heart encompasses the sinus venosus and the atrium [1]. In the 4th week of development, the sinus venosus consists of a left and a right part receiving blood from the common cardinal vein, the omphalomesenteric and umbilical veins. The sinoatrial fold marks the border with the common atrium. Asymmetrical expansion of the common atrium corresponds with a rightward shift of the connection of the sinus to the atrium. The right-sided part of the sinus venosus including its tributing cardinal veins enlarge to form the caval veins that will incorporate into the right atrium [2]. The left-sided part in human development largely obliterates and remodels to form the coronary sinus in adults (Fig. 30.1).

The inner lining of the myocardial tube consists of endocardial cells that are continuous with the endothelium of the blood vessels. As a consequence of left–right patterning, the heart tube is asymmetric and loops to the right in normal development. Major parts of both the arterial and venous pole are derived from the

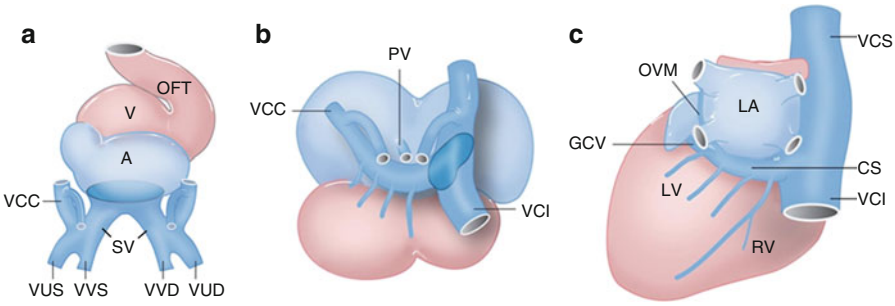


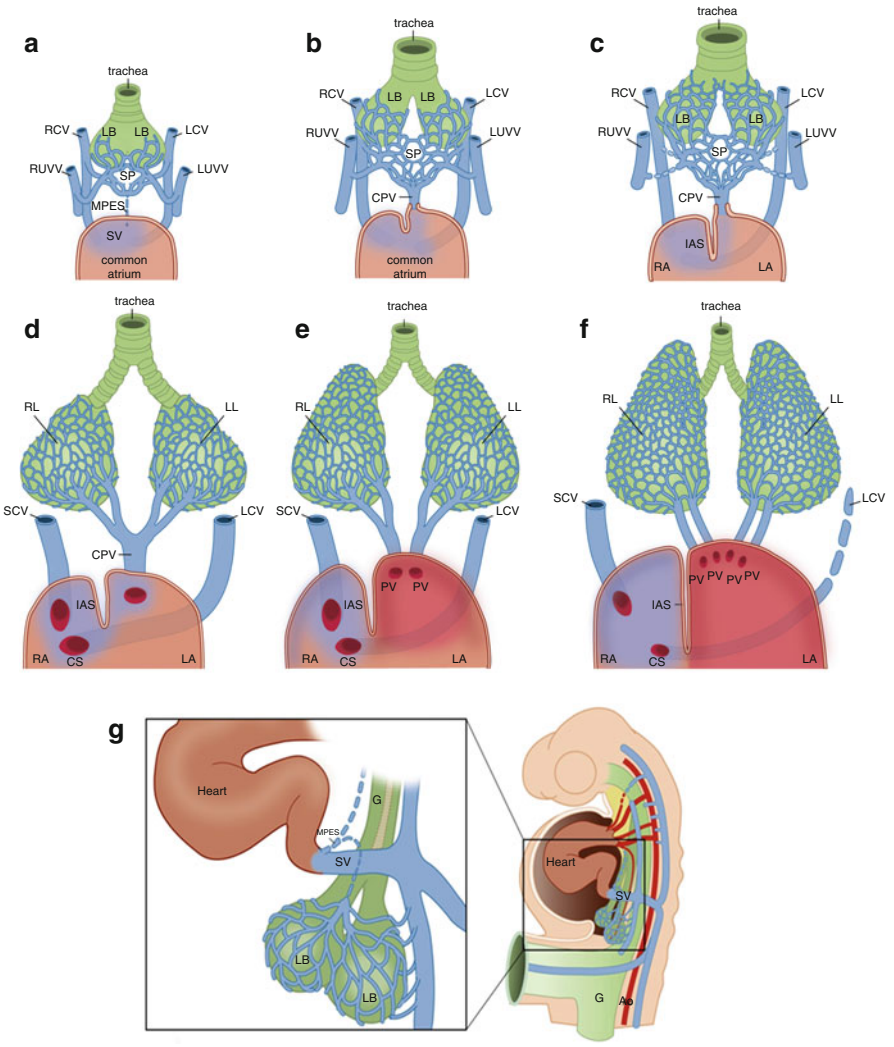
Fig. 30.1 Schematic representation of subsequent stages of cardiac development. (**a**) The venous pole of the heart encompasses the sinus venosus (*SV*) and the atrium (*A*). Initially, the SV consists of, more or less symmetrical, *left* and *right* horns that receive blood from the common cardinal vein (*VCC*), the *left* and *right* vitellin veins (*VVS* and *VVD*, resp.) and the *left* and *right* umbilical veins (*VUS* and *VUD*, resp.). (**b**, **c**) Due to an asymmetrical expansion of the common atrium, a rightward shift of the connection of the sinus to the atrium occurs. The right-sided cardinal vein will form the superior caval vein (*VCS*). The *left side* in human will largely obliterate, the remaining part will form the coronary sinus (*CS*) and the vein of Marshall (*OVM*, remant of the *left* cardinal vein). Initially, the single pulmonary vein Anlage will drain rather central in the atrium. After septation, the pulmonary veins (*PV*) will drain into the *left* atrium. *GCV* great cardiac vein, *LV* left ventricle, *OFT* outflow tract, *OVM* oblique vein of Marshall, *RV* right ventricle, *V* common ventricle, *VCI* inferior caval vein (Source: [41])

splanchnic mesoderm behind the heart during development, referred to as contributions from the second heart field (SHF). The primitive heart tube thus expands by intrinsic growth patterns of the cardiac tube, but also by extrinsic additions at both the arterial and the venous pole at expense of the SHF splanchnic mesoderm, as shown by expression patterns of, e.g. Tbx (T-box) 5, GATA (GATA binding protein) 4–6 and NKX2-5 (NK2 homeobox 5) [3], Islet1 (ISL LIM homeobox 1) [4] and Id2 (inhibitor of DNA binding 2) [5]. During heart development, cardiac chambers and transitional zones appear, the latter involved in septation, valvulogenesis and formation of the conduction system. The future atrial and ventricular components are separated by the atrioventricular canal. At the venous pole, recruitment of myocardium has been reported to depend on Pitx2c (paired-like homeodomain 2), Nkx2-5, Tbx18, Shox2 (short-stature homeobox 2) and Pdpn (podoplanin) [6–10]. This region located posterior to the primary heart tube, as part of the SHF is referred to as the posterior second heart field being involved in addition of myocardium to the sinus venosus, the pulmonary veins, the cardinal veins, as well as to the body of the atria.

30.2 Pulmonary Plexus and Vein Development

During the 4th week of embryonic human development, the primary lung buds appear at the apex of the endodermal respiratory diverticulum, surrounded by splanchnic mesoderm (Fig, 30.2). The lung buds differentiate into the bronchial system and their bifurcations in the future lungs. The splanchnic mesoderm

provides the pulmonary vascular smooth muscle and connective tissues as well as the capillaries, the latter developing into a splanchnic plexus by which the primitive lungs drain into the systemic circulation. The splanchnic plexus is connected to the venous pole of the primitive heart tube by a strand of endothelial cells, the so-called mid-pharyngeal endothelial strand that becomes the common pulmonary vein (Fig. 30.2a, g). The MPES is initially not lumenized, and the main route of drainage to the venous pole of the heart is via connections of the pulmonary splanchnic plexus to the embryonic systemic veins (cardinal, umbilical and vitellin veins). This early drainage pattern has also been referred to as a *peripheral* drainage pattern.



Upon lumenization of the MPES, the lungs initially drain both centrally to the atrium and peripherally to the systemic veins, the so-called *intermediate* drainage period (Fig. 30.2b). After atrial septation, the pulmonary–systemic connections are lost and regress (Fig. 30.2c), the beginning of the *central* drainage period [11] (Fig. 30.2d–g). Persistence of early pulmonary-to-systemic connections is likely the substrate for anomalous pulmonary venous connections. Several forms of anomalous pulmonary venous connections can be attributed to a deficiency in development towards the establishment of a central drainage pattern (Table 30.1).

30.3 Myocardialization of the Pulmonary Veins

The entrance of the common pulmonary vein to the sinus venosus is bordered by two muscular ridges (Fig. 30.3). The right-sided pulmonary ridge merges with the so-called dorsal mesenchymal protrusion (DMP), important for formation of the base of the atrial septum [12, 13]. As a consequence, the pulmonary vein between the two muscularized ridges will at the left side be incorporated in the posterior wall of the left atrium, thereby contributing to the histological characteristics of the left atrial body, consisting partly of vascular smooth muscle cells and partly of myocardium [14]. The DMP is derived from the embryonic second heart field that

Fig. 30.2 Schematic overview of pulmonary vein development, frontal views. (**a**) Peripheral draining period. During the 4th week of embryonic development, 2 lung buds (*LB*) can be discriminated at the caudal end of the respiratory diverticulum of the foregut. These lung buds into the bronchi and their bifurcations in the future lungs. The splanchnic mesenchyme forms a splanchnic plexus (*SP*) of veins that is already connected to the endocardium of the primitive heart tube, by means of a strand of endothelial cells, the mid-pharyngeal endothelial strand (*MPES*), the Anlage of the common pulmonary vein. Initially this strand is not lumenized yet; during early embryology, the normal route of drainage is from the pulmonary venous plexus to the systemic veins via pulmonary-to-systemic venous connections. (**b**) Intermediate draining period. At this stage, the MPES has lumenized to become the common pulmonary vein (*CPV*), allowing drainage of the splanchnic plexus not only to the systemic veins but also into heart. (**c**) As the CPV grows and dilates and becomes the main route of drainage of the pulmonary venous blood, the primitive pulmonary-to-systemic connections will gradually regress. (**d**) Central drainage period. During normal development, the primitive pulmonary-to-venous connections will regress completely, and the only route of drainage of the pulmonary venous blood is now directly into the heart. (**e**, **f**) During further development, bifurcations of the CPV will be incorporated into the left atrium (*LA*), usually to an extent that four separate pulmonary venous ostia (*2 right sided* and *2 left sided*) can be distinguished, although variations occur [17]. The right cardinal vein (*RCV*) has become the superior caval vein (*SCV*), and the left cardinal vein (*LCV*) will partly become the coronary sinus (*CS*), whereas the extracardiac part of the LCV will regress to become the ligament of Marshall. (**g**) Lateral view of the embryo with a magnification of the boxed area. A splanchnic vascular network surrounds the lung buds (*LB*) that grow out from the foregut (*G*). *G* foregut, *IAS* interatrial septum, *LB* lung buds, *LL* left lung, *LUVV* left umbilical and vitellin veins, *RA* right atrium, *RL* right lung, *RUVV* right umbilical and vitellin veins, *SV* sinus venosus

Table 30.1 Abnormal pulmonary vein development in relation to anatomical level, time and clinical entities

Anatomical level	Anomaly	Developmental impairment	Time relation (in relation to atrial septation)	Pulmonary-to-systemic connections	Clinical entity
Common PV/MPES	Absence	Absent connection PV-LA (absent MPES)	Before	Will persist	TAPVC (extracardiac type)
	Atresia	Non-lumenization of the MPES (PV Anlage)	Before	Will persist	TAPVC (extracardiac type)
	Stenosis	Secondary stenosis of an initially lumenized common PV	Before/during	Will disappear	Cor triatriatum
	Atresia	Secondary obliteration of an initially lumenized PV	After	Have disappeared	TAPVC (lethal)
First or second PV bifurcation	Atresia	Non-lumenization of 1 or more individual PV(s)/ tributaries	During	Will persist	PAPVC (extracardiac type)
	Stenosis	Secondary stenosis of an initially lumenized individual PV	Before/during After	Will disappear Have disappeared	Congenital PV stenosis Acquired PV stenosis
	Atresia	Obliteration of 1 or more initially lumenized PVs	After	Have disappeared	(Solitary) PV atresia
Veno-atrial junction	Variant number of PVs	Abnormal PV incorporation	After	Have disappeared	Unilateral common
	<4	Incomplete			PV ostium[a]
	>4	Extreme			>4 PV ostia[a]
Dorsal mesenchymal protrusion	Hypoplasia	Abnormal mesenchymal contribution from SHF	During	Will disappear	Sinus venosus defect with PAPVC (cardiac type) A(V)SD
	Absence	Absent mesenchymal contribution from SHF	During	Will mostly disappear	TAPVC (cardiac type) A(V)SD

Source: Reproduced with permission from [23]
MPES mid-pharyngeal endothelial strand, *PAPVC* partial anomalous, PV connection, *PV(s)* pulmonary vein(s), *TAPVC* total anomalous PV connection, *SHF* second heart field
aNote: not regarded as pathological but rather as anatomical variations

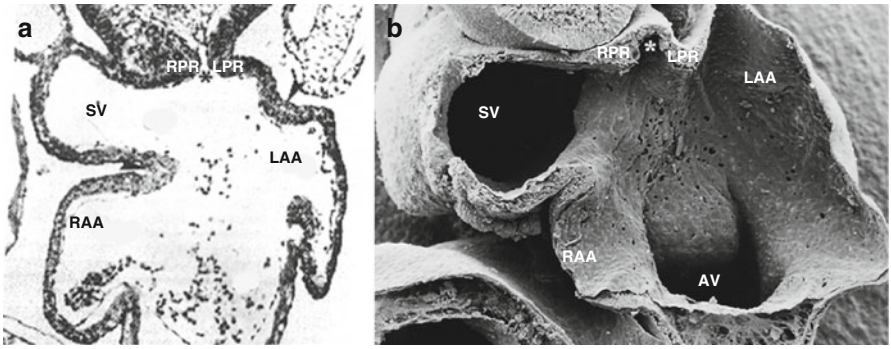


Fig. 30.3 Transverse light microscopic section (**a**) and electron microscopic (**b**) section at the level of the sinus venosus (*SV*) and atrial appendages (*RAA* and *LAA*) of a chick embryo. The entrance of the common pulmonary vein (*asterisk*) to the SV is bordered by two muscular ridges, the *left* (*LPR*) and *right* pulmonary ridge (*RPR*). The latter merges with the so-called dorsal mesenchymal protrusion (not shown), at the base of the interatrial septum, causing the pulmonary vein to be located at the *left side* after atrial septation (Source: Panel a adapted from [42])

comprises an area of mesenchyme behind the heart that will contribute to the embryonic heart during further development [3].

The common pulmonary vein becomes myocardialized and gives rise to separate venous orifices in the posterior left atrial wall. The degree of incorporation is variable, and not all individuals have four distinct pulmonary venous ostia, while common ostia of particularly the left pulmonary veins as well as additional pulmonary venous ostia are often observed [15–17].

The wall of the pulmonary veins will develop a myocardial sleeve as is the case with the cardinal (caval) veins. The myocardial continuity of the atrial and venous walls, varying in different species, has been thoroughly investigated by several groups and has led to different opinions regarding the origin and differentiation of the involved cell populations [7, 14, 18–20].

30.4 Histological Characterization During Normal Development

Pulmonary vein development in human embryos takes place between 7 and 22 weeks (crown rump length 19–170 mm). Using various specific antibodies for muscle actins, alpha-smooth muscle actin and atrial myosin light chain, three different areas can be distinguished in the posterior left atrial wall: (1) the smooth-walled atrial body with incorporated pulmonary vein smooth muscle cells, (2) the trabeculated appendage without vascular smooth muscle cells and (3) the transitional zone between body and appendage (see Fig. 19.2). The transitional zone histologically resembles the sinus venosus and presents with a very thin and even absent myocardium, from which it was hypothesized that during incorporation of the pulmonary

vein into the body of the left atrium, the area of the sinus venosus myocardium was reduced to the narrow zone encircling the entrance to the atrial appendage [14]. These observations are relevant for interpreting the definition of the sinoatrial transition and the sinus venosus, but also for the interpretation of areas of preferential induction of arrhythmias in later life. Interestingly, the body of the right atrium, the right atrial appendage and the right-sided face of the atrial septum are not lined by vascular smooth muscle cells.

30.5 Origin and Incorporation into the Left Atrium

The origin and incorporation of myocardium and smooth muscle cells into the pulmonary vein and left atrium has been investigated using various approaches. Based on overlapping gene expression patterns in the SHF of Pitx2c, Islet1, Tbx18 and Nkx2-5, clues are provided to the origin of myocardial cells enveloping the caval and pulmonary veins together with segments of the atrium (elegantly summarized by Lescroart et al. [19]). The topic of connection of the pulmonary vein to the sinus venosus and subsequent incorporation into the left atrium is marked by a fierce debate [21–25], but it is unnecessary to repeat the various arguments here. It suffices to note that myocardium associated with the left-sided veins (pulmonary vein and left cardinal vein, including the dorsal left atrium) shares a common lineage, as does myocardium associated with the right-sided veins including the dorsal right atrium [19]. Therefore, we favour an original left-sided connection of the common pulmonary vein to that part of the sinus venosus that will be incorporated in the dorsal wall of the left atrium. With this concept in mind, we must realize that patterning of the various developmental players (second heart field, DMP, myocardium, smooth muscle cells) will be reflected by the (epi)genetic regulation networks and in case of TAPVC by misregulation.

It is evident that different areas of gene expression can be recognized, as Pitx2c is expressed in the myocardium of the pulmonary vein, left superior caval vein and (left) part of the atrium, Nkx2-5 in the pulmonary vein myocardium/mesenchyme and atrial myocardium, Tbx18 only in the myocardium surrounding the caval veins and Nppa (natriuretic peptide A) only in the atrial wall. Podoplanin is expressed in the myocardium and smooth muscle cells of the pulmonary veins, as well as in the left atrial dorsal wall including the left and right venous valves. The temporospatial expression pattern of these genes in SHF progenitors based on the use of reporters and transgenes in mice is intricate [7, 9, 10, 26]. A recently published approach initiated by Buckingham et al. [27] exploits retrospective clonal analysis based on rare random events of recombination of a nonfunctional *nLaacz* sequence into functional *nLacZ,* targeted to the alpha cardiac actin gene [19], the recombination event being independent of gene expression. They conclude that there are left and right myocardial sublineages at the venous pole of the heart. The pulmonary and left superior caval vein share common progenitors with dorsal left atrial myocardium (Fig. 30.4), whereas the right superior caval vein and dorsal right atrial myocardium share their own common progenitors, illustrating the difference between

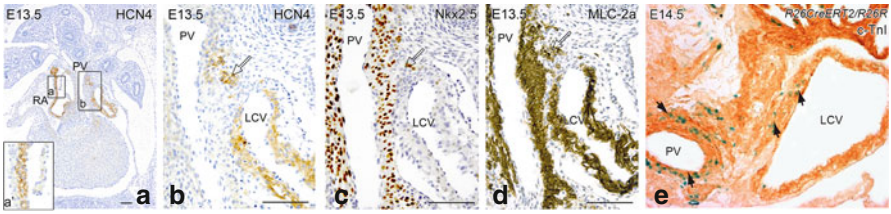


Fig. 30.4 Cellular continuity between myocardium forms the left cardinal vein (*LCV*) that includes a transient left sinoatrial node (SAN) and pulmonary veins (*PV*). (**a**–**d**) Cells from the region of the left-sided SAN can be traced section to section towards the myocardium surrounding the pulmonary veins (*PV*). (**a**) Overview section of an E13.5 heart, showing expression of the marker HCN4 at the level of the sinus venosus. (**b**, **c** and **d**) are enlargements of the boxed area in (**a**). Expression of HCN4 (**b**) is observed as the cluster of cells (*open arrows* in **b**–**d**) that can be followed section to section from the *left* SAN region towards the PV myocardium. These cells also express the myocardial marker MLC-2a (**d**) and mostly lacking expression of Nkx2-5 (**c**). (**e**) Section of an E14.5 heart of a *R26CreERT2/R26R* embryo showing a clone of β-galactosidase-positive cells in the LSCV, PV and LA. A c-TnI antibody was used to stain the myocardium (Source: Panels **a**–**d** are reproduced with permission from [43]. Source: Panel **e** is modified with permission from [19])

genetic tracing experiments and lineage analysis. Furthermore, these results suggest that the caval vein myocardium does not have a separate clonal origin from the pulmonary myocardium.

30.6 Total Anomalous Pulmonary Venous Connection (TAPVC)

At the end of the 5th week in human development, the common pulmonary vein has established the connection of the lungs with the atrium allowing regression of the earlier splanchnic connections and the MPES to the systemic circulation (Fig. 30.2). In hearts with TAPVC, a connection between the splanchnic plexus and the left atrium is lacking, leading to persistence of pulmonary drainage via (one of) the cardinal veins. This can be distinguished clinically in infracardiac, cardiac and supracardiac connections. The left atrial wall lacks vessel wall tissue as incorporation of the pulmonary vein has not been established in neonate and adult patients [28] (Fig. 30.5a–d). In mouse embryos, the differentiation of the sinus venosus and pulmonary myocardium has been investigated showing that both share expression of podoplanin (expressed in the posterior second heart field) and the cation channel protein HCN4 (hyperpolarization-activated cyclic nucleotide-gated potassium channel 4) but lack Nkx2-5 [8, 29]. During subsequent development, Nkx2-5 becomes upregulated and HCN4 (a conduction-related gene) downregulated in the pulmonary vein until the latter becomes restricted to the sinus node, suggesting that early conduction properties may relate to the enhanced susceptibility of this area for arrhythmias later in life. The low incidence of arrhythmias in patients with TAPVC might be explained by impaired myocardial sleeve formation surrounding the

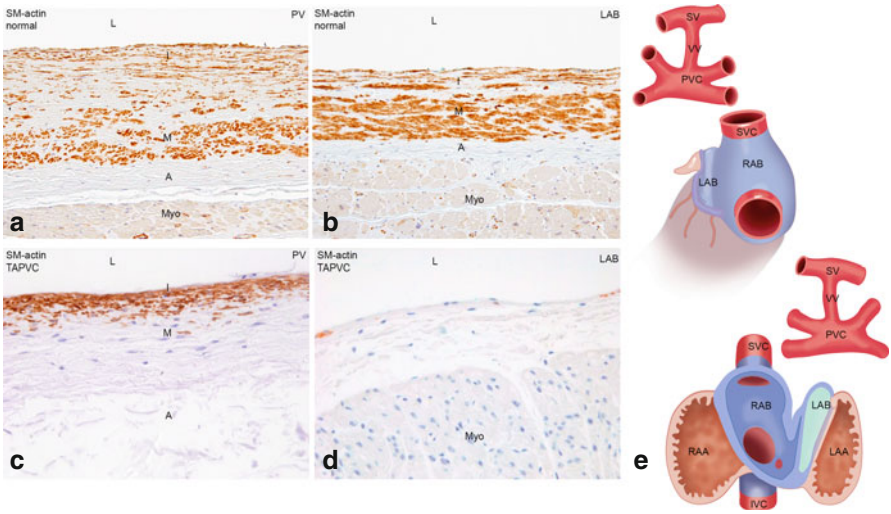


Fig. 30.5 Histology of total anomalous pulmonary venous connection (*TAPVC*). (**a**, **b**) Sections of a normal adult heart stained with alpha SM actin, showing the histological structure of pulmonary venous and *left* atrial wall. In the PV (**a**) as well as in the LAB (**b**), an identical and characteristic vessel wall, with myocardial covering (*myo*) is found. Profound intimal thickening (*I*) is observed most obviously in the PV in this adult heart. (**c**, **d**) Sections of neonatal heart–lung specimens stained with 1A4 against alpha-smooth muscle actin (*SM actin*), showing the histological structure of pulmonary venous and *left* atrial wall in hearts with total anomalous pulmonary venous connection (*TAPVC*). (**c**) Pulmonary venous confluence representative for TAPVC showing vessel wall consisting of a tunica intima (*I*), media (*M*) and a tunica adventitia (*A*) without myocardial covering. (**d**) Smooth-walled *left* atrial body (*LAB*) with a slightly thickened (sub)endocardial layer of collagen, elastic fibres and occasional smooth muscle cells covered by myocardium (*Myo*). No vessel wall tissue was found. (**e**) Schematic depiction of outer (**a**) and inner (**b**) side of atrial chambers and pulmonary veins (*PV*) in hearts with extracardiac type of TAPVC. The pulmonary veins (*PV*) drain via a vertical vein (*VV*) into a systemic vein (*SV*) and are not covered by myocardium. The *left* atrium body (*LAB*) consists smooth-walled primary myocardium, without secondary addition of myocardium from the posterior heart field. Because of the absence of PV connection and incorporation into the LA, the LAB is small and does not contain vessel wall tissue. *CS* coronary sinus, *IVC* inferior caval vein, *L* lumen, *PVC* pulmonary venous confluence, *SVC* superior caval vein (Source: Modified with permission [28])

pulmonary veins [28]. Furthermore, as a consequence of impaired pulmonary vein incorporation, resulting probably in diminished flow and hemodynamics, the left atrium is hypoplastic (Fig. 30.5e). Optimizing results of TAPVC correction is often accompanied by surgical augmentation of left atrial function [30].

30.7 Animal Models

Several reporter and transgenic mouse models have been employed to study the development of the venous pole of the heart, focusing on the left (pulmonary) side. Only conditional deletion and transgenesis of transcription factors governing many aspects of cardiogenesis including Islet1, GATA4-6, BMPs (bone morphogenic proteins),

Nkx2-5, SRF (serum response factor), Tbx family members and others can address the function of these genes in specific parts of heart development. Reporter models rely heavily upon specific expression patterns in defined time-windows and as such have their limitations. Nevertheless, in this field, much evidence has been gained. In this chapter, specific animal models for pulmonary venous development will be highlighted. Genes involved in the formation of the posterior heart field and subsequent recruitment of myocytes to the venous pole of the heart will often result in a phenotype when mutated. The sinus venosus myocardium forms a horseshoe-shaped band lining the base of the left and right cardinal veins. It is unique in that it does not express the precardiac marker Nkx2-5 in early developmental stages [7, 8].

The PDGF (platelet-derived growth factor) family of growth factors comprises regulators involved in various stages of cardiac development. In the posterior heart field and venous pole, expression of PDGF-receptor alpha and the ligands PDGF-A and PDGF-C has been shown in, e.g. chicken [31] and mouse embryos [32] showing temporal and spatial patterns consistent with a role in pulmonary vein development. Knockdown of PDGFRA function in a mouse model may result in a spectrum of cardiac inflow defects including TAPVR in humans [32]. Loss of PDGFRA function causes TAPVR with low penetrance (~7 %) reminiscent of that observed in the human population. Mutation analysis of the PDGFRA gene in TAPVR patients revealed a sequence variant on human chromosome 4q12 suggestive for a putative TAPVR gene.

An important component of the atrium is the posterior heart field-derived dorsal mesenchymal protrusion (DMP), involved in development of the dorsal wall of the atrium and more specifically in atrial septation. Furthermore, the DMP is essential for partitioning the pulmonary vein from the systemic venous system as shown in the abovementioned Pdgf signalling [32]. Abnormal developing DMP from impaired podoplanin signalling results in atrioventricular septum defects, combined with a diminished incorporation of the vascular smooth muscle cells, but also in deficient pulmonary vein myocardium and connection [26, 32] (Fig. 30.6). Furthermore, sonic hedgehog [33] and the Tbx5-hedgehog molecular network [34] are essential for second heart field and DMP formation. Nevertheless, pulmonary vein differentiation is unaffected when DMP formation is hampered in Tbx5 mutants [34] suggesting that the DMP and pulmonary vein develop independently and in parallel from the SHF.

Shox2, homologous to *SHOX*, involved in short-stature syndrome in humans, is expressed in the sinus venosus myocardium. *Shox2* knockout mice showed embryonic lethality between 11.5 and 13.5 dpc, and sinus venosus myocardium was markedly hypoplastic. Although primarily investigated for the anomalies at the right side of the venous pole including the sinoatrial node, the left-sided pulmonary vein was also diminished in size [6]. As this mouse model did not survive beyond E13.5, further development of the pulmonary vein could not be investigated.

The role of Id2 (inhibitor of differentiation/inhibitor of DNA binding) in chicken, Xenopus and mouse has been described in the second heart field [29]. Id1-4 proteins are negative regulators of basic loop–helix proteins, promoting cell proliferation and keeping the cells in an undifferentiated state [35] dependent on GATA4 and NKX2-5. Knockout mouse embryos (embryonic day, ED 11.5) present with severe abnormalities including dilated cardinal veins and an underdeveloped sinus venosus resulting in displaced venous drainage. The pulmonary veins are connected to the

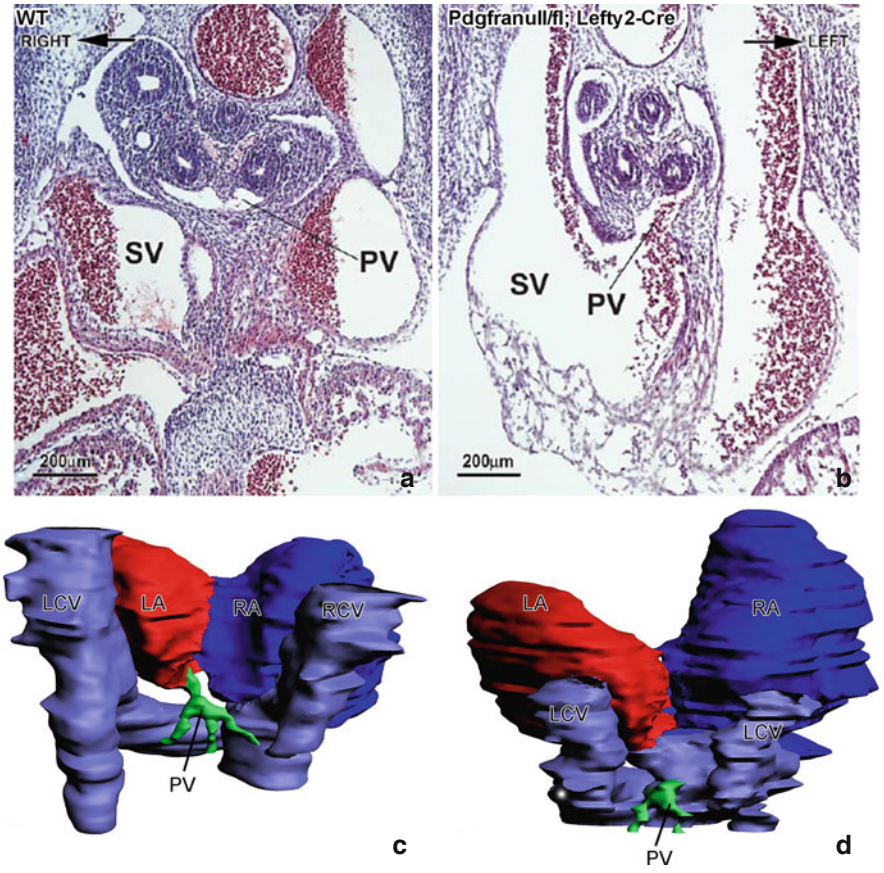


Fig. 30.6 TAPVR in the Pdgfra mouse mutant. A restricted deficiency of PDGFRA was achieved here in Pdgfra$^{flox/flox}$; H Lefty 2-Cre embryos, to avoid the confounding effects of earlier PDGFRA deficiency during gastrulation or early cardiogenesis [32]. Transverse section (**a**) and dorsal view of a 3D reconstruction (**b**) of a wild-type E11.5 mouse embryo. The primitive pulmonary vein (indicated in green in the 3D reconstruction) with its tributaries connects to the *left* atrium (red area in b). (**c**, **d**) Similar levels in the Pdgfra $^{null/fl}$; Lefty 2-Cre embryo. The PV (indicated in green in the 3D reconstruction) connects to the sinus venosus, consisting of the *right* and *left* cardinal veins (*RPV* and *LCV*, resp.) and their confluence (indicated in purple in the 3D reconstruction). The *right* atrium (*RA*) is indicated in blue (Source: Modified with permission after [32])

atrium caudally to that of the left cardinal vein into the sinus venosus. At ED 12.5, a less well-developed interatrial septum is present, while the cardinal and pulmonary veins hardly show any myocardial layer. Furthermore, the intracardiac part of the pulmonary vein is surrounded by differentiated atrial myocardium lacking the transitional zone characteristic for wild-type embryos. In ED 13.5 and 14.5, the left atrium appears smaller, reminiscent of human patients with TAPVC. However, the DMP does not seem to be affected in the Id2 mutant which is in line with the observation that atrioventricular defects have not been found. Remarkably, the myocardialization of the DMP appears more advanced than in the wild type after loss of Id2 control. Nevertheless, myocardialization of the systemic veins was diminished from

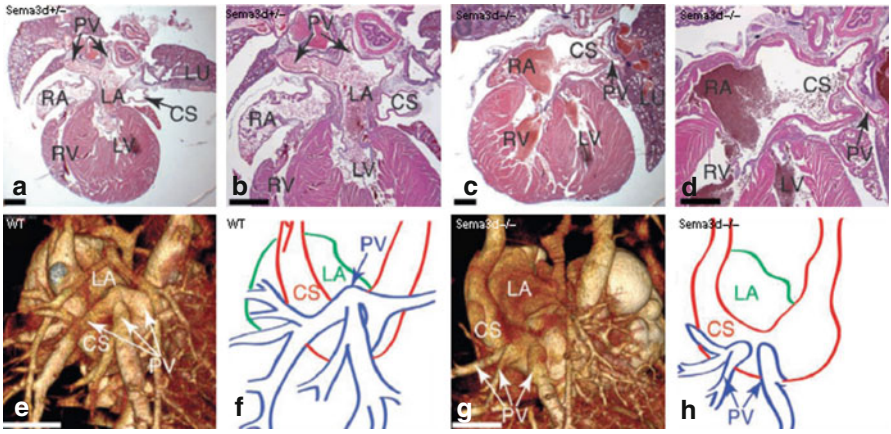


Fig. 30.7 Pulmonary venous connections in Semaphorin 3d heterozygous (Sema3d+/–) and Semaphorin 3d mutant (Sema3d–/–) adult mice. The pulmonary veins (*PV*) normally connect to the *left* atrium (*LA*) in the Sema3d+/– mouse (**a**, **b**). In the mutant mouse, an (**c**, **d**) anomalous pulmonary venous connections to the coronary sinus is observed (**e**–**h**). Volume-rendered micro-computed tomography (microCT) images (**e**, **g**, dorsal views) and schematic drawings of the pulmonary venous connections (**f**, **h**). The pulmonary veins normally enter the LA in a newborn wild-type (*WT*) mouse (**e**, **f**) and connect to the coronary sinus in a newborn mutant mouse (**g**, **h**) (Source: Adapted with permission [40])

which may be concluded that in Id2 mutants the balance between differentiated and undifferentiated cells is lost [29].

In animal models, it has been shown that many genes are involved in atrial and venous return development. Myocardial progenitor cells in the left or right posterior heart field contribute to the left or right atrium, respectively [19]. Manipulation of Pitx2c [36] demonstrates repression of either right atrial identity or of proliferation of the left sinus venosus [37]. Islet1 is an important regulator in the posterior heart field [4]. The Islet1 SHF enhancer contains three consensus binding sites suggestive for regulation by Forkhead transcription factors [38] and is part of a more complex regulative network also including sonic hedgehog, Fgf8 (fibroblast growth factor 8), Tbx1 and retinoic acid [39]. The pulmonary vein gains access to the Isl1 expressing medial dorsal side of the caudal heart tube that is no longer Tbx18 positive. As the remainder of the venous pole expresses Tbx18, it has been suggested [10] that the ancestors to the pulmonary and systemic veins are strictly separated. This gene expression-based evidence contrasts strongly with the findings of Galli [37] and Lescroart [19] who describe distinct left- and right-sided lineages in contrast to systemic and pulmonary lineages.

TAPVC occurs in Semaphorin 3d (Sema3d) mutant mice [40], despite normal formation of the MPES. In 35 % of mutant mouse embryos, the developing splanchnic plexus surrounding the lung buds does not anastomose uniquely with the MPES. Normally, Sema3d provides a migration repulsive signal to endothelial cells in early stages of development (E9.5 in mouse), establishing a boundary between the pulmonary plexus and the systemic circulation, whereas in the mutants, endothelial tubes form aberrant connections traversing this normally vascular-free area. Furthermore, atrial septation is impaired in these mutants providing further evidence for the co-development of the atrial septum and the pulmonary vein (Fig. 30.7).

Conclusion

In summary, it is evident that several gene networks act in the differentiation of the pulmonary veins, starting already early in development before formation of the myocardial and smooth muscle sleeves. The formation of the myocardial wall of the pulmonary vein is accompanied by incorporation into the dorsal wall of the right atrium, a differentiation that can be followed during development. Maldevelopment of the connection between pulmonary vein and left atrium may result in persisting connections to the systemic circulation as seen in patients with TAPVC.

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Part VIII

Tetralogy of Fallot and Double Outlet Right Ventricle

Clinical Presentation and Therapy of Tetralogy of Fallot and Double Outlet Right Ventricle

31

David J. Driscoll

Contents

31.1 Tetralogy of Fallot and Pulmonary Atresia with Ventricular Septal Defect

Tetralogy of Fallot (TOF) consists of (1) ventricular septal defect, (2) pulmonary stenosis (which may be valvular, subvalvar, and/or supravalvar), (3) an aorta that "overrides" the ventricular septal defect, and (4) right ventricular hypertrophy (Fig. 31.1). TOF represents 4–8 % of congenital cardiac defects. Many consider pulmonary atresia with ventricular septal defect (PA/VSD) as a "severe form" of TOF. The difference between the two is that in PA/VSD, there is no outlet of the

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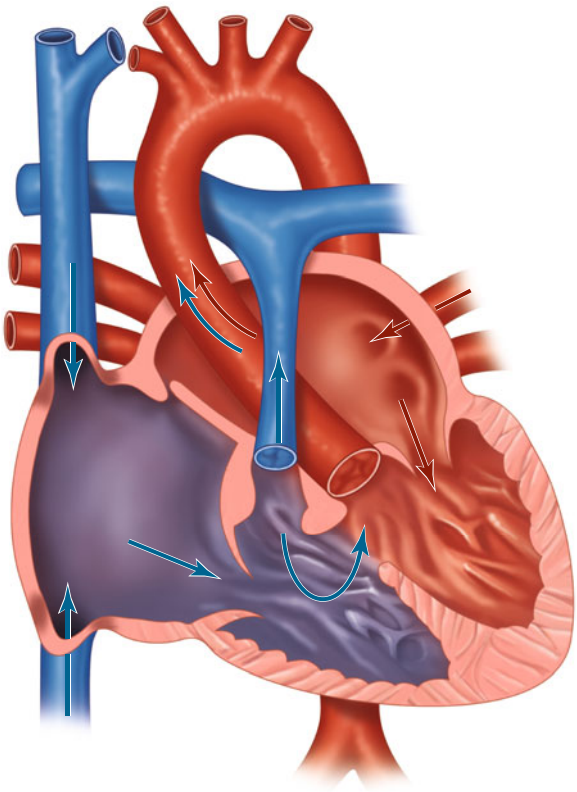

Fig. 31.1 Diagrammatic representation of tetralogy of Fallot. There is VSD and obstruction to pulmonary blood flow by subpulmonary and pulmonary valve stenosis

right ventricle. In TOF, there is an outlet but it is stenotic. In PA/VSD, the atresia can involve only a short segment of the main pulmonary artery, or, in severe cases, the atresia can include the entire main pulmonary artery and, in severe cases, the central as well as the distal pulmonary arteries.

In both TOF and PA/VSD, there may be major aortopulmonary collateral arteries (MAPCAs) which are vessels arising from the aorta or the subclavian arteries that supply segments of the pulmonary arterial tree. These are seen much more commonly in PA/VSD than in TOF.

31.1.1 Pathologic Physiology

Newborns and infants with TOF or PA/VSD are cyanotic due to the right-to-left shunt through the ventricular septal defect and decreased pulmonary blood flow. The degree of hypoxemia is proportional to the volume of pulmonary blood flow which is related to the severity of right ventricular outflow tract obstruction (RVOTO) and any additional sources of pulmonary blood flow (including a patent ductus arteriosus and systemic-to-pulmonary artery collateral vessels). Because patients with PA/VSD have no forward blood flow from the right ventricle into the

pulmonary artery, survival is totally dependent upon pulmonary blood flow from a patent ductus arteriosus and/or MAPCAs. This may or may not be the case in TOF depending upon the severity of RVOTO.

31.1.2 Clinical Presentation

Infants with tetralogy of Fallot or PA/VSD come to medical attention because of cyanosis or a murmur soon after birth.

31.1.3 Physical Examination

Most patients with tetralogy of Fallot or PA/VSD will be cyanotic. However, in some, the degree of cyanosis may be minimal (the so-called pink tetralogy) and not apparent on physical examination. The right ventricular impulse will be increased at the right lower sternal border. The first heart sound will be normal but the second heart sound will be increased in intensity and frequently single. There is an increased right ventricular impulse at the lower left sternal border. In TOF here is a systolic ejection murmur along the left sternal border. In PA/VSD because there is not a patent RV outflow tract, there is not a systolic ejection murmur. However, one may hear a systolic murmur created by flow through the MAPCAs.

31.1.4 Chest Radiograph

In approximately 25 % of patients, a right aortic arch will be apparent on the chest radiograph. Hypoplasia of the main pulmonary artery and right ventricular hypertrophy may produce the "coeur en sabot" (boot shaped) configuration of the cardiac silhouette, but this is not a particularly helpful sign in an infant

31.1.5 Echocardiographic and Cardiac Catheterization Issues

The diagnosis of tetralogy of Fallot or PA/VSD, the presence and size of the patent ductus arteriosus, and the size of the main and central right and left pulmonary arteries can be established using two-dimensional echocardiography. Angiography may be necessary to ascertain the size and distribution of the peripheral pulmonary arteries, the presence or absence of peripheral pulmonary stenosis, the presence and anatomy of MAPCAs, and the presence of additional ventricular septal defects.

31.1.6 Treatment

Initial management of patients with TOF and PA/VSD involves establishing important details of the anatomic diagnosis and treating the hypoxemia and acidosis if

they are significant. Severely hypoxemic infants should be treated with an infusion of prostaglandin E_l to reopen the ductus arteriosus or maintain its patency. If pulmonary blood flow is inadequate, a systemic-to-pulmonary artery anastomosis should be established surgically. A Blalock-Taussig (subclavian artery to pulmonary artery anastomosis) or modified Blalock-Taussig (interposition of a Gore-Tex tube from the subclavian artery to the pulmonary artery) is the procedure of choice. Intracardiac repair of tetralogy of Fallot or PA/VSD usually is performed between ages 3 and 12 months. Intracardiac repair involves closure of the ventricular septal defect and relief of the RVOTO or atresia. The latter may necessitate patch enlargement of the right ventricular outflow tract, pulmonary annulus, or both or insertion of a valve containing conduit from the right ventricle to the pulmonary artery.

31.1.7 Outcome

Hypercyanotic or "tetralogy" spells can occur in all forms of congenital heart disease in which there is obstruction to pulmonary blood flow and a communication between the subpulmonary and subaortic ventricles. These are much more common in TOF than in PA/VSD. These spells initially were associated with tetralogy of Fallot, hence, the name. Hypercyanotic spells consist of the abrupt onset of increased cyanosis, hypoxemia, dyspnea, and agitation. If left untreated, they can lead to profound hypoxemia, acidosis, seizures, and death. They rarely occur before the age of 2 months.

The long-term outcome of treatment of TOF or PA/VSD depends primarily on the size and anatomy of the pulmonary arteries and whether or not a competent native pulmonary valve remains after operation. Patients with normal size pulmonary arteries that are normally distributed and a competent pulmonary valve postoperatively have an excellent long-term prognosis. For patients who require insertion of a right ventricle to pulmonary conduit (which includes all patients with PA/VSD), the conduits will eventually fail and require replacement several times during the patient's lifetime. Patients with TOF who, after operation, have pulmonary valve insufficiency may require insertion of a prosthetic valve latter in life.

There is an ongoing risk of arrhythmias and late sudden death for patients with repaired tetralogy of Fallot or PA/VSD. The highest risk is in patients with residual VSD, residual RVOTO, poor ventricular function, and a QRS duration >180 msec.

31.2 Double-Outlet Right Ventricle

As the name indicates in double-outlet right ventricle (DORV), both great arteries arise totally or primarily from the right ventricle (Fig. 31.2). Invariably, there is a VSD. If there is not VSD, the left ventricle would be hypoplastic and then the anomaly would be considered as a form of functional single ventricle which will not be discussed in this chapter.

Since there essentially always is VSD, one of the great arteries may be related to the VSD. Indeed that great artery can be "overriding" the VSD. If the great artery is

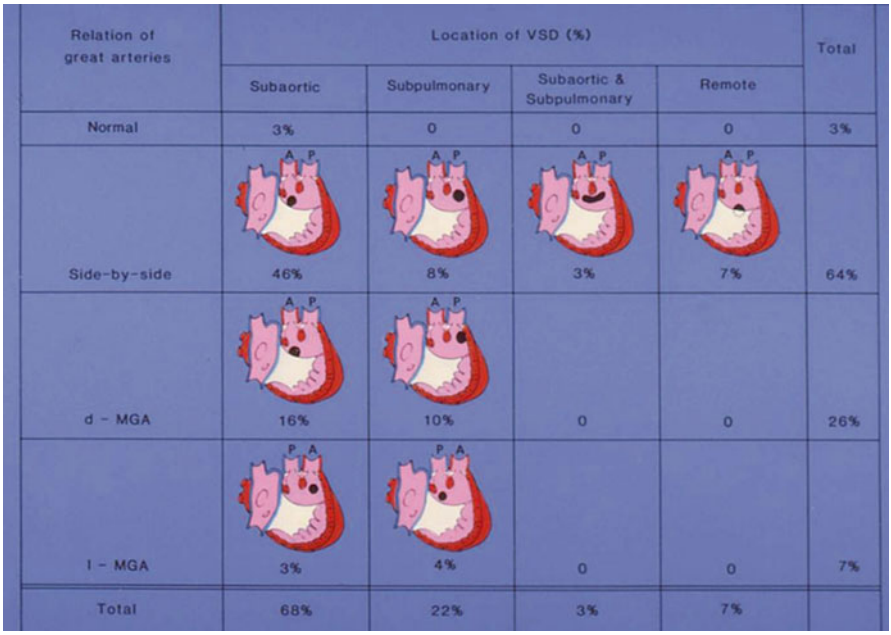

Fig. 31.2 Table of the various types of double-outlet right ventricle (Reproduced or adapted from Hagler, D., Double-Outlet Right Ventricle and Double-Outlet Left Ventricle, in *Moss and Adams' Heart Disease in Infants, Children, and Adolescents*, 7th edition 2008, Lippincott Williams & Wilkins, 2006, with permission of the author and publisher.)

overriding the VSD, then the defect can only be considered DORV if at least 51 % of the vessel is committed to the right ventricle. In addition, if the defect is considered DORV, there cannot be mitral annular fibrous continuity with the annulus of the nearest of semilunar valve. In DORV, there are four relationships that can exist between the VSD and the great arteries: (1) subaortic, (2) subpulmonary, (3) doubly committed, and (4) remote.

In DORV, the great arteries can be in a normal relationship to each other or malposed relatively to each other. Despite the fact that the great arteries can be malposed relative to each other, DORV should never be classified as "d-transposition" since both arise from the RV, and, by definition, the great arteries must arise from the wrong ventricle to be labeled "transposition."

31.2.1 Pathologic Physiology and Operative Approach

Which great artery is positioned nearest the VSD and whether or not there is pulmonary stenosis will dictate the physiology of the defect. If the aorta is committed to the VSD and there is pulmonary stenosis, then the physiology and operative repair is similar to that of TOF. If the aorta is committed to the VSD and there is no pulmonary stenosis, then the physiology and operative repair is similar to that of a

VSD. However, if the pulmonary artery is committed to the VSD, then the physiology and operative approach is similar to that of d-transposition of the great arteries (d-TGA) either with or without pulmonary stenosis. Indeed the last situation carries the moniker of a "Taussig-Bing anomaly."

31.2.2 Physical Examination

Since the physiology of DORV depends upon the relative position of the great arteries to the VSD, the physical findings also will depend upon the position of the great arteries and the presence or absence of pulmonary stenosis (see Sect. 31.1.3 above for examination findings for TOF and Chap. 34 for findings with d-TGA).

31.2.3 Echocardiographic and Cardiac Catheterization Issues

The diagnosis of DORV, the relationship of the great arteries to the VSD, and the presence or absence and severity of pulmonary stenosis, and the presence and size of the patent ductus arteriosus can be ascertained by 2- dimensional echocardiography.

31.2.4 Outcome

As noted above, the operative approach will be similar to that of TOF, VSD, or TGA, and hence, the outcomes will be similar to that of those defects. However, there is an important additional consideration that effects outcome. In many cases, the aorta is related to the VSD but may be a considerable distance from it. In these cases, a complicated baffle must be sewed in the RV to direct blood from the left ventricle through the VSD in into the aorta. These baffles can become narrow requiring reoperation.

Human Genetics of Tetralogy of Fallot and Double Outlet Right Ventricle

32

Cornelia Dorn, Andreas Perrot, and Silke Rickert-Sperling

Contents

Abstract

Tetralogy of Fallot (TOF) and double outlet right ventricle (DORV) are conotruncal defects resulting from disturbances of the second heart field and the neural crest, which can occur as isolated malformations or as part of multiorgan syndromes. Their etiology is multifactorial and characterized by overlapping genetic causes. In this chapter, we present the different genetic alterations underlying the two diseases, which range from chromosomal abnormalities like aneuploidies

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and structural mutations to rare single nucleotide variations affecting distinct genes. For example, mutations in the cardiac transcription factors *NKX2-5*, *GATA4*, and *HAND2* have been identified in isolated TOF cases, while mutations of *TBX5* and 22q11 deletion, leading to haploinsufficiency of *TBX1*, cause Holt-Oram and DiGeorge syndrome, respectively. Moreover, genes involved in signaling pathways, laterality determination, and epigenetic mechanisms have also been found mutated in TOF and/or DORV patients. Finally, genome-wide association studies identified common single nucleotide polymorphisms associated with the risk for TOF.

32.1 Introduction

Tetralogy of Fallot (TOF) is the most common form of cyanotic congenital heart disease (CHD) and affects about 0.28 children per 1000 live births [1, 2], while double outlet right ventricle (DORV) is diagnosed in approximately 0.03–0.09 of 1000 live births [3]. Both malformations are conotruncal defects, which result from disturbances of the second heart field and the neural crest, and show overlapping genetic causes. The etiology of TOF is multifactorial, with about 25 % of cases occurring in the context of chromosomal abnormalities and syndromic disorders [4]. Genetic causes for isolated TOF include rare single gene defects, copy number variations (CNVs), as well as associations with common single nucleotide polymorphisms (SNPs). The majority of cases probably have a multigenic background, with individual combinations of risk alleles occurring in each patient [5, 6]. For DORV, the proportion of cases with chromosomal abnormalities is higher (>40 %), and the majority of patients show additional extracardiac symptoms [3]. Furthermore, epigenetic and environmental factors including DNA methylation, exposure to teratogens, and insufficient folate supply can also impact on the etiology of cardiac malformations like TOF and DORV [7, 8].

32.2 Recurrence Risk in Affected Families

The genetic basis of CHD has been demonstrated by the study of affected families and by an increased recurrence risk in siblings and offspring of CHD patients. In 1972, one of the first large studies for TOF determined a recurrence risk of 1 % for TOF and 2 % for any form of CHD in the siblings of 100 index patients [9]. In 2014, a significant reduction in reproductive fitness was observed in a large cohort of 543 adult TOF patients. Moreover, the offspring had a recurrence risk of 4.8 % for any form of CHD and of 2.3 % for severe cardiac malformations [10]. Familial recurrence of DORV was reported in 5 % (7/149) of cases [3]. Finally, both TOF and DORV have been demonstrated to be associated with parental consanguinity [11].

32.3 Chromosomal Abnormalities in TOF and DORV

32.3.1 Aneuploidies

Chromosomal aneuploidies were the first recognized genetic cause of CHD and represent a major pathogenic burden. Trisomy 21 (Down syndrome) is present in 5 % of TOF cases, usually associated with atrioventricular canal defect [12, 13], and in 4 % of DORV patients [3]. CHD is also a common feature of trisomy 13 (Patau syndrome), with more than 90 % of patients affected by cardiac malformations (among them 11 % DORV and 6 % TOF) and trisomy 18 (Edwards syndrome) with 85 % of patients with CHD (among them 13 % DORV and 9 % TOF) [14]. Moreover, TOF has been reported in several cases of monosomy X (Turner syndrome) [15], while DORV has been diagnosed in a patient with a 47, XYY genotype [16].

32.3.2 Chromosomal Structural Mutations

Besides numerical chromosomal changes, structural aberrations like deletions, duplications, and inversions play a major role in the etiology of CHD. 22q11 deletion syndrome (DiGeorge/velocardiofacial syndrome) is the most common cause of TOF and can be detected in up to 16 % of patients [17]. Moreover, it is present in 7 % of DORV cases [3]. The cardiac phenotype is caused by hemizygosity of the cardiac transcription factor T-box protein 1 (*TBX1*) [18], which has also been found to be mutated in affected patients without the 22q11 deletion [12, 19]. Another major cause of CHD are abnormalities of chromosome 8, which have been detected in 10 % of DORV patients [3] and can also result in TOF [20]. Moreover, the malformations have been identified in cases of cri du chat syndrome (5p15.2 deletion, with TOF and DORV [21]), cat eye syndrome (22q11 inversion/duplication, with TOF [22]), Williams-Beuren syndrome (7q11.23 deletion, with TOF [23]), Jacobsen syndrome (11q terminal deletion, with TOF and DORV [24, 25]), and 1p36 deletion syndrome (with TOF [26]).

32.3.3 Copy Number Variations

Submicroscopic structural variations changing the genomic copy number (copy number variation, CNV) have been studied in several large cohorts of isolated TOF patients or broad panels of CHD cases using SNP arrays and whole exome sequencing. The estimated burden of rare *de novo* CNVs (5–10 %) was significantly higher than in healthy controls [27–30]. Different gene prioritization approaches were used to identify novel candidate genes located in the CNV regions, and moreover, well-known CHD genes including jagged 1 (*JAG1*) [27], GATA binding protein (*GATA*) *4* [28], and *TBX1* [27, 29] were found affected by copy number changes. Furthermore, a study of nearly 2500 patients with CHD showed a strong association of 1q21.1 microduplications with sporadic isolated TOF, implicating the *GJA5* gene encoding

connexin 40 in the pathology of cardiac malformations [31]. In general, the overlap of CNVs identified in the patients is very low, demonstrating the genetic heterogeneity of TOF [32].

32.4 Single Gene Defects

Over the last decades, more than 50 human genes involved in isolated or syndromic forms of CHD have been identified [6]. Most CHD gene mutations cause haploinsufficiency or a dosage reduction of the encoded protein, while mutations increasing gene activity or dosage are less common [8]. Genes playing a role in the pathogenesis of TOF and DORV encode cardiac transcription factors, components of signaling cascades, or other regulatory proteins such as epigenetic regulators. A detailed overview of the genes and studies can be found in Table 32.1. Here we only list those genes for which a clear genetic diagnosis could be made in a patient with TOF or DORV. However, mutations in additional genes might be causative in the respective syndromic disorders.

32.4.1 Cardiac Transcription Factors

Cardiac development is a finely tuned process regulated by transcriptional networks that are governed by a core set of cardiac transcription factors (see Chap. 11), many of which are associated with isolated CHD [6]. The gene *NKX2-5* encoding the transcription factor NK2 homeobox 5 is found mutated in about 4 % of TOF patients [33] and also rarely in DORV patients. Mutations in GATA4, which interacts with NKX2-5 at cardiac promoters, primarily cause septal defects [6, 33] but have also been identified in several cohorts of sporadic TOF patients as well as one study of familial TOF [34]. Moreover, mutations in TOF patients have also been reported for the *GATA6* gene [35] and *HAND2* encoding heart and neural crest derivatives expressed 2 protein [36], a downstream target of GATA4. The activity of GATA proteins is modulated by the FOG family of transcription factors and mutations in the zinc finger protein FOG family member 2 (*ZFPM2*/*FOG2*) have been found in various studies on TOF and DORV patients [37, 38]. Furthermore, T-box transcription factors also play an important role in cardiac developmental processes. Haploinsufficiency of *TBX1* is the primary cause of CHD in DiGeorge syndrome, and mutations in this gene have also been identified in patients without the 22q11 deletion [12, 19]. Furthermore, mutations in *TBX5* cause Holt-Oram syndrome, for which 20 cases with TOF have been described so far [39] and which can also present with DORV.

32.4.2 Genes Associated with Laterality Determination

The bilateral symmetry of the developing embryo is first broken during cardiac development, and left-right patterning is established during early embryogenesis by the interplay of different signaling pathways and by the action of nodal cilia [6]

Table 32.1 Single gene defects involved in isolated or syndromic TOF and/or DORV

Gene	Protein function	Phenotype	Status	References
Transcription factors and cofactors				
BCOR	Transcriptional corepressor	OFCD syndrome (with DORV)	2 or more independent reports	[60, 61]
FOXC1	Forkhead TF	TOF	2 or more patients	[62]
FOXC2	Forkhead TF	TOF	Single case report	[62]
FOXH1	Forkhead TF	TOF	2 or more patients	[44]
GATA4	GATA binding TF	TOF	2 or more independent reports	[63–66]
GATA6	GATA binding TF	TOF	2 or more independent reports	[35, 67, 68]
HAND2	Helix-loop-helix TF	TOF	2 or more independent reports	[36, 62]
HOXA1	Homeobox TF	BSAS (with TOF), ABDS (with TOF)	2 or more independent reports	[69, 70]
NKX2-5	Homeobox TF	TOF	2 or more independent reports	[12, 71–73]
		DORV, heterotaxy (with DORV)	2 or more independent reports	[72–74]
NKX2-6	Homeobox TF	TOF	Single case report	[75]
		DORV	Single case report	[75]
PITX2	Homeobox TF	DORV	Single case report	[76]
SALL4	Zinc finger TF	Okihiro syndrome (with TOF)	Single case report	[77]
TBX1	T-box TF	TOF, DiGeorge syndrome (with TOF)	2 or more independent reports	[12, 19, 62, 78]
TBX5	T-box TF	Holt-Oram syndrome (with TOF)	2 or more independent reports	[39, 79]
		Holt-Oram syndrome (with DORV)	Single case report	[80]
TBX20	T-box TF	TOF	Single case report	[81]
TFAP2B	AP-2 TF	TOF	Single case report	[82]
ZFMP2	Zink finger TF	TOF	2 or more independent reports	[37, 38, 83]
		DORV	2 or more independent reports	[38, 83, 84]
ZIC3	Zink finger TF	DORV, heterotaxy (with DORV)	2 or more independent reports	[43, 85]

(continued)

Table 32.1 (continued)

Gene	Protein function	Phenotype	Status	References
Genes involved in signaling pathways				
ACVR2B	Activin receptor	Heterotaxy (with DORV)	Single case report	[86]
ALDH1A2	Aldehyde dehydrogenase	TOF	2 or more patients	[87]
BRAF	Serine/threonine protein kinase	LEOPARD syndrome (with TOF)	Single case report	[88]
CFC1	Ligand (EGF family)	TOF	2 or more patients	[44]
		DORV	Single case report	[89]
GDF1	Ligand (BMP/TGF-beta family)	TOF	2 or more patients	[90]
		DORV	Single case report	[90]
JAG1	Notch ligand	TOF, Alagille syndrome (with TOF)	2 or more independent reports	[12, 47–49, 91]
MAP2K1	MAP kinase kinase	CFC syndrome (with TOF)	Single case report	[92]
NODAL	Ligand (TGF-beta family)	TOF	2 or more patients	[40]
		DORV	2 or more independent reports	[40, 93]
NOTCH2	Notch receptor	Alagille syndrome (with TOF)	Single case report	[94]
RAF1	Serine/threonine protein kinase	Noonan syndrome (with TOF)	Single case report	[52]
TDGF1	Co-receptor (TGF-beta signaling)	TOF	2 or more patients	[44]
Other genes				
CHD7	DNA helicase	CHARGE syndrome (with TOF)	2 or more patients	[55]
		CHARGE syndrome (with DORV)	2 or more patients	[55]
GJA5	Gap junction protein	TOF	2 or more patients	[31]
NPHP4	Ciliary protein	DORV, heterotaxy (with DORV)	2 or more patients	[45]
SH3PXD2B	Adapter protein	FTHS (incl. DORV)	2 or more patients	[95]

Only those genes are listed, for which a clear genetic diagnosis was made in the respective case(s)
ABDS Athabascan brainstem dysgenesis syndrome, *BSAS* Bosley-Salih-Alorainy syndrome, *CFC syndrome* cardiofaciocutaneous syndrome, *DORV* double outlet right ventricle, *FTHS* Frank-ter Haar syndrome, *OFCD syndrome* oculofaciocardiodental syndrome, *TF* transcription factor, *TOF* tetralogy of Fallot

(see Chap. 7). Components of the Nodal signaling pathway are critical in determining organ laterality, and disruption of these genes causes a wide variety of cardiac malformations, often associated with laterality defects [6] (see Chap. 38). Many DORV patients show laterality defects such as heterotaxy, and a number of mutations have been identified in the Nodal pathway [3]. Nodal growth differentiation factor (*NODAL*) mutations were reported in two studies on DORV patients and could also be identified in TOF patients [40]. The *CFC1* gene, encoding the CRYPTIC protein (a co-receptor in Nodal signaling), was found to be mutated in isolated TOF and DORV cases. Moreover, non-synonymous variants that could act as susceptibility alleles were identified in patients with DORV and heterotaxy [41, 42] (see Chap. 38). Further genes encoding signaling molecules of the Nodal pathway that have been found mutated in DORV and/or TOF patients are growth differentiation factor 1 (*GDF1*), teratocarcinoma-derived growth factor 1 (*TDGF1*), and activin A receptor, type IIB (*ACVR2B*). Furthermore, mutations in the transcription factors Zic family member 3 (*ZIC3*; acting upstream of Nodal signaling) and forkhead box H1 (*FOXH1*; a possible link between the BMP and Nodal pathways) could be found in several DORV and TOF patients, respectively [43, 44]. Finally, the cilia-related gene *NPHP4* encoding nephronophthisis 4 protein was identified in a linkage analysis of a consanguineous family with laterality defects and DORV and was subsequently found mutated in further DORV patients [45] (see Chap. 38).

32.4.3 Genes Involved in Signaling Pathways

Several signaling pathways are involved in the coordination of cardiac development, and mutations of signaling molecules have been identified in isolated and syndromic forms of CHD [6, 8]. Notch signaling is required for patterning of the cardiac chambers and valves and moreover regulates cardiomyocyte proliferation and differentiation [46]. Mutations in the Notch ligand gene *JAG1* cause the multisystem disorder Alagille syndrome (AGS), which includes CHD in about 90 % of patients [6]. Approximately 10 % of AGS patients present with TOF [47], and moreover, *JAG1* mutations have also been identified in isolated TOF cases [48] and in one study on familial TOF [49]. Interestingly, a *JAG1* mutation also was found in a patient with DiGeorge syndrome and TOF, indicating that it might act as a modifier of the phenotype [50]. About 1 % of AGS cases are caused by mutations in *NOTCH2* [6], which has been shown for one TOF patient so far. Mutations in the rat sarcoma viral oncogene homolog/mitogen-activated protein kinase (Ras/MAPK) pathway, which regulates cell proliferation, differentiation, and survival, cause Noonan syndrome (NS) and other distinct but overlapping syndromes (see Chap. 23). NS presents with TOF in about 4 % of patients [8, 51], and a microduplication encompassing v-raf-1 murine leukemia viral oncogene homolog 1 (*RAF1*) has been identified in a patient with NS phenotype including TOF [52]. Moreover, mitogen-activated protein kinase kinase 1 (*MAP2K1*) and v-raf murine sarcoma viral oncogene homolog B (*BRAF*) mutations were reported in one TOF patient with

cardiofaciocutaneous syndrome and one with LEOPARD syndrome, respectively. Finally, retinoic acid (RA) signaling is both a regulator and target of TBX1, thus playing a role in pharyngeal arch development [6]. Mutations in *ALDH1A2*, encoding an aldehyde dehydrogenase involved in RA signaling, were identified in two non-syndromic TOF patients.

32.4.4 Epigenetic Regulators

Epigenetic mechanisms represent an important layer of transcriptional regulation and play a central role in cardiac development [53]. Mutations in the *CHD7* gene, encoding chromodomain helicase DNA binding protein 7 whose binding correlates with H3K4 methylation, cause CHARGE syndrome. Cardiac malformations occur in 75–80 % of the patients, with TOF being the most common defect. Moreover, DORV with atrioventricular canal is also frequently seen in CHARGE patients [54, 55]. Furthermore, an enrichment of *de novo* mutations in histone-modifying enzymes could recently be demonstrated in a large CHD cohort including TOF and DORV patients and their parents [56].

32.5 Oligogenic Defects

The majority of TOF cases are isolated, non-syndromic cases – the precise causes of which are yet to be discovered. This is true for the majority of CHDs and many serious non-Mendelian diseases with a clear genetic component. It has been assumed that CHDs might also be caused by rare autosomal recessive variations in concert with private variations [8, 96], which might individually show minor functional impairment but in combination could be disease causing [97]. In this concept, multiple mutations in different genes can lead to disturbances of a molecular network that result in a common phenotypic expression. TOF was the first CHD for which this concept was discovered to be correct [5]. Rare and private mutations in neural crest, apoptotic, and sarcomeric genes were shown to define the genetic background of isolated, non-syndromic TOF (see Fig. 32.1). A great challenge, however, is the discrimination of causative genes. Recently, this challenge was successfully addressed using a burden analysis approach named GMF (gene mutation frequency), which considers the frequency at which a gene is affected by selected deleterious variations in a cohort [5]. Interestingly, besides known developmental genes, cardiomyopathy genes have been confirmed to form part of the genetic basis of TOF. This discovery harbors the possibility that the genetic background of TOF might be correlated to its clinical long-term outcome. Large-scale sequencing projects are currently ongoing and their outcome is of utmost interest.

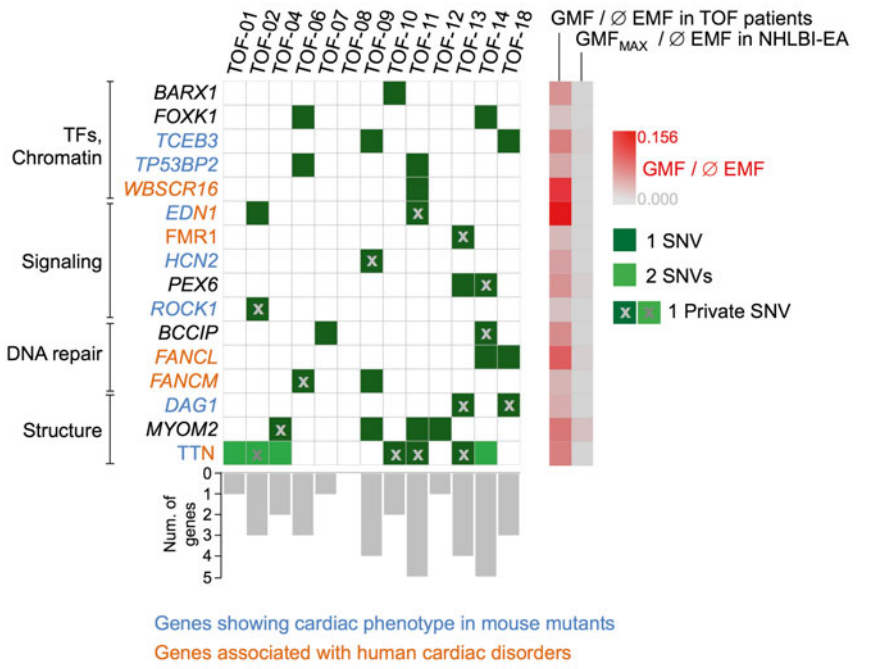

Fig. 32.1 Distribution of mutations found in 16 significantly affected genes ($P > 0.05$) in TOF patients. Private mutations are marked by '*x*'. Gene-wise frequencies of mutations are represented by *gray bars*. Gene mutation frequency (GMF) in TOF cases and European-American controls (NHLBI-EA) are indicated by a gray-to-red gradient. For titin (TTN), the average exon-mutation frequency (EMF) over all significantly over-mutated exons is given. *EMF* exon mutation frequency, *GMF* gene mutation frequency, *SNV* single nucleotide variation (adapted from Grunert et al. [5])

32.6 Associations with Common Variations

Besides rare damaging mutations in cardiac regulators and signaling pathways, common SNPs have also been found associated with CHD in several genome-wide association studies (GWAS). Two associated loci (12q24 and 13q32) could be identified in a two-stage study of more than 1600 TOF patients, demonstrating that common genetic variation influences the risk of TOF [57]. However, the majority of CHD-associated variants are individually unique, which results in allelic heterogeneity and reduces the power of GWAS for CHD [58].

Conclusion

Huge advances have been made in understanding the etiology of congenital heart malformations. However, the underlying causes for the majority of CHDs

still remain unclear. Estimates suggest that 80 % of cardiac malformations are caused by the interaction of various genetic, epigenetic, and environmental factors [59], which complicates studies aiming to identify single contributors. About 25 % of TOF patients have chromosomal abnormalities such as trisomy 21 or 22q11 deletion, while the majority are isolated cases caused by mutations in cardiac transcription factors, signaling pathways, or most likely combinations of different genes. For DORV, the proportion of patients with chromosomal abnormalities is higher. Moreover, the malformation often occurs in combination with laterality defects like heterotaxy, which is also reflected by the high number of DORV-associated genes playing a role in laterality determination. Taken together, further studies will be required for a deeper understanding of CHD etiology, which will hopefully offer novel preventive and therapeutic strategies and help to improve genetic counseling for affected families.

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Molecular Pathways and Animal Models of Tetralogy of Fallot and Double Outlet Right Ventricle

33

Robert G. Kelly

Contents

Abstract

Tetralogy of Fallot and double outlet right ventricle are outflow tract (OFT) alignment defects situated on a continuous disease spectrum. A myriad of upstream causes can impact on ventriculoarterial alignment that can be summarized as defects in either (1) OFT elongation during looping morphogenesis or (2) OFT remodeling during cardiac septation. Embryological processes underlying these two developmental steps include deployment of second heart field cardiac progenitor cells, establishment and transmission of embryonic left/right information driving OFT rotation, and OFT cushion and valve morphogenesis. The formation and remodeling of pulmonary trunk infundibular myocardium are critical components of both steps. Importantly, OFT alignment is mechanistically distinct from neural crest-driven OFT septation, although neural crest cells impact indirectly on alignment through their role in modulating signaling during SHF development. As yet poorly understood

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non-genetic causes of alignment defects that impact the above processes include hemodynamic changes, the uterine environment, and stochastic events. The heterogeneity of causes converging on alignment defects characterizes the OFT as a hotspot of congenital heart defects.

33.1 Introduction

Division of the embryonic outflow tract (OFT) during cardiac septation creates an exclusive connection between the ascending aorta and left ventricle that is essential to isolate the pulmonary and systemic circulatory systems at birth. Tetralogy of Fallot (TOF) and double outlet right ventricle (DORV) are OFT (or conotruncal) congenital heart defects (CHDs) resulting from defects in correct alignment of the ascending aorta and pulmonary trunk with the left and right ventricles. In the unseptated embryonic heart, the OFT is aligned with the future right ventricle. Subsequent remodeling events associated with cardiac septation, including rotation of the dividing OFT, cushion and valve morphogenesis, regional cell death, and a leftward shift of the OFT, align the ascending aorta and pulmonary trunk and with the left and right ventricles to create independent left and right ventriculoarterial connections. Defects in initial elongation of the OFT, by addition of myocardial progenitor cells from the second heart field (SHF) and/or in the subsequent remodeling events, lead to a failure of the ascending aorta to make an exclusive connection with the left ventricle (Fig. 33.1). Thus defects in a wide range of underlying morphogenetic processes converge on alignment defects. These processes include progenitor cell

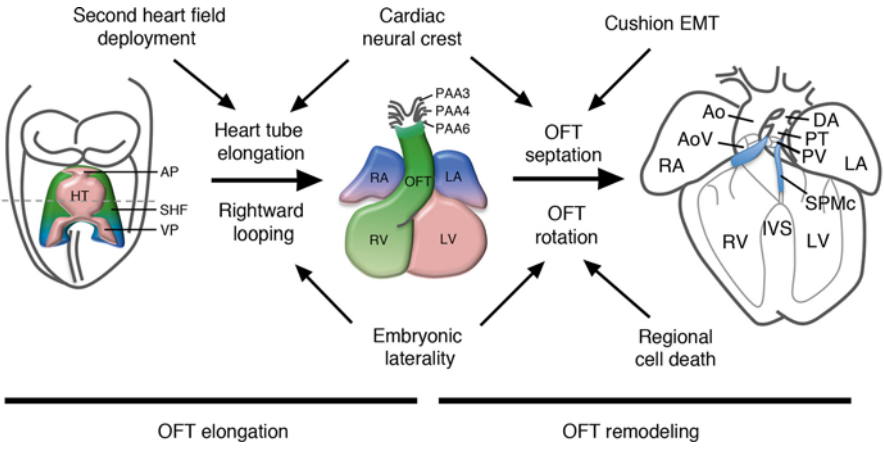

Fig. 33.1 Schema illustrating the progressive developmental steps during formation and remodeling of the outflow tract (*OFT*), together with the major cell types and developmental processes involved. *HT* heart, *AP* arterial pole, *VP* venous pole, *SHF* second heart field, *PAA* pharyngeal arch artery, *RA* right atrium, *LA* left atrium, *RV* right ventricle, *LV* left ventricle, *Ao* aorta, *AoV* aortic valve, *DA* ductus arteriosus, *PT* pulmonary trunk, *PV* pulmonary valve, *SPMc* subpulmonary myocardium, *IVS* interventricular septum

specification and deployment, embryonic laterality pathways, cushion morphogenesis and epithelial-to-mesenchymal transition (EMT), planar cell polarity, and non-genetic factors such as the uterine environment and stochastic events that may impact on hemodynamics in the developing heart. A spectrum of morphological variation is commonly seen in animal models of alignment defects extending from overriding aorta to transposition of the great arteries (TGA). Furthermore, TOF and DORV are associated with other forms of CHD including an obligatory ventricular septal defect (VSD), as well as aortic arch and atrioventricular septal defects. This complexity of etiology and phenotype is reflected in the frequency of alignment defects in human CHD patients and in the wide range of genes and pathways that have been implicated in TOF and DORV in patients and animal models. In this chapter we will first consider the relationship between TOF and DORV and then review examples of current knowledge about the molecular pathways driving the processes of OFT elongation and OFT remodeling.

33.2 TOF and DORV are Part of a Spectrum of Outflow Tract Alignment Defects

Addition of cardiac progenitor cells from the SHF in pharyngeal mesoderm to the elongating poles of the heart drives heart tube elongation during looping morphogenesis [1]. The entire OFT is derived from the SHF, and direct or indirect impairment of SHF deployment results in OFT shortening [2]. Maximal OFT extension creates the template for subsequent septation and is critical for correct ventriculoarterial alignment. Septation is driven by coincident cushion morphogenesis and influx of neural crest-derived mesenchyme that together divide the OFT into the ascending aorta and pulmonary trunk [3]. During this process, the OFT rotates in a counterclockwise direction (with respect to the direction of blood flow), and the future pulmonary trunk is positioned in a ventral position above the right ventricle, the ascending aorta becoming wedged between the atrioventricular valves and aligned with the left ventricle (Fig. 33.2). Extensive evidence suggests that septation and alignment are distinct, though coincident, processes: failure of OFT septation will result in common arterial trunk (or persistent truncus arteriosus), while failure of alignment will result in a spectrum of alignment defects, ranging from mild to severe [4, 5]. At the mild end of the spectrum, failure of the aorta to make an exclusive connection with the left ventricle results in overriding aorta (OA), where the aorta connects with both ventricles above a membranous VSD. The VSD arises due to malalignment of the ventricular and outflow tract septa. When associated with pulmonary stenosis or atresia, overriding aorta results in a TOF phenotype. If >50 % of the aorta lies over the right ventricle, overriding aorta becomes DORV, with a subaortic VSD. Total failure of OFT rotation, in the absence of a leftward shift of the OFT, results in a dextroposed aorta that connects with the right ventricle in a side-by-side configuration with the pulmonary trunk (Fig. 33.2). This defect, DORV with a subpulmonary VSD, is known as Taussig-Bing syndrome and is a configuration related hemodynamically to TGA. TOF and DORV thus lie within a disease spectrum of alignment defects and are distinguished by the degree of aortic dextroposition.

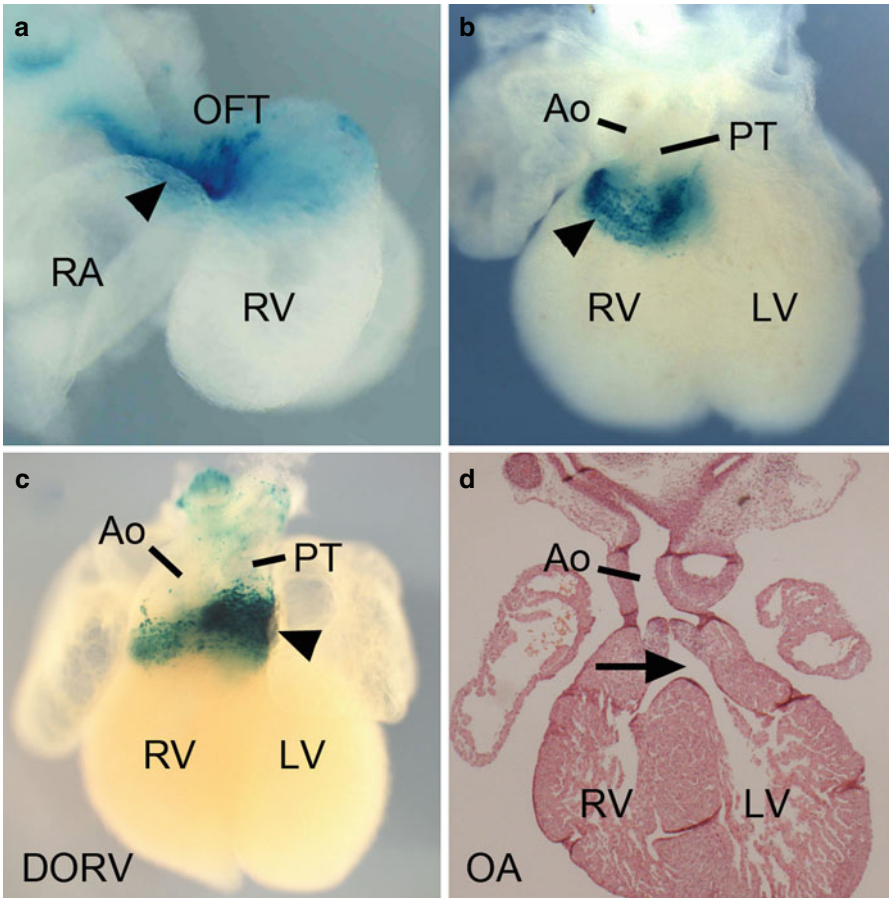

Fig. 33.2 Mouse hearts illustrating rotation of the myocardial OFT wall using a transgene enhancer trap (line *y96-Myf5-nlacZ-16*) expressed in the inferior wall of the midgestation OFT (**a**) and in subpulmonary myocardium after septation (**b**). Transgene-driven β-galactosidase activity is visible (arrowheads) in blue after incubation in X-gal solution. Expression of the *y96-Myf5-nlacZ-16* transgene in a fetal *Tbx3*$^{-/-}$ heart (**c**) with double outlet right ventricle (*DORV*) and a leftward positioned pulmonary trunk (*arrowhead*). Histological section through an *Fgfr2IIIb*$^{-/-}$ heart (**d**) with overriding aorta (*OA*), showing the aorta aligned with a ventricular septal defect (*arrow*). *RA* right atrium, *RV* right ventricle, *OFT* outflow tract, *Ao* aorta, *PT* pulmonary trunk, *LV* left ventricle

33.3 The Second Heart Field and Outflow Tract Elongation

Failure to maximally elongate the embryonic OFT results in an OFT that is too short to make an exclusive connection between the ascending aorta and left ventricle. Defects in SHF progenitor cell deployment range from defects in addition of cells to the distal OFT to total failure to extend the linear heart tube, producing a

consequent spectrum of mild to severe OFT defects, including ventriculoarterial alignment defects [2, 6]. Addition of cells giving rise to myocardium at the base of the pulmonary trunk, known as subpulmonary (or infundibular) myocardium, is of particular importance for alignment defects. Indeed, underdevelopment of subpulmonary myocardium is thought to be a primary cause of TOF [7]. Ablation of the SHF in chick embryos results in a reduction of subpulmonary myocardium and OA with pulmonary stenosis (TOF) [8]. Future subpulmonary myocardium is the last myocardial derivative of the SHF to be added to the OFT, giving rise to the inferior OFT wall [9]. Subpulmonary myocardium originates in the posterior SHF, adjacent to progenitor cells that contribute to the venous pole of the heart and are critical for atrioventricular septation [10]. Clonal analysis and lineage experiments have shown that subpulmonary and venous pole myocardium are clonally related, identifying a common progenitor cell population in the SHF that segregates to the cardiac poles to give rise to parts of the heart affected in common forms of CHD [11–13].

Many transcriptional regulators and signaling pathways have been implicated in the control of proliferation and progressive differentiation of SHF progenitor cells [2, 14]. In mouse embryos, addition of subpulmonary myocardium is dependent on the T-box containing transcription factor Tbx1, encoded by the major gene implicated in del22q11.2 (or DiGeorge) syndrome [15]. Haploinsufficiency for *TBX1* in human patients is the most common known genetic cause of TOF, accounting for 15 % of TOF [6, 16]. Tbx1 is required for proliferation and delayed differentiation in the SHF [17, 18]. Whereas homozygous *Tbx1* null mouse embryos have common arterial trunk with failure of subpulmonary myocardial addition, mice carrying hypomorphic *Tbx1* alleles with <25 % of normal *Tbx1* levels have a range of alignment defects including DORV and OA [19]. Among other transcriptional regulators of SHF development, NK2 homeobox 5 (*Nkx2-5*) is essential for elongation of the early heart tube. As in the case of *Tbx1*, hypomorphic embryos with <25 % of normal *Nkx2-5* levels display alignment defects including DORV and OA, preceded by a shortened OFT and decreased proliferation in the SHF [20]. Embryos doubly heterozygous for GATA binding protein (*Gata*) 4 or *Gata6* plus *Gata5* also display DORV and overriding aorta [21]. *Gata3* mutant embryos also have a shortened OFT and DORV and common arterial trunk phenotypes [22]. Loss of function of the Gata co-factor friend of GATA 2 (Fog2; or zinc finger protein, FOG family member 2; ZFPM2), or the domain of Gata4 that interacts with Fog2, also results in CHD, including OA and DORV phenotypes [23, 24]. Mutation of homeobox A1 (*Hoxa1*), expressed in subpulmonary myocardial progenitors in the SHF, has recently been shown to result in an OA phenotype [11, 25].

Intercellular signaling pathways play an important role in SHF development. Fibroblast growth factor (FGF) signaling controls SHF proliferation downstream of Tbx1 [16]. Mouse embryos lacking components of the FGF signaling pathway display OFT shortening and alignment defects, including Fgf receptor *Fgfr2IIIb* null embryos, mesodermal conditional *Fgfr1* and *Fgfr2* null embryos, and *Fgf8* hypomorphic and mesodermal conditional mutant embryos [26–29]. The incidence of alignment defects in mesodermal *Fgf8* mutant embryos is increased when alleles of *Fgf10* are additionally removed [30]. *Fgf15* null embryos also display DORV and OA [31].

In chick embryos, treatment with Fgf8-blocking antibodies or an FGF receptor antagonist results in DORV and OA phenotypes with pulmonary stenosis or atresia [32]. Additional intercellular signaling pathways regulating SHF development include Notch signaling: conditional Notch mutant embryos and embryos lacking the Notch target genes Hes family bHLH transcription factor 1 (*Hes1*) and Hes-related family bHLH transcription factor with YRPW motif 1 (*Hey1*) display OFT shortening and DORV and OA with pulmonary stenosis and are associated with impaired FGF signaling and proliferation in the SHF [33–35]. Deletion of *Jag1* in the SHF results in DORV (in addition to common arterial trunk and atrial septal defects) and Jagged 1 (*Jag1*) *Notch2* compound heterozygous embryos display OA with pulmonary stenosis [35, 36]. Endothelial specific *Jag1* deletion also results in OA, highlighting the multiplicity of roles different intercellular signaling pathways play during OFT development [37]. DORV is also observed in embryos lacking an enzyme encoded by the gene presenilin 1 (*Psen1*), required to activate the Notch receptor [38].

Proliferation in the SHF is also regulated by canonical Wnt and hedgehog signaling pathways [2, 14]. Indeed, mouse embryos cultured in the presence of an activator of the canonical Wnt signaling pathway have elongated OFTs and expanded numbers of SHF cells; conditional loss of function of canonical Wnt signaling leads to a reduction in progenitor cell numbers [39]. Chick embryos cultured in the presence of the Shh antagonist cyclopamine have decreased proliferation in the SHF resulting in OA [40]. Genetic tracing in the mouse has shown that SHF cells exposed to Shh signaling contribute to subpulmonary myocardium [41]. Mouse embryos lacking Shh display a single ventricular outlet, corresponding to total pulmonary atresia [42]. Retinoic acid signaling is also active in future subpulmonary myocardial progenitor cells. Analysis of retinoic acid receptor mutant embryos have revealed a role in renewal of SHF cells during OFT elongation leading to DORV and OA phenotypes [43]. Exposure of mouse embryos to retinoic acid also results in DORV (together with common trunk and TGA) phenotypes, revealing critical RA dosage requirements for SHF deployment [44, 45]. The planar cell polarity pathway regulates polarized cell movements and has also been implicated in the regulation of SHF deployment and OFT alignment [46]. Mouse embryos lacking components of this pathway encoded by genes such as dishevelled segment polarity protein 2 (*Dvl2*), VANGL planar cell polarity protein 2 (*Vangl2*), or Scribble (*Scrb*) have shorter OFTs and consequent alignment defects [46–48]. Double heterozygous *Vangl2+/−;Scrb+/−* mutant mice also have DORV [49]. Planar cell polarity plays an important role in epithelial SHF cells during their deployment to the elongating OFT [47]. *Vangl2* mutant mice display altered epithelial properties in the distal OFT during myocardial differentiation that may be essential for maximal OFT elongation [48]. The noncanonical wingless-type MMTV integration site family (Wnt) ligand encoding genes *Wnt5a* and *Wnt11* have been implicated upstream of PCP during SHF deployment and OFT morphogenesis [47, 50, 51]. Embryos lacking either of these genes display DORV, as well as common arterial trunk and TGA phenotypes; double mutant mice have a severe reduction of SHF progenitors [52]. *Wnt5a* is a direct target of Tbx1 in the SHF and has been shown to phenocopy the subpulmonary myocardial deficit of *Tbx1* null embryos [53, 54].

Intercellular signals controlling SHF development are both autocrine in origin, as in the case of Notch and FGF signaling, and from surrounding cell types in the pharyngeal region, including pharyngeal endoderm and ectoderm and neural crest-derived mesenchyme [2, 14]. Together these signals define and regulate the niche of SHF progenitor cells during heart tube extension. Experiments in chick embryos have revealed the critical requirement for neural crest-derived cells in SHF deployment. Ablation of the cardiac neural crest results not only in common arterial trunk due to failure of formation of the outflow tract septum but also to alignment defects such as TOF and DORV. These arise due to perturbation of critical signal exchange between crest and SHF cells in the pharyngeal region prior to the addition of these cell populations to the heart [4]. Neural crest cells have been shown to modulate FGF signaling in the pharyngeal region, thus decreasing SHF proliferation during the terminal stages of heart tube elongation [32]. Neural crest ablation rescues the alignment defects observed in embryos cultured in the presence of Fgf8-blocking antibodies [32]. The precise regulation of proliferation in the SHF is a critical step in progenitor cell deployment, and loss of cardiac neural crest cells perturbs the balance between proliferation and differentiation leading to decreased SHF incorporation and a shorter OFT that fails to wedge and align correctly [5]. Consistent with these findings, mutations in genes affecting cardiac neural crest development in the mouse result in a spectrum of OFT defects that include both septation (direct) and alignment (indirect) defects. Mouse embryos lacking paired box 3 (*Pax3*), expressed early in the neural crest lineage, for example, display a range of OFT defects including septation defects (common arterial trunk) and alignment defects (DORV) [55]. Mice lacking *Tbx3*, expressed in different cell types in the pharyngeal region including neural crest-derived mesenchyme, also have a high incidence of DORV [56, 57]. Mutation of pre-B-cell leukemia homeobox (*Pbx*) 1–3 result in OA, possibly through *Pax3* regulation [58, 59]. Together these experiments reveal how perturbation of any of a large number of convergent pathways leads to OFT alignment defects through impaired SHF deployment.

33.4 Outflow Tract Remodeling and Alignment

OFT elongation is clearly not the only prerequisite for correct ventriculoarterial alignment. Maximal elongation of the OFT by addition of SHF progenitor cells creates the template for a series of coincident processes that remodel the OFT into separate right and left ventricular outlets. These processes include OFT rotation, cushion morphogenesis, and differential growth and cell death. Evidence from analysis of developing mouse, chick, and human hearts has shown that the OFT wall rotates in a counterclockwise direction during the septation process and that this is essential for the correct positioning of the great arteries [60–63]. Maximal OFT elongation appears to be required for normal OFT rotation to occur. During the rotation process, the myocardial OFT gives rise to subaortic and subpulmonary myocardium, derived from the superior and inferior OFT walls, respectively [64]. Subpulmonary myocardium assumes a ventral position at the base of the pulmonary

trunk (Fig. 33.2). Different degrees of perturbation of OFT rotation result in arrest of this process and lead to positioning of the aortic valve over the right ventricle or to the right of the pulmonary valve, generating alignment defects including OA, DORV, and TGA.

Counterclockwise OFT rotation is driven by the embryonic left-right laterality pathway. Left-right laterality is initiated in the early embryo by cilia-driven flow of signaling molecules to the left side of the node at the anterior end of the primitive streak [65]. Mutation of genes involved in cilia morphogenesis and function result in a range of CHD including alignment defects. These include dynein, axonemal, heavy chain 11 (*Dnah11*), encoding a dynein molecule mutant in *iv/iv* mice, together with *inv* mutant mice, which display DORV, OA, and TGA [66, 67]. Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2 (*Cited2*) also regulates early establishment of laterality, and loss of function leads to a range of laterality and alignment defects including DORV and TGA, together with common arterial trunk [68, 69]. Genes involved in ciliogenesis have been identified from mutation screens in mice with alignment defects and TOF patients [70, 71]. Conditional mutagenesis will reveal the extent to which cilia also contribute to alignment defects through later roles in SHF deployment or in flow detection in the developing heart. The failure to establish embryonic laterality results in heterotaxy phenotypes by which independent laterality decisions are made by different components of the heart, leading to a large spectrum of CHD including alignment defects. Asymmetric growth factor signaling at the node establishes distinct left and right domains of gene expression in lateral mesoderm, including left-sided expression of genes encoding the Nodal ligand and the transcription factor paired-like homeodomain 2 (Pitx2) [65]. Pitx2 confers laterality to organs that develop asymmetrically, including the heart. Although not the driver of rightward looping, *Pitx2* is expressed in the left side of the SHF and controls OFT rotation [72]. Indeed, *Pitx2* mutant embryos are characterized by DORV and TGA, together with common trunk, and subpulmonary myocardium is malpositioned in the absence of *Pitx2* [62]. A recent study suggests that the terminal stages of SHF addition are asymmetric and that left-sided addition of subpulmonary myocardial progenitors expressing *Nkx2-5* may be a driving force in OFT rotation, a process termed the pulmonary-push model [73]. This would promote counterclockwise rotation of the pulmonary trunk around the aorta. Interestingly, this process is impaired in embryos lacking the Vegf164 isoform: insufficient addition of subpulmonary myocardium in these embryos is likely to account for subsequent DORV and pulmonary hypoplasia phenotypes [73]. Furthermore, compound heterozygosity for *Pitx2* and *Tbx1* results in alignment defects including DORV and pulmonary stenosis, further indicating how SHF deployment and laterality pathways intersect during OFT morphogenesis [74].

Multiple mechanisms intervene in OFT remodeling and impact on alignment. Rotation of the OFT myocardial wall is accompanied by spiraling of the underlying endocardial cushions that contribute to aortic and pulmonary valve leaflets. OFT cushions contain mesenchyme derived from endocardial EMT in the proximal OFT and neural crest cell influx in the distal OFT; SHF cells may also directly contribute to cushion mesenchyme [3]. Fusion of endocardial cushions plays an essential role in

division of the OFT into the ascending aorta and pulmonary trunk. Defects in EMT are thought also to lead to alignment defects through disruption of cushion growth. Diverse signaling pathways are involved in cushion EMT and valve morphogenesis, including bone morphogenetic protein (BMP), transforming growth factor beta (TGFβ), and Notch signaling pathways [75]. OFT cushion defects are observed in TGFβ2 mutant mice characterized by DORV and TGA phenotypes [76]. Hemodynamics is also an important contributor to cushion remodeling: altered blood flow patterns lead to alignment defects including DORV, OA, and TGA [77]. Flow also impacts on aortic arch remodeling downstream of Pitx2 function in the OFT, suggesting that OFT rotation itself may be genetically determined rather than flow driven [78]. Differential growth of subaortic and subpulmonary myocardium has also been proposed to be essential for OFT alignment. Accordingly, either deficiency of subpulmonary myocardium or overgrowth of subaortic myocardium after progenitor cell addition to the heart would lead to a side-by-side configuration of the ascending aorta and pulmonary trunk [7, 79]. Analysis of genes differentially expressed in subaortic versus subpulmonary myocardium may generate new insights into the remodeling processes [10]. Independently of OFT rotation, a leftward shift of the OFT has been proposed to play an important, although poorly understood, role in aligning the ascending aorta with the left ventricle [80, 81]. The absence of such a leftward shift would distinguish DORV from TGA in the context of a lack of OFT rotation. Myocardial cell shape defects have also been proposed to potentially contribute to alignment defects [46]. For example, altered cell polarity in the remodeling OFT has been observed in *Wnt11* null embryos, and mutation of the gene encoding non-muscle myosin IIB results in an OA phenotype associated with myocardial hypertrophy [51, 82]. Finally, programmed cell death also plays a major role in the remodeling step, in particular at the base of the ascending aorta, where patterned apoptosis is required to align the aorta with the left ventricle [83]. Blocking cell death in chick embryos using caspase inhibitors or by expression of an inhibitor of apoptosis results in DORV (TGA type) and TGA phenotypes [84].

The generation and remodeling of OFT myocardium are thus both essential for correct ventriculoarterial alignment. It is important to point out that the range of OFT phenotypes observed in mouse models and human CHD patients highlights the importance of genetic modifiers of mutant phenotypes. In addition, non-genetic factors, including teratogens, maternal diabetes or illness, epigenetic factors, and even stochastic events, can impact on either or both steps of OFT elongation and remodeling and result in alignment defects such as DORV and TOF [85].

Conclusion

In conclusion, DORV and TOF are part of a disease spectrum of OFT alignment defects arising through multiple upstream causes, reflected in the diversity of cell types, genes, and embryological processes involved in OFT extension and remodeling. While OFT alignment is distinct from neural crest-driven OFT septation, the two processes are coincident and together ensure the separation of systemic and pulmonary circulatory systems at birth. The mechanistic complexity underlying these processes is reflected in the range of associated CHDs and genetic heterogeneity observed in human patients.

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Part IX

d-Transposition of the Great Arteries

Clinical Presentation and Therapy of d-Transposition of the Great Arteries

34

David J. Driscoll

Contents

34.1 Introduction

d-Transposition of the great arteries (d-TGA) is the most common form of congenital heart disease that presents with cyanosis in a newborn. The aorta arises from the right ventricle and the pulmonary artery arises from the left ventricle. It constitutes 3.8 % of all congenital cardiac defects. Forty percent of patients with d-TGA have an associated ventricular septal defect. Among patients with d-TGA, 6 % of those with intact ventricular septum and 31 % of those with ventricular septal defect have associated pulmonary stenosis.

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34.2 Pathologic Physiology

In d-TGA systemic venous return (blood with low oxygen content) returns to the right ventricle and then is pumped to the body via the aorta without passing through the lungs for gas exchange (Fig. 34.1). Pulmonary venous return (oxygenated blood) returns to the left ventricle and then is pumped back to the lungs (Fig. 34.1). The effective pulmonary blood flow (the volume of deoxygenated blood that participates in gas exchange in the lungs) is low, albeit total pulmonary blood flow is increased. This is incompatible with life unless a communication exists between the two circuits to allow mixture of the oxygenated and deoxygenated blood. This mixture occurs at the patent foramen ovale (or atrial septal defect), the ductus arteriosus (if patent), and the ventricular septal defect (if present). This is a tenuous situation for a patient with an intact ventricular septum and no true atrial septal defect because mixing of the two circuits will decrease as the patent ductus arteriosus closes and the patent foramen ovale becomes sealed.

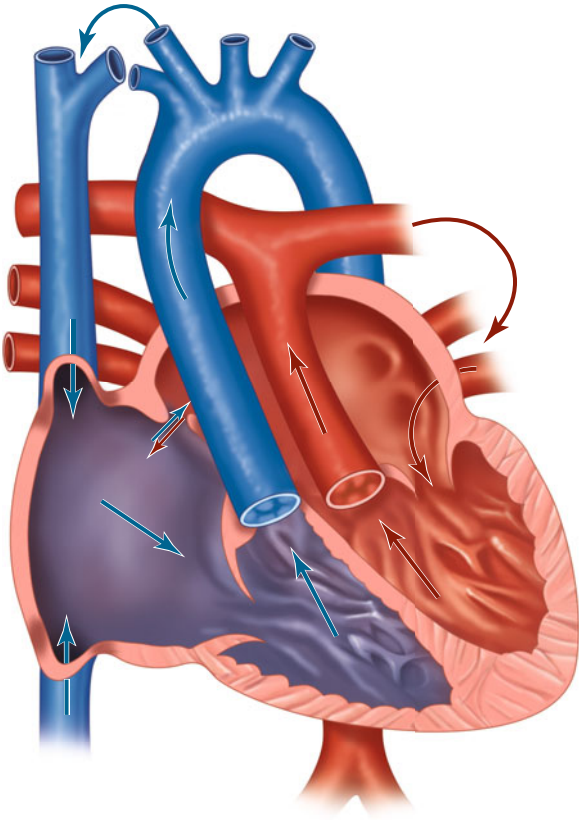

Fig. 34.1 Diagrammatic representation of transposition of the great arteries. Note that the aorta arises from the right ventricle and the pulmonary artery arises from the left ventricle. If there are no communications between the left and right side of the heart, it would be impossible to supply oxygenated blood to the body which is incompatible with life

Technically, double outlet right ventricle (DORV) cannot be classified as TGA because in TGA both great arteries must arise from the wrong side of the ventricular septum. However, DORV with a subpulmonary ventricular septal defect results in transposition physiology and is repaired in a manner similar to d-TGA.

34.3 Clinical Presentation

A newborn with d-TGA represents a medical emergency. It is critical to establish or exclude this diagnosis and to document and to insure adequate sites for mixing between the systemic and pulmonary circuits which, in d-TGA, are in parallel rather than in series.

34.4 Physical Examination

The primary physical finding is cyanosis. The right ventricular impulse will be increased. Depending upon the associated defects, there may or may not be a murmur. If a VSD or pulmonary stenosis is present, there will be a systolic murmur. If there is associated coarctation of the aorta, the femoral pulses will be weak or absent.

34.5 Echocardiographic and Cardiac Catheterization Issues

The diagnosis of d-TGA and most associated malformations can be established noninvasively with two-dimensional echocardiography. The presence of a patent ductus arteriosus can be determined, but the ductus arteriosus cannot be relied on as a stable source of mixing because it likely will close. The ductus arteriosus can be maintained patent by infusion of prostaglandin E-l, and this should be done if the baby is extremely hypoxemic ($PaO_2 < 25$ mmHg), is acidotic, or is to be transferred to another institution.

34.6 Treatment: Management of d-TGA with Intact Ventricular Septum

34.6.1 Arterial Switch (Jatene) Procedure

This procedure represents the best option for managing infants with d-TGA, intact ventricular septum or a VSD, and a normal pulmonary valve. In this operation, the aorta and pulmonary artery are transected cephalad to their respective valves. The ostia of the coronary arteries are removed from the stump of the aorta and sewed to the stump of the pulmonary artery (the neoaorta). The distal portion of the aorta is anastomosed to the proximal stump of the pulmonary artery, and the distal portion of the pulmonary artery is anastomosed to the stump of the aorta.

The mortality after this procedure is 10 % or less and the long-term results are quite good. This procedure must be done early (<3 weeks of age), before the thickened left ventricular walls involute as pulmonary arterial resistance decreases. In many institutions, the operation is performed without preoperative cardiac catheterization. Prostaglandin E_l is infused preoperatively to maintain patency of the ductus arteriosus and preserve the acid–base stability of the patient.

34.6.2 Atrial Switch (Senning or Mustard) Procedure

The Senning and Mustard operations were described in the 1960s and predate the arterial switch operation. These are alternatives to the arterial switch procedure. They are used if the pulmonary valve is abnormal such that it would not work as the neoaortic valve following a Jatene (arterial switch) procedure. With these procedures systemic and pulmonary venous returns are rerouted in the atriums. That systemic venous return from the superior and inferior venae cavae is directed through the mitral valve and into the left ventricle (and subsequently to the pulmonary artery). The pulmonary venous return is directed through the tricuspid valve (and subsequently to the aorta). This is accomplished by sewing a baffle in the atrium. In essence, transposition of the great arteries is treated by creating "transposition" of the venous return to the heart.

The Senning and Mustard operations have a low operative mortality but significant intermediate and long-term problems. These include obstruction of systemic and pulmonary venous returns by the baffle, atrial arrhythmias, tricuspid insufficiency, and right ventricular failure.

The Senning and Mustard operations usually are performed in patients 6 months to 1 year of age but some surgeons will perform the operation in the newborn period. If one of these methods of operation is to be utilized, an adequate interatrial communication must be established in the newborn period. This is accomplished by doing a Rashkind balloon atrial septostomy. A catheter with an inflatable balloon at its tip is advanced from the femoral or umbilical vein to the right atrium through the patent foramen ovale and into the left atrium. The balloon is inflated in the left atrium and withdrawn rapidly into the right atrium, producing a rent in the atrial septum.

34.7 Outcome

34.7.1 Arterial Switch (Jatene) Procedure

The operative mortality for the arterial switch is in the range of 2–10 %. The long-term outcome is excellent. Some patients will require reoperation for pulmonary artery stenosis. However this has become less common with the introduction of the LeCompte maneuver.

34.7.2 Atrial Switch (Senning or Mustard) Procedure

The operative mortality for the atrial switch operation is in the range of 1–5 %. There are numerous long-term problems with the atrial switch procedure. Since the right ventricle remains the systemic ventricle after this operation, late right ventricular failure occurs. Because of the extensive suturing in the atria, late atrial arrhythmias and sudden death occur.

Human Genetics of d-Transposition of the Great Arteries

35

Patrice Bouvagnet and Anne Moreau de Bellaing

Contents

Abstract

Dextro-transposition of the great arteries (d-TGA) is one of the rare congenital heart diseases (CHD) which benefits from early neonatal diagnosis because d-TGA requires rapid postnatal catheter procedure. In that respect, detecting parental genetic predisposing factors would contribute to focusing prenatal echographical attention to the early detection of d-TGA cases. A high male to female ratio and a high recurrence risk of d-TGA in the context of heterotaxy suggest the impact of genetic factors although familial cases of d-TGA are exceptional. Since the late 1990s, a growing list of genes and chromosomal regions was associated with d-TGA among which the *ZIC3* gene. Although this gene is located on the X chromosome, *ZIC3* (Zic family member 3) does not explain the male preponderance in d-TGA. d-TGA causal genes are involved in many different cellular pathways and can be provisionally sorted in two groups: those which disrupt the function of the embryonic node cilia and those which are downstream

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of this major embryological process of lateralization. Many more genes or gene factors remain to be discovered in d-TGA and related CHD because only a small percentage of d-TGA is yet genetically resolved.

35.1 Introduction

Among congenital heart defects (CHD), dextro-transposition of the great arteries (d-TGA) has one of the lowest recurrence risk suggesting that genetic factors are not preponderant in this particular CHD to the point that Digiglio et al. reported in 2001 a series of familial d-TGA to show that familial d-TGA could be observed. Besides exceptional familial cases, the fact that the male to female ratio of d-TGA cases is high suggests that sexual chromosomes have an impact on d-TGA genesis. In contrast to isolated d-TGA, d-TGA in a context of heterotaxy has a relative high recurrence risk when any CHD and/or heterotaxic sign are considered. In this chapter, we will review all genes and gene regions associated to d-TGA. These genes are presented in Table 35.1.

35.2 Epidemiology

Although in most cases, as for other congenital heart defects (CHD), it is a sporadic disease, there are a few familial cases [16]. The prevalence of d-TGA is about twice as common in males as in females [17], suggesting that recessive genetic factors on the X chromosome could play a role. This hypothesis has been reinforced by the fact that parental consanguinity is not higher in d-TGA cases than in the control population [18], a situation compatible with X-linked inheritance. d-TGA usually occurs as an isolated congenital heart defect but occasionally may be part of a heterotaxia complex. The relative proportion of these two groups of d-TGA is unknown, but the relative risk of recurrence for first-degree relatives is much higher in the framework of heterotaxy than in cases unassociated with heterotaxy, suggesting a different influence of genetic factors between these two groups [19]. Thus, it has been hypothesized that d-TGA could be, in some cases, the sole expression of heterotaxy.

35.3 Molecular Genetics

35.3.1 d-TGA Without Heterotaxy

Among all gene mutations so far identified in d-TGA, only one *MED13L* (mediator complex subunit 13-like; alias *PROSIT240*, *THRAP2*) was identified outside of the framework of heterotaxy. It was found interrupted in a patient with mental retardation and d-TGA who had a reciprocal translocation [7]. The subsequent mutational screening of a series of 97 d-TGA patients revealed three missense mutations that could be causal, although in one case, the mutation was found in a normal mother.

Table 35.1 Genes and phenotypic description of d-TGA patients

Gene name	d-TGA plus other cardiac anomalies	Extracardiac anomalies	Familial/sporadic	Reference
ACVR2B	P1, interrupted IVC, AVC; P2, CAVC, sub-PS, PS, interrupted IVC, azygous to SVC	P1, R-sided stomach; P2, midline liver, polysplenia	P1, DNA variant present in unaffected mother, African-American origin	P1: [1]; P2: [2]
CFC1	P1, dextrocardia; P2, dextrocardia, bilateral SVC	P1, L-isomeric lungs, R-sided stomach, transverse liver, intestinal malrotation, polysplenia; P2, R-isomeric lungs, R-sided stomach, asplenia	–	P1, P2: [3]
CFC1	P1, dextrocardia, bilateral SVC	P1, R-isomeric lungs, R-sided stomach, asplenia; P2, pyloric stenosis	P1, DNA variant present in one of the phenotypically normal parents; P2, sporadic	P1: [3]; P2: [4]
GDF1	P1, TAPVR, common atrium, PS	P1, transverse liver, R-isomeric lungs, asplenia	P1, familial compound mutation	P1: [5]; P2, P3: [6]
MED13L	Three patients	–	P1, DNA variant present in unaffected mother	P1, P2, P3: [7]
NODAL	P1, SV; P2, CAVC, P3, DILV, PA; F1, 6 relatives with diverse CHD	P1, abdominal SI; P2, abdominal SI, midline liver, asplenia; F1, abdominal SI, asplenia	P1, sporadic; P2, phenotypically normal mother; F1, phenotypically normal father	P1–P3, F1: [8]
ZIC3	P1, CAVC, PA; P2, MA, PS; P3, MA, SV	P1, abdominal SI, asplenia, arhinencephaly, posteriorly placed anus; P2, extrahepatic atresia, abdominal SI, sacral agenesis, rectal stenosis, club feet; P3, asplenia	P1–P3, family LR1	[9]
ZIC3	AVC, PA	Midline stomach, transverse liver, intestinal malrotation	Familial, 1 affected brother	[10]
ZIC3	LSVC, ASD-SV, AVC, HypoRV, PS	Hypertel, large nasal bridge, sagittal suture fusion	Familial, male incomplete penetrance	[11]

(continued)

Table 35.1 (continued)

Gene name	d-TGA plus other cardiac anomalies	Extracardiac anomalies	Familial/sporadic	Reference
ZIC3	P1, bilat SVC, TAPVR, CAVC; P2, interrupted IVC, ASD, VSD, PS; P3, TAPVR, SV, CAVC, PS, PDA; P4, TAPVR, R-atrial isomerism, HLHS	P1, cholestasis with biliary atresia, asplenia; P2, EHBA, imperforate anus, club feet, fused lumbar vertebrae, posterior embryotoxon, low-set ears; P3, horseshoe kidney, asplenia, low-set ears; P4, intestinal malrotation, abnormal liver lobation, asplenia, webbed neck, R-isomeric lungs	P1–P3, familial, all affected are males; P4, familial, 2 fetuses	P1–P4: [12]
ZIC3	SV, PA, MA	Asplenia	–	[13]
ZIC3	–	–	–	[14]
ZIC3	P1, PDA; P2, HypoLV, MV, VSD, ventricular inversion; P3, CAVC, PA	P3, abdominal SI, asplenia	P1, P2, sporadic; P3, 2 sibs with Ivemark, 1 with heart defect	P1–P3: [15]

Abbreviations: *ASD* atrial septal defect, *ASD-SV* ASD sinus venosus type, *AVC* atrioventricular canal, *CAVC* canal atrioventricular complete, *DILV* double inlet right ventricle, *Dup.* duplication, *EHBA* extrahepatic biliary atresia *F1* family 1, *HLHS* hypoplastic left heart syndrome, *Hypertel.* hypertelorism, *HypoRV* hypoplastic right ventricle, *HypoLV* and *HypoLV* hypoplastic left and right ventricle, *IVC* interrupted vena cava, *IVS* inferior vena cava, *LSVC* left superior vena cava, *LV* left ventricle, *MA* mitral atresia, *MV* mitral valve, *P1, P2, P3* patient number, *PA* pulmonary atresia, *PDA* patent ductus arteriosus, *PS* pulmonary stenosis, *SI* situs inversus, *SV* single ventricle, *SVC* superior vena cava, *TAPVR* total anomalous pulmonary venous return, *VSD* ventricular septal defect

35.3.2 d-TGA with Heterotaxy

All other mutations were found either in patients with isolated heterotaxy or heterotaxy with ciliary dysfunction. So far, *MED13L* has not yet been tested on a series of heterotaxic patients.

35.3.2.1 d-TGA Without Cilia Dysfunction

In heterotaxy without cilia dysfunction, gene mutations have been found in six genes in patients with d-TGA: *ACVR2B* (activin receptor type II B), *CFC1* (CRYPTIC), *GDF1* (growth differentiation factor 1), *LEFTY2* (left-right determination factor 2), *MYH6* (alpha-cardiac myosin heavy chain), *NODAL* (nodal growth differentiation factor), and *ZIC3* (Zic family member 3) (see Chap. 38). The causality of *ACVR2B* is dubious in d-TGA because the two studies reporting variants [1, 2] found the same variant (p.Arg40His), which is found relatively frequently (0.5 %) in the world population. The two mutations found in *CFC1* in d-TGA patients are strong candidates for disease-causing mutations because they change the reading frame [3] or alter a splicing site [4]. The frameshift mutation (c.522delC, p.Gly174del) is associated with heterotaxic anomalies including one case of right and one of left lung isomerism but also was found in a normal mother. In the study of Goldmuntz et al. [4], the *CFC1* gene was screened in a series of 58 patients with d-TGA, and only the mutation causing abnormal splicing was found, yielding a prevalence of 2 % for *CFC1* mutations in patients with d-TGA. Three *GDF1* mutations were found in patients with d-TGA: two in a systematic screen (p.Cys227X and p.Ala318Thr) [6] and compound heterozygous mutations in a family with autosomal recessive inheritance and heterotaxy including right atrial isomerism [5]. Interestingly, one of the compound mutations is the p.Cys227X mutation. This mutation was found in controls suggesting that alone this mutation has either no impact or that it can predispose to isolated d-TGA, a phenotype less severe than heterotaxy, as observed when this mutation is associated with another mutation in trans.

The gene encoding the alpha-cardiac myosin heavy chain gene (*MYH6*) was found with a mutation in a girl with a d-TGA. This mutation changed a highly conserved histine to a glutamine (p.His252Gln). Her mother with a patent foramen ovale and her unaffected grandmother carried the mutation [20]. No other of the 49 TGA cases had a *MYH6* mutation. Four putative heterozygous mutations were found in d-TGA patients in the *NODAL* gene (p.Glu203Lys, p.Gly260Arg, p.Arg275Cys, and a deletion/insertion) with demonstrated experimental deleterious consequence [8]. Surprisingly, the p.Gly260Arg mutation was found in five cases of Hispanic origin. This latter mutation and the p.Arg275Cys were also found in unaffected parents.

ZIC3 is the most studied gene in this series. It initially was identified through a positional cloning effort in families suffering from heterotaxy with X-linked inheritance [9]. At least nine additional ZIC3 mutations have been found in d-TGA patients [11–15, 21]. *ZIC3* mutations have several specificities. They often are associated with midline anomalies (anal stenosis or imperforation, sacral agenesis, horseshoe kidney), extrahepatic biliary atresia, club feet, and mild facial dysmorphy. The inheritance is X-linked affecting boys, but there were examples of non-penetrance in males [11, 22] and on the contrary variable expression in females due to biased

X-inactivation either revealing a recessive mutation (by inactivation of the normal allele) [13] or switching off a dominant mutation (by inactivation of the mutant allele) [1]. In this latter family, affected children had heterotaxy and CHD but no d-TGA. In addition to these nucleotide mutations, a cryptic chromosomal deletion was instrumental for the discovery of the role of ZIC3 gene in heterotaxy [10].

Whole exome sequencing of trios composed of an affected child and unaffected parents revealed an astonishing ~10 % of putatively causal *de novo* mutations [23]. In this series, 47 d-TGA were included and deleterious variants were found in four genes: *NAA15* (N-alpha-acetyltransferase 15, NatA auxiliary subunit), *MKRN2* (makorin ring finger protein 2), *SMAD2* (SMAD family member 2), and *RAB10* (RAB10 member RAS oncogene family). All genes were mutated twice except the *RAB10* gene. SMAD2 is downstream of NODAL signaling enabling transcriptional activation of poised promoter/enhancers by demethylating H3K27me.

Copy number variations (CNV) have been implicated in heterotaxy [24]. Among 262 heterotaxic patients, three had a d-TGA and a duplication of a gene: *NUP188*, *NEK2*, or *TGFBR2*. The involvement of these genes in lateralization was further demonstrated in *Xenopus* developmental experiments. More recently, 39 trios with a d-TGA child and unaffected parents were tested for CNVs [25]. Five small-sized *de novo* CNVs were found involving genes never previously involved in CHD (deletions, *CENPU* and *PRIMPOL*, *EIF3J* and *SPG11*, and *MACROD2*; duplication, *NTRK3* and *DDX53*). Finally, a single heterozygous deletion of the *ISL1* gene was found in a series of 169 d-TGA cases but no *ISL1* point mutations [26]. Parental DNAs were unavailable. In contrast, isolated d-TGA is never seen in 22q11 deletion patients [27].

35.3.2.2 d-TGA with Embryonic Node and Primary Cilia Dysfunction

Mutations in *NPHP4* (nephronophthisis 4) are causal in nephronophthisis, an autosomal recessive kidney disease which represents the most common genetic cause of end-stage renal disease in children and young adults. The protein encoded by *NPHP4* is located in the primary cilia of renal epithelial cells. A recent study carried out in a family with autosomal recessive occurrence of heterotaxy with complex cardiovascular malformations including two cases of d-TGA found a homozygous mutation in *NPHP4* (p.Arg1044His). Several other heterozygous mutations were found in d-TGA patients [28]. Interestingly, this protein has been shown to be indispensable for normal rotatory cilia beating in Küppfer's vesicle, the equivalent of the embryonic node in the fish embryo. Other genes of the primary cilium encode proteins shared with the embryonic node and can result in heterotaxy (*NPHP2* (alias *INV*), *NPHP3*, *NPHP12* (alias *IFT139*), *OFD1* (oral-facial-digital syndrome 1), *CEP290* (alias *NPHP6*), *BBS* (Bardet-Biedl syndrome 1), *BBS7*, *BBS8*, *BBS10*, *BBS12*, *LZTFL1* (leucine zipper transcription factor-like 1), and *MKS1* (Meckel syndrome type 1)), but the related syndromes are very rare and include multivisceral anomalies.

35.3.2.3 d-TGA with Respiratory Cilia Dysfunction

Cilia are cytoplasmic cellular protrusions. There are three types of cilia in the human body: (1) Immotile cilia with nine pairs of peripheral microtubules. They have a sensory function and are present on many different cell types. (2) Cilia

with a rotatory movement. These cilia are found in the embryonic node and play an essential role in a primordial step of lateralization development. They have nine pairs of peripheral microtubules with dynein arms that are responsible for cilia movement. (3) Cilia with a windshield type of movement which are located in the upper and lower respiratory tracts. These respiratory cilia are essential for the clearance of inhaled particles including microbes. They have nine pairs of microtubules with dynein arms and a central microtubule pair surrounded by a complex of proteins and radial spokes. Each of these cilia types has specific proteins and shares some proteins with the other cilia types. Only the dysfunction of the nodal cilia may result in heterotaxy and hence d-TGA, but if the defective protein is shared with another cilia type, additional symptoms will be present. In the context of cilia dysfunction, the most frequently associated anomaly is recurrent airway infection due to respiratory ciliary dyskinesia. This disease is called primary ciliary dyskinesia (PCD) (see Chap. 38). In particular, proteins which are components of the dynein arms and those which are essential for the assembly and regulation of dynein arms – the motor of moving cilia – are common to respiratory and embryonic node cilia. In contrast, mutation in genes encoding proteins of the central complex and radial spokes is never responsible for heterotaxy and hence d-TGA. When a protein common to embryonic node and respiratory cilia is abnormal, the occurrence of heterotaxy is about 54 % with 45 % of complete situs inversus and about 10 % of situs ambiguous (incomplete left/right inversion leading to CHD) (see Chap. 38). In about 45 % of PCD cases, there is no situs anomaly (situs solitus) [29, 30]. In the most recent study, only one patient had a d-TGA out of a series of 37 PCD with situs ambiguous [30]. This patient had no outer dynein arm and no mutations were found in *DNAI1* and *DNAH5*. In a series of 43 heterotaxic patients, Nakhleh et al. [31] found ciliary dysfunction in about 42 % including three patients with d-TGA and associated cardiac malformations. Nevertheless, no homozygous or compound heterozygous mutations were found after testing ten PCD causal genes (*DNAI1*, *DNAI2*, *DNAH5*, *DNAH11*, *DNAL1*, *CCDC39*, *CCDC40*, *DNAAF1* (alias *LRRC50*), *DNAAF2* (alias *KTU*), and *NME8* (*TXNDC3*)) which could result in heterotaxy. As of today, there are five additional genes which can cause PCD and heterotaxy (*DNAAF3*, *CCDC103*, *CCDC114*, *CCDC164*, and *HEARTR2*).

Conclusions

There are already numerous d-TGA causal genes, but altogether, these causal genes account for only a small percentage of d-TGA. In particular, the genetic factor – presumably located on the X chromosome – which is responsible for the excess of male cases of d-TGA is still elusive since it is not the *ZIC3* gene [13, 15]. Here, we reported only on gene mutations which result in d-TGA, but it can be anticipated that any gene mutation responsible for heterotaxy could be causal for d-TGA. It is also possible that a substantial percentage of isolated d-TGA is the consequence of mutation in genes which do not lead to heterotaxy when mutated.

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Molecular Pathways and Animal Models of d-Transposition of the Great Arteries

36

Amy-Leigh Johnson and Simon D. Bamforth

Contents

Abstract

During normal cardiovascular development, the outflow tract becomes septated and rotates so that the separate aorta and pulmonary trunk are correctly aligned with the left and right ventricles, respectively. However, when this process goes wrong, the aorta and pulmonary trunk are incorrectly positioned resulting in oxygenated blood being directly returned to the lungs, with deoxygenated blood being delivered to the systemic circulation. This is termed transposition of the great arteries (TGA). The precise etiology of TGA is not known, but the use of animal models has elucidated that genes involved in left–right determination of the embryonic body play key roles. Other factors such as retinoic acid levels are also crucial. This chapter reviews the animal models that can be manipulated genetically or with exogenous agents to present with TGA.

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36.1 Introduction

Transposition of the great arteries (TGA), which is better known as discordant ventriculo-arterial connections, is a congenital heart defect affecting the emergence of the aorta and pulmonary trunk from the heart. Whereas in the normal configuration, the aorta will arise from the left ventricle to deliver oxygenated blood to the systemic circulation, and the pulmonary trunk will arise from the right ventricle to take blood to the lungs for oxygenation; in TGA, these major vessels have been transposed meaning that the aorta takes deoxygenated blood from the right ventricle to the systemic circulation while the pulmonary trunk will shuttle blood to the lungs where it is immediately returned to the left atrium. Therefore, this is a serious heart defect, and survival is dependent on the mixing of blood within the heart, usually by septal defects. TGA appears to be caused by a failure of the outflow tract to rotate so the aorta and pulmonary trunk do not spiral and form two parallel vessels, abnormally connected to the ventricles.

Animal models have been produced that mimic TGA (Fig. 36.1), and these are restricted to the mouse and chick as the anatomy of the heart outflow tract is analogous to that of the human. However, the development of TGA is often not highly penetrant and not seen consistently within the same animal model. This makes the molecular pathways underlying the cause of TGA very difficult to identify. A number of causes for TGA in animal models have been proposed, including environmental factors such as those affecting retinoic acid levels and genetic mutations affecting the left–right patterning pathway.

36.2 Left–Right Patterning

Despite many human syndromes presenting with some form of cardiovascular defect, TGA is extremely rare in any syndrome except for heterotaxy [2]. However, TGA may be sporadically associated with VACTERL syndrome. Transgenic mice with a deletion of a gene associated with this syndrome, proprotein convertase subtilisin/kexin type 5 (*Pcsk5*), have TGA and double-outlet right ventricle (DORV) [3]. Heterotaxy is a rare congenital defect where the major organs are positioned abnormally within the body (see Chaps. 37 and 38). In the normal body, the position of the organs is called *situs solitus*, but when the organs are found in the mirror image of normal, it is known as *situs inversus*. However, when the reversal of organs is incomplete, this is known as *situs ambiguous* and can have profound effects on the formation of the heart and lungs. Indeed, it is considered that defects in the establishment of the left–right axis almost always result in complex congenital heart malformations [4].

The establishment of laterality in the embryo is conducted through the asymmetric and transient expression of transforming growth factor (TGF) β-related proteins that are both positively and negatively regulated (Fig. 36.2). This pathway begins in the node, a pit at the end of the primitive streak containing posterior-tilting ciliated cells that rotate. In the early mouse embryo (around E7.5 of gestation), these rotating cilia cause a leftward nodal flow that initiates the left–right axis determination

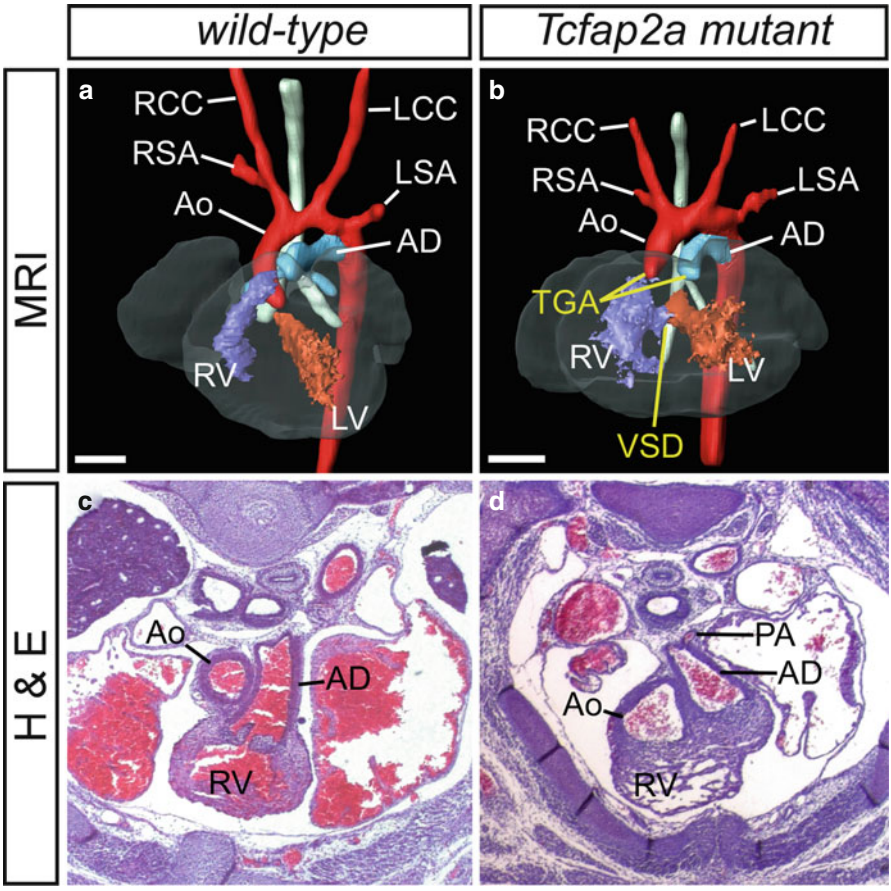

Fig. 36.1 Transgenic mouse model displaying transposition of the great arteries (*TGA*). 3D reconstructions from magnetic resonance imaging (*MRI*) data sets [1] (**a**, **b**) and hematoxilin and eosin-stained (*H&E*) sections (**c**, **d**) of E15.5 fetuses. (**a**, **c**) By E15.5, the heart and its associated great vessels have developed into the mature configuration with the aorta (*Ao*) arising from the left ventricle (*LV*) and the arterial duct (*AD*) arising from the right ventricle (*RV*). (**b**, **d**) The heart of a transgenic fetus mutated for transcription factor AP-2alpha (*Tcfap2a*) presents with TGA, whereby the aorta arises from the RV and the DA arises from LV. Note how the two arteries lie in parallel (**b**) rather than spiral around each other (**a**). The presence of a pulmonary artery (*PA*) joining the AD is indicated (**d**) as is a VSD (**b**). Other abbreviations: *LCC* left common carotid, *LSA* left subclavian artery, *RCC* right common carotid, *RSA* right subclavian artery. Scale, 500 μm

process. In a mutant mouse line known as *iv/iv* (*inversus viscerum*), half of all these mice present with situs inversus, associated with complex cardiac malformations, including TGA [6]. In *iv/iv* embryos, the nodal cilia are immotile because of a mutation in the gene encoding the motor protein, axonemal dynein heavy chain 11 (*Dnah11*) [7]. The nodal flow is therefore absent resulting in randomized laterality of visceral organs [8, 9].

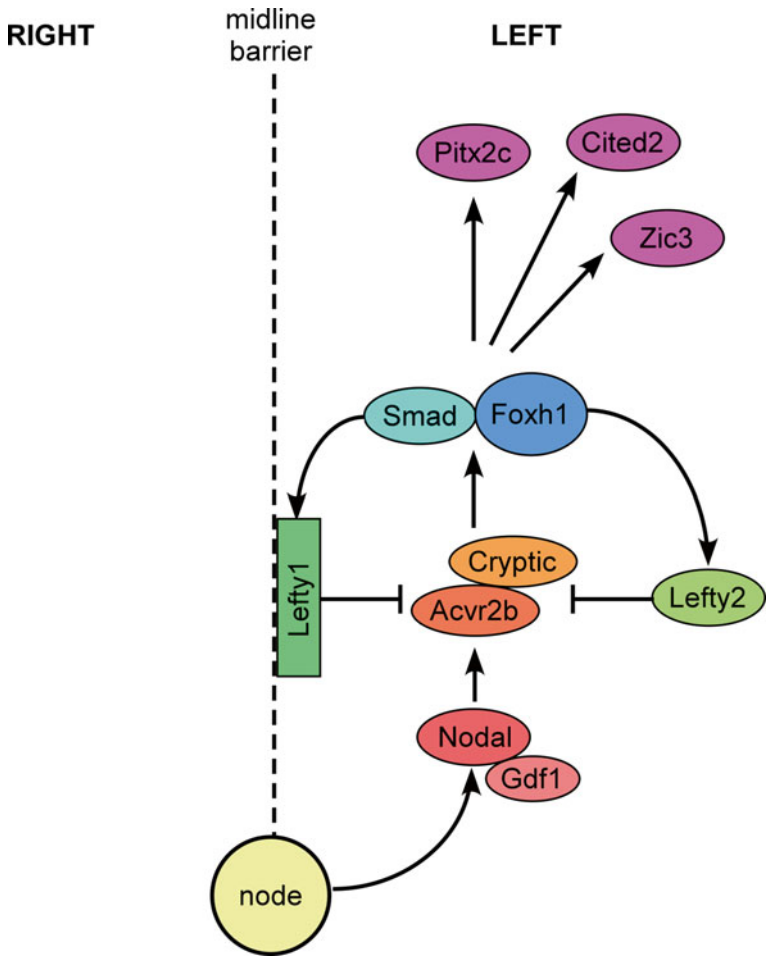

Fig. 36.2 Signaling molecules involved in the establishment of laterality. Nodal is expressed in the node from where it is transferred to the left lateral plate mesoderm (LLPM) forming a growth differentiation factor 1 (*Gdf1*)–Nodal heterodimer. Nodal signals through type I and type II transforming growth factor (TGF) β receptors (e.g., activin A receptor, type IIB; Acvr2b) and the co-receptor Cryptic. Smad proteins, along with forkhead box H1 (*Foxh1*), transduce the Nodal signal, and in response, left–right determination factor (*Lefty*) 1 and Lefty2 are expressed asymmetrically in the LLPM. The Lefty proteins inhibit Nodal signaling by competitively interacting with Cryptic and the type II TGFβ receptor, thereby acting as antagonists of Nodal and restricting its expression to the LLPM. The Nodal signaling pathway results in the induction of genes involved in left–right patterning, e.g., paired-like homeobox 2c (*Pitx2c*), Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2 (*Cited2*) and Zic family member 3 *(Zic3)* (Adapted from Shiratori et al. [5])

The cilia found in the node are localized on the posterior portion of the apical surface of each of the node's cells and display a morphological planar polarization. This planar cell polarity (PCP) regulates the polarity of epithelial cells in relation to their apical–basal axis [10], and PCP proteins are responsible for regulating planar

polarity within the node [11]. As the polarity and function of the nodal cilia are critical for establishing the left–right signaling pathway, it is perhaps not surprising that left–right patterning defects leading to TGA can be found in some transgenic mouse models mutated for PCP genes. For example, half of all mice deficient for the PCP gene *Dishevelled 2* (*Dvl2*) die from defects in outflow tract development that include TGA, DORV, and common arterial trunk [12] (More information on common arterial trunk is provided in Chap. 48). The defects observed in *Dvl2* mutant mice are more penetrant in mice doubly homozygous for *Dishevelled 1* and *Dvl2*. Cardiac outflow tract defects are also seen in mice mutated for *Scrib* (*Circletail*, *Crc*), another PCP gene [13]. The defects observed include TGA, as well as DORV, aberrant right subclavian artery, and atrioventricular septal defect.

Nodal is expressed in the node where it is subsequently transferred to the left lateral plate mesoderm (LLPM). In transgenic mice specifically lacking *Nodal* activity within the node, left–right patterning defects occur affecting the location of the stomach, right pulmonary isomerism, and heart looping randomization resulting in TGA [14]. Growth differentiation factor 1 (*Gdf1*) is co-expressed with Nodal in the node and is essential for Nodal expression in the LLPM, forming a Gdf1–Nodal heterodimer. *Gdf1* is closely related to the *Xenopus Vg1* gene, which influences the left–right patterning of the heart and viscera in the frog [15]. Although mouse Gdf1 and frog Vg1 proteins have limited sequence homology, the two proteins have functional and regulatory similarities [16]. In mice, *Gdf1* is expressed in the primitive node, the ventral neural tube, and the LLPM of developing mouse embryos. Loss of *Gdf1* is associated with the incorrect formation of the left–right axis, affecting the location of the stomach, spleen, pancreas, intestines, kidneys, and adrenal gland, and right pulmonary isomerism is also observed. *Gdf1*-null embryos are not viable, with only two-thirds of mutants surviving until birth, which then die within the immediate perinatal period. Mutant embryos present with cardiovascular defects including TGA as well as ASD, VSD, or a common atrioventricular canal [17]. Loss of *Gdf1* results in the absence of left–right determination factor (Lefty) 1 in the left side of the floor plate and Lefty2 and Nodal in the LLPM suggesting that *Gdf1* is likely to be directly or indirectly upstream of these genes [17]. A zebra fish model has been used to demonstrate the functional consequences of *GDF1* mutations found in human patients with congenital heart disease, including TGA, tetralogy of Fallot, DORV, and interrupted aortic arch [18] (see Chap. 42 for more information on interruption of the aortic arch). These experiments showed that each of the mutations were either hypomorphic or resulted in a complete loss of function of *Gdf1*.

Expression of Gdf1 in perinodal cells requires sonic hedgehog (Shh) signaling, since Gdf1 expression is lost in the Smoothened (*Smo*) mutant mouse [19]. *Smo* is an essential component of the hedgehog signaling pathway. Downregulation of Gdf1 expression, which is also observed in transgenic mice with aberrant ciliogenesis [20], likely accounts for the lack of Nodal expression in the LLPM in these mutants with impaired hedgehog signaling. Moreover, transgenic mouse experiments have shown that Shh signaling in the ventral foregut endoderm is required for cardiac morphogenesis at the arterial pole of the heart as mice with a conditional deletion of *Smo* from the second heart field also display TGA [21].

Nodal is a ligand that can signal through type I and type II TGFβ receptors, one of which is the activin receptor, *Acvr2b*. Mice lacking *Acvr2b* have left–right patterning defects and TGA [22]. Activins are dimeric growth and differentiation factors which belong to the TGFβ superfamily. Cryptic, an EGF–CFC (epidermal growth factor–Cripto-FRL1-Cryptic) family protein, is a Nodal co-receptor, and transgenic mice lacking *Cryptic* have left–right patterning defects and cardiovascular abnormalities including TGA [23, 24]. Smad proteins, along with Foxh1, transduce the Nodal signal, and transgenic mice lacking *Smad2* die early in development from defects in gastrulation [25]. However, transgenic mice doubly heterozygous for *Smad2* and *Nodal* (i.e., *Smad2+/−*; *Nodal+/−*) display TGA associated with right pulmonary isomerism of the lung, in more than 50 % of cases [25].

In response to Nodal signaling, Lefty1 and Lefty2 are expressed asymmetrically in the LLPM. The Lefty proteins inhibit Nodal signaling by competitively interacting with Cryptic and the type II TGFβ receptor, thereby acting as antagonists of Nodal and restricting its expression to the LLPM. Lefty1 is expressed at the midline and is thought to act as a physical barrier preventing the movement of left-sided proteins across to the right LPM. Transgenic mice lacking *Lefty1* have left–right patterning defects, such as left pulmonary isomerism and misalignment of the cardiac outflow tract resulting in TGA, as well as DORV [26]. Other cardiovascular malformations include VSD, ASD, and common atrioventricular canal. Mice null for *Lefty2* die early during embryogenesis, but this can be overcome through the creation of an alternative allele in transgenic mice. Deletion of the *Lefty2* asymmetric enhancer element prevents these early gastrulation defects, but also prevents the asymmetric expression of Lefty2 in the LLPM at the early somite stage [27]. In these mutant mice, visceral situs abnormalities were identified along with cardiovascular defects including TGA, as well as DORV and aberrant great arteries.

The Nodal signaling pathway in the LLPM results in the induction of genes that, when mutated in transgenic mice, result in left–right patterning defects. One of these genes important for establishing a left-sided identity within the developing embryo is *Pitx2c*. The paired-like homeobox transcription factor gene, *Pitx2*, has four known isoforms: *Pitx2a* and *Pitx2b* are expressed symmetrically in the brain, whereas the *Pitx2c* isoform is expressed asymmetrically in the LLPM [28, 29]. *PITX2D* has only been identified in humans [30]. *Pitx2c* plays a major role in the establishment of left–right asymmetry of the body and is a direct target of Nodal [31]. Transgenic mice null for *Pitx2c* display outflow tract defects with rotational anomalies, leading to TGA [32]. This suggests that embryonic laterality affects outflow tract rotation and consequently may be the cause of some instances of congenital heart disease such as TGA.

Other genes downstream of the left–right signaling pathway include Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2 *(Cited2)* and Zic family member 3 *(Zic3)*. The *Cited2* null mouse presents with left–right patterning defects and cardiovascular abnormalities including TGA [33, 34], as do mice null for *Zic3* [35]. Indeed, mutations in *ZIC3* have been found in patients with heterotaxy [36].

TGA has also been identified in transgenic mouse mutants not necessarily directly linked to a left–right patterning defect. These include mice mutated for *Perlecan*, a heparan sulfate proteoglycan [37], in which a high incidence of TGA is seen in fetuses with an intact ventricular septum but in the absence of any other laterality defect. It appears that TGA in this model is a consequence of excess mesenchyme preventing proper outflow tract rotation and thereby producing discordant ventriculoarterial alignment. Other such examples are the removal of PR domain-containing protein 1 (*Prdm1)* from the second heart field [38] and transgenic mice null for the endothelin A receptor (*Ednra*), which display craniofacial, aortic arch and outflow tract defects, which include TGA [39]. Mice mutated for fibroblast growth factor 8 (*Fgf8)* [40, 41] also display TGA, and in the chicken, TGA can be induced by blocking Fgf8 signaling with an Fgf8 receptor antibody [42]. This prevents adequate myocardium being added to the outflow tract resulting in a malalignment of the arterial pole.

36.3 Retinoic Acid

It appears that the most reliable method of causing TGA in a mouse model is to administer pregnant mice with retinoic acid or with retinoic acid inhibitors [2]. Retinoic acid (RA) is a very important molecule during development, and disruptions in the amounts produced, whether increased or decreased, can be severely teratogenic. The cardiovascular system is particularly sensitive to RA levels, and a range of phenotypes affecting the heart and great arteries can be observed in affected mouse embryos. However, as discussed above, TGA is only seen in patients with heterotaxy, but mouse models with disrupted RA levels do not appear to show any other aspects of a heterotaxy-like phenotype. The administration of all *trans-retinoic* acid to pregnant females at E8.5 of gestation has been demonstrated to result in three-quarters of the fetuses presenting with TGA [43]. A smaller proportion had DORV and defects in the great arteries were also observed. TGA is also produced by a reduction in RA levels through the administration of BMS-189453 to pregnant females [44]. This is a synthetic retinoid that acts as an antagonist of the retinoic acid receptors alpha, beta, and gamma. Following administration of this compound to pregnant females, cardiovascular malformations were identified in three-quarters of the embryos, with two-thirds of these displaying TGA. The transposition phenotype is much more prevalent than DORV. This suggests that RA depletion is a key factor in the misalignment of the outflow tract. Timing of the administration of BMS-189453 appears to be critical as exposure earlier or later than ~E7.5 results in much lower rates of TGA. Interestingly, of those embryos with TGA, the aorta was displaced to a right-sided, anterior position in comparison to the pulmonary trunk in the majority of embryos (60 %), while a smaller proportion showed the great arteries positioned side by side (40 %) [44].

In a further study, treating pregnant females with folic acid (FA) during pregnancy was found to reduce the teratogenic effects of BMS-189453 [45]. Cardiovascular malformations, including the incidence of TGA, were significantly

reduced in embryos collected from the FA-treated pregnant females compared to BMS-treated controls. Additionally, a substantial reduction in the levels of neural tube defects and thymus defects was also observed. To further understand the genetic changes induced by varying the levels of RA signaling during development, a microarray experiment was designed to analyze the genes that were differentially expressed between wild-type and BMS-treated embryos and between BMS-treated offspring and those from females that were treated with FA during pregnancy [46]. HIF-1α was found to be downregulated in BMS-treated embryos, compared to wild-type, but upregulated in those embryos pretreated with FA. Additionally, an RA response element (RARE) was identified in the promoter region of mouse HIF-1α, suggesting that this is a downstream target of RA signaling, which can be rescued by FA treatment [46]. In a separate study, mice null for *Folr1*, a folate-binding protein gene, died at mid-embryogenesis from extensive cell death. However, folic acid supplementation of pregnant females greatly reduces this early embryonic lethality and allows for *Folr1*-null mice to be born and bred [47]. Examination of *Folr1−/−* fetuses from matings between *Folr1*-null mice identified cardiovascular defects including TGA.

Conclusion

TGA occurs in the developing mammalian heart when the outflow tract fails to rotate correctly resulting in the great arteries becoming abnormally connected to the ventricles. Experimental models have demonstrated that the developing embryo is extremely sensitive to retinoic acid levels; an excess of, or too little, retinoic acid results in a high incidence of TGA. In transgenic mouse models, the genetic manipulation of factors necessary for establishing the left–right asymmetry pathway often results in a malrotation of the developing outflow tract resulting in TGA. As some transgenic mouse models also display TGA without heterotaxy, this suggests that the manifestation of TGA may be the sole phenotype of a left–right patterning defect.

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Part X

Defects of Situs

Clinical Presentation and Therapy of Defects of Situs

37

David J. Driscoll

Situs is derived from Latin and is the place where something exists or originates. In human anatomy situs can be solitus (derived from Latin, meaning "normal"), inversus, or ambiguus. Determining whether situs is normal, inversus, or ambiguus depends primarily on the location of unpaired organs such as the spleen, the liver, the stomach, and intestines. Situs is solitus if the left atrium, spleen, stomach, and the trilobed lung are on the left side and the liver and bilobed lung are on the right side. Situs ambiguus is present if the location of unpaired structures is random or indeterminant even after detailed and appropriate imaging.

"Heterotaxia" is derived from Greek and means different ("hetero") arrangement (taxis) and is used to describe anomalous position of the viscera. Two types of heterotaxia include polysplenia and asplenia. Polysplenia sometimes is referred to as bilateral left sidedness and asplenia is sometimes referred to as bilateral right sidedness. Another term describing sidedness is "isomerism." The term left-sided isomerism indicates that both atria are morphologically like a left atrium. This is associated with polysplenia and bilateral bilobed lungs. The term right-sided isomerism implies that both atria are morphologically like a right atrium. This is associated with asplenia and bilateral trilobed lungs.

The sidedness of lungs is best determined by the relationship of the pulmonary arteries to their respective bronchi. Normally, for the morphologic right lung, the pulmonary artery travels anterior to the upper and intermediate bronchi. For the morphologic left lung, the left pulmonary artery courses over the main bronchus and posterior to the upper lobe bronchus. Also, the mainstem bronchus of the morphologically left lung is 1.5–2 times longer than the mainstem bronchus of the right

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lung. This frequently can be appreciated on a chest radiogram. With bilateral right or bilateral left sidedness, the mainstem bronchi will be equal in length.

Situs inversus totalis means that there is complete (mirror-image) reversal of both thoracic and abdominal organs. It is uncommon for situs inversus totalis to be associated with congenital heart defects. However, essentially all other forms of situs abnormalities usually are associated with significant, and usually quite complex, forms of congenital heart defects.

In the past, it was thought that certain cardiac defects were associated with asplenia ("right isomerism") and other defects were associated with polysplenia ("left isomerism"). In general, asplenia was associated with anomalies of systemic and pulmonary venous return such as bilateral superior vena cavae, bilateral hepatic venous connections, complete atrioventricular septal defects (about 2/3 of cases), transposition of the great arteries, double outlet right ventricle, pulmonary stenosis and pulmonary atresia. The risk of overwhelming infection is increased in the absence of a spleen.

Polysplenia was associated with dextrocardia, atrial situs ambiguus, ventricular inversion, and VA concordance with left posterior aorta, partial anomalous pulmonary venous return, atrioventricular septal defects (about 1/3 of cases), and interruption of the inferior vena cava with azygos continuation to the superior vena cava. Although there may be segregation of certain defects toward either asplenia or polysplenia, there is considerable crossover.

As one can appreciate from the forgoing discussion, cardiac malformations associated with abnormal situs are protean. Rather than discussing the pathologic physiology, clinical presentation, physical examination, echocardiographic and cardiac catheterization issues, and treatment and outcome of this wide range of defects in this chapter, the reader is referred to the chapters in which the specific defects are discussed.

Human Genetics of Defects of Situs

38

Andreas Perrot and Silke Rickert-Sperling

Contents

Abstract

Defects of situs are associated with complex sets of congenital heart defects in which the normal concordance of asymmetric thoracic and abdominal organs is disturbed. The cellular and molecular mechanisms underlying the formation of the embryonic left-right axis have been investigated extensively in the past decade. This has led to the identification of mutations in at least 24 different genes in humans with heterotaxy and situs defects. Those mutations affect a broad range of molecular components, from transcription factors, signaling molecules, and chromatin modifiers to ciliary proteins. A substantial overlap of these genes is observed with genes associated with other congenital heart diseases such as tetralogy of

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Fallot and double outlet right ventricle, d-transposition of the great arteries, and atrioventricular septal defects. In this chapter, we present the broad genetic heterogeneity of situs defects including recent human genomics efforts.

38.1 Introduction

Situs inversus is found in about 0.01 % of the population, or about 1 person in 10,000. In the most common situation, the relationship between the organs is unchanged, and most people with situs inversus have no medical symptoms or complications [1]. In cases of situs ambiguus, the arrangement of the thoracoabdominal organs across the left-right (LR) axis of the body is variable. Frequently, the term heterotaxy is used synonymously for situs ambiguus. This broad term includes patients with a wide variety of very complex cardiac lesions. A right-sided heart position is called dextroposition. If the apex of the heart points to the right, it is called dextrocardia.

Heterotaxy shows a high 79 % recurrence risk ratio among first-degree relatives, which underlines a strong genetic basis of the disease [2]. Recent studies concerning its etiology have yielded new insights into the genetic architecture and will be summarized here [3]. A number of specific gene studies in patients were based on insights obtained by the study of left-right patterning in the early embryo of model organisms. However, multiple genetic variations, RNA and protein expression, and the interaction of these molecules impacts on the ultimate phenotype. Furthermore, this individual-specific mix of genes and expression patterns collides with the environment (teratogens, maternal exposures, and infectious agents; see Chap. 16). We will focus on the genetic causes and provide insight into the extensive genetic heterogeneity of situs defects.

38.2 Copy Number Variations

Copy number variations (CNVs) are sub-chromosomal changes in genome structure and generally comprise structural variants of intermediate size that range from about 1000 to 5×10^6 bases of DNA [3, 4]. These deletions, duplications, and inversions lead to gain or loss of chromosomal segments often containing multiple genes. Recent studies have identified multiple CNVs that contribute to non-syndromic CHD [3].

Fakhro et al. analyzed more than 250 patients with heterotaxy with high-resolution genotyping and estimated from these data copy number frequencies. They found 45 CNVs in 39 cases of which five where further assessed and confirmed by Morpholino knockdown in *Xenopus tropicalis*, the clawed frog [5]. Knockdown of these genes, *NEK2* (NIMA-related kinase 2), *ROCK2* (Rho-associated, coiled-coil containing protein kinase 2), *TGFBR2* (transforming growth factor, beta receptor II), *GALNT11* (polypeptide N-acetylgalactosaminyltransferase 11), and *NUP188* (nucleoporin 188 kDa), leads to strongly disrupted morphological

LR development and altered expression of Pitx2 (paired-like homeodomain 2), a molecular marker of LR patterning [5].

Glessner et al. studied several hundred CHD trios with two complementary high-resolution techniques, single nucleotide polymorphism (SNP) arrays and whole exome sequencing [6]. A significant increase in CNV burden was observed when comparing CHD trios with healthy trios. Rare *de novo* CNVs were identified in 51 probands, among them three patients with heterotaxy. The CNVs of these patients were located on chromosomes 6q21.2, 17p11.2, and 22q13.2, and size ranged between 5 and 680 kilobases. They affected genes such as *CDKN1A* (cyclin-dependent kinase inhibitor 1A), *FAM27L* (family with sequence similarity 27-like), and *CYP2D6* (cytochrome P450, family 2, subfamily D, polypeptide 6), respectively [6].

38.3 Single Gene Defects

A number of different genes have been implicated in situs defects indicating that there is an extensive genetic heterogeneity. To date, 24 genes have been shown to be involved in situs abnormalities; but this number should be seen as preliminary and is likely to rise with future genetic studies. Genes playing a role in heterotaxy frequently encode cardiac transcription factors, components of signaling pathways, or histone-modifying proteins, along with other proteins (Table 38.1 lists all known genes).

Table 38.1 Genes where mutations in patients with heterotaxy and situs defects have been described

Gene	Protein function	Phenotype	Status	References
Transcription factors (TF)				
NKX2-5	Homeobox TF	Heterotaxy, situs inversus	Two or more independent reports	[7, 8]
GATA4	GATA binding TF	Dextrocardia	Single case report	[9]
ZIC3	Zink finger TF	Heterotaxy, situs ambiguus, situs inversus, dextrocardia	Two or more independent reports	[10–12]
Genes involved in signaling pathways				
ACVR2B	Activin receptor (TGFbeta family)	Heterotaxy, dextrocardia	Two or more patients	[13]
GDF1	Ligand (TGFbeta family)	Situs inversus, situs ambiguus, dextrocardia	Two or more patients	[14]
CFC1	Ligand (EGF-CFC family)	Heterotaxy, dextrocardia	Two or more independent reports	[15–17]
LEFTY A	Ligand (TGFbeta family)	Dextrocardia	Two or more patients	[18]

(continued)

Table 38.1 (continued)

Gene	Protein function	Phenotype	Status	References
NODAL	Ligand (TGFbeta family)	Dextrocardia, situs inversus	Two or more patients	[19]
Histone-modifying genes				
RNF20	Histone-modifying gene	Heterotaxy, dextrocardia	Single case	[20]
SMAD2	Histone-modifying gene	Heterotaxy, dextrocardia	Two or more patients	[20]
MED20	Histone-modifying gene	Heterotaxy, dextrocardia	Single case report	[20]
NAA15	Histone-modifying gene	Heterotaxy, dextrocardia	Single case report	[20]
Ciliary genes				
DNAI1	Dynein arm	Situs inversus	Two or more independent reports	[21, 22]
DNAH5	Dynein arm	Situs inversus	Two or more patients	[23]
NPHP2	Ciliary gene	Situs inversus	Two or more patients	[23]
NPHP3	Ciliary gene	Situs inversus	Two or more patients	[24]
NPHP4	Ciliary gene	Dextrocardia, situs inversus	Two or more patients	[25]
CCDC11	Ciliary gene	Situs inversus	Single case report	[26]
Other genes				
PKD2	Polycystin 2	Dextrocardia, situs inversus	Two or more independent reports	[27, 28]
SHROOM3	Cytoskeletal protein	Dextrocardia, situs inversus	Two or more patients	[29]
CRELD1	Cell adhesion protein	Heterotaxy	Two or more patients	[30]
MKRN2	E3 ubiquitin ligase	Situs inversus	Single case report	[20]
OBSCN	Sarcomeric protein	Heterotaxy, dextrocardia	Single case report	[20]
UMODL1	Urinary protein	Heterotaxy, dextrocardia	Single case report	[20]

38.3.1 Cardiac Transcription Factors

The expression of cardiac transcription factors occurs in highly specified temporal-spatial patterns throughout development [3] (see Chap. 12). Transcription factors orchestrate heart development, and many of them are associated with isolated CHD [3, 31] (see, e.g., Chap. 30). Transcriptional focal points include NK2 homeobox 5

(*NKX2-5*) and GATA binding protein 4 (*GATA 4*) which are known to be involved in situs defects.

Watanabe et al. identified a deletion frameshift mutation in *NKX2-5* in a familiar CHD case with heterotaxy as well as atrial septal defect (ASD) [7]. Izumi et al. described a further deletion frameshift mutation in *NKX2-5* (along with three other variants of unknown significance in genes associated with ciliary disease; see Sect. 38.3.4) in a patient with complex CHD including heterotaxy using clinical exome sequencing [8]. In *GATA4*, a frameshift mutation was identified by Hirayama-Yamada et al. in a family with multiple ASD cases of which one showed additional dextrocardia [9]. However, affection of other contributing genes might be likely in this case.

The X-linked form of heterotaxy is caused by mutations in the zinc finger transcription factor *ZIC3* (Zic family member 3) and affects approximately 1 % of sporadic heterotaxy cases [1]. Mutations often cause loss of function and, in some cases, result in abnormal subcellular localization and trafficking [1]. *ZIC3* was the first gene unequivocally associated with human situs abnormalities [32, 33]. To date, a number of different *ZIC3* point mutations (missense, nonsense, and frameshift) have been described in X-linked familial heterotaxy cases as well as in sporadic heterotaxy and isolated CHD cases [10–12, 32, 34]. Patients from these studies show the whole spectrum of situs defects such as situs inversus, heterotaxy (situs ambiguus including asplenia and polysplenia), and dextrocardia. In summary, *ZIC3* is the most frequent disease gene for laterality defects.

38.3.2 Genes Involved in TGFβ Signaling Pathways

Heart development involves coordination of a number of signaling pathways [31]. Several mutations in signaling molecules have been detected in different forms of CHD associated with situs defects.

Activins and their receptors are members of the transforming growth factor beta (TGFβ) family of signaling molecules. Two missense mutations in *ACVR2B* encoding the activin receptor type II B were found in three patients showing heterotaxy and complex CHD [13]. Kaasinen et al. described a family with right atrial isomerism associated with situs inversus, situs ambiguus, dextrocardia, and asplenia showing mutations in the growth differentiation factor 1 (*GDF1*) [14]. Two truncating mutations of *GDF1* were observed to segregate with the phenotype in an autosomal recessive manner [14]. Of note, this study also identified 11 carriers of heterozygous truncating mutations in *GDF1* in control subjects without CHD indicating a high frequency and compatibility with normal development and health.

CFC1 encoding CRYPTIC protein is a member of the EGF (epidermal growth factor)-CFC (Cripto, Frl1, and Cryptic) family encoding extracellular proteins important for intercellular signaling pathways during vertebrate embryogenesis. Bamford et al. were the first to describe loss-of-function mutations in human *CFC1* in patients with heterotaxic phenotypes [15]. They identified nine patients carrying four different missense mutations and one deletion with various forms of

heterotaxy associated with CHDs such as d-transposition of the great arteries (d-TGA), ventricular septal defect (VSD), and ASD; five of the patients showed dextrocardia. The mutant proteins had aberrant cellular localization in transfected cells and showed functional effects in a zebrafish model [15]. Selamet Tierney et al. found three non-synonymous variants in *CFC1* in patients with laterality defects and CHD and suggested that these may act as susceptibility alleles in conjunction with other genes and/or environmental factors [16]. Further, Roessler et al. screened *CFC1* in a cohort of 251 patents with laterality defects and identified two mutations [17].

A further member of the TGFβ family, the left-right determination factor 2 (*LEFTY2*, also known as *LEFTY A*), was shown to be mutated in heterotaxy patients and is well known for its role in left-right patterning during mouse development [18]. Kosaki et al. found one nonsense and one missense mutation in *LEFTY A* in two patients with LR-axis malformations and CHD [18]. However, they stated that the *LEFTY A* mutant alleles may be necessary, but not sufficient, to give an LR phenotype in these affected individuals, because each mutation was found to be carried by one of the parents [18].

Analysis of a cohort of 269 patients with heterotaxy and/or isolated cardiovascular malformations revealed four different missense mutations in *NODAL* (Nodal growth differentiation factor) [19]. *NODAL* mutations were found in 14 unrelated subjects consisting of one in-frame insertion/deletion and two conserved splice site mutations. About one third of these patients showed dextrocardia and situs inversus (as well as asplenia in some cases) associated with a wide spectrum of CHD including pulmonary atresia, d-TGA, ASD, and VSD.

38.3.3 Histone-Modifying Genes

Histone proteins package chromosomal DNA into structural units called nucleosomes. They act as "spools" around which DNA winds and play an important role in gene regulation. Histone-modifying proteins have been identified for a number of distinct forms of modifications such as acetylation and methylation/demethylation.

Zaidi et al. used exome sequencing in parent-offspring trios to determine and compare the incidence of *de novo* mutations in 362 severe CHD cases (including heterotaxy) and 264 controls [20]. They found an excess of *de novo* mutations in genes involved in the production, removal, and reading of histone 3 lysine 4 (H3K4) methylation, an activating chromatin mark, and H3K27 methylation, an inactivating chromatin mark [20]. In heterotaxy or dextrocardiac patients, they found mutations in *SMAD2* (SMAD family member 2), *MED20* encoding mediator complex subunit 20, and *RNF20* encoding RING finger protein 20, an E3 ubiquitin ligase that regulates chromosome structure by monoubiquitinating histone H2B. The latter case showed additional asplenia as also seen in a further case with a frameshift mutation in *NAA15* (N-alpha-acetyltransferase 15, NatA auxiliary subunit) [20].

38.3.4 Ciliary Genes

There are two general types of cilia, namely, primary cilia serving as sensory organelles and motile cilia exerting mechanical force. Motile cilia are critical to the development of proper organ laterality and represent a central pathway disturbed in heterotaxy [35]. Primary ciliary dyskinesia (PCD) is one of the most widely recognized ciliopathies and a genetically heterogeneous disorder resulting from loss of function of different parts of the primary ciliary apparatus, often components of the dynein motor complex [1] (see Chap. 39). In cases where dextrocardia, situs ambiguus, or situs inversus are present in the infant, PCD should be considered as a highly possible diagnosis [36]. To date, PCD-causative mutations in 29 genes are known, and the number of causative genes is bound to rise (as reviewed by Kurkowiak et al. [36]). In this section, we will present a selection of important PCD genes associated with situs defects.

Kartagener syndrome is characterized by the combination of PCD and situs inversus and occurs in approximately 20 % of patients with PCD [35]. The absence of normal ciliary movement results in a lack of definitive patterning; thus whether the viscera take up the normal or reversed left-right position during embryogenesis is driven by stochasticity [1]. The syndrome is caused by mutations in *DNAI1* encoding axonemal dynein intermediate chain 1, an outer dynein arm component that is essential for ciliary function at the node [21]. Guichard et al. identified compound heterozygous DNAI1 gene defects in three independent patients from a cohort of 34 patients with Kartagener syndrome [21]. A further study in more than 300 PCD patients showed that half of them had situs inversus and at least 6 % of patients with PCD have heterotaxy, and most of those have CHDs such as systemic vein abnormalities, aortic coarctation, atrioventricular septal defect (AVSD), and double outlet right ventricle [22]. Further, the patients carried more mutations in ciliary outer dynein arm genes (*DNAI1* and *DNAH5* encoding axonemal dynein heavy chain 5) as compared with PCD patients with situs solitus [22].

Nephronophthisis (NPHP), an autosomal recessive cystic kidney disease, belongs to the ciliopathies and is characterized by cilia-related defects. Mutations in *nephronophthisis-2 (NPHP2* also named *INVS* for inversin) can result in NPHP with situs inversus and mild cardiac defects [23]. Bergmann et al. showed that *NPHP3* (also named nephrocystin-3) mutations can cause a broad clinical spectrum of early embryonic patterning defects comprising situs inversus and structural heart defects as well as kidney disease [24]. Interestingly, a genome-wide linkage analysis identified *nephronophthisis-4 (NPHP4)* mutations in patients with cardiac laterality defects but without NPHP from a consanguineous family [25]. Further sequencing of this gene identified eight additional missense mutations in a cohort of unrelated patients with dextrocardia, situs inversus, and asplenia/polysplenia [25].

Performing homozygosity mapping in a consanguineous family with laterality defects, Perles et al. identified a homozygous splice site mutation in *CCDC11* encoding coiled-coil domain containing 11 protein which is preferentially expressed in ciliated cells [26]. The patient carrying the mutation was characterized by situs inversus and severe cardiac malformations [26].

38.3.5 Other Genes

PKD2, encoding polycystin 2 and belonging to the superfamily of transient receptor potential channels, causes autosomal dominant polycystic kidney disease. Bataille et al. reported an association of that disease resulting from *PKD2* mutations with left-right laterality defects [27]. One large gene deletion, one single-exon duplication, and one in-frame duplication, respectively, were found in three unrelated patients characterized by dextrocardia or situs inversus in addition to kidney disease [27]. Another patient with complete situs inversus and autosomal dominant polycystic kidney disease caused by a *PKD2* missense mutation (leading to a premature truncation of polycystin 2) was identified by Oka et al. [28].

CRELD1 encoding the cell adhesion molecule cysteine-rich with EGF-like domains 1 was analyzed in a cohort of 11 patients with AVSD and heterotaxy [30]. One missense mutation was detected in a patient characterized by dextrocardia, right ventricle aorta with pulmonary atresia, and a right aortic arch in addition to partial AVSD [30]. However, Robinson et al. suggested that *CRELD1* mutations might increase the risk of developing a heart defect, rather than being directly causative [30].

Using whole exome sequencing, Tariq et al. found mutations in *SHROOM3* (Shroom family member 3), an actin-binding protein, which is responsible for early cell shape during morphogenesis through a myosin II-dependent pathway [29]. They first identified a mutation in a patient with complex heterotaxy phenotype (including dextrocardia and situs inversus) by exome sequencing and subsequently analyzed a cohort of sporadic heterotaxy patients in *SHROOM3* leading to the identification of further two mutations that were predicted to be pathogenic [29].

Besides alterations in histone-modifying enzymes, the study of Zaidi et al. (see Sect. 38.3.3) identified three *de novo* mutations in *MKRN2* (encoding makorin RING finger protein 2, a probable E3 ubiquitin ligase), in *OBSCN* (encoding obscurin, a giant sarcomeric protein important for myofibrillogenesis), and in *UMODL1* (encoding uromodulin-like 1, a protein similar to uromodulin which is associated with various kidney diseases). Interestingly, Zaidi et al. also described a second *de novo* missense mutation in all of the three genes in a CHD patient without heterotaxy or situs defect [20].

Conclusion

As in other forms of congenital heart disease, defects of the situs and heterotaxy have a complex genetic etiology. The findings from the mentioned studies are entirely consistent with a complex, oligogenic disease model. One can speculate that rare heterozygous mutations identified in the sporadic cases have probably an epistatic effect with additional genetic modifiers within other developmental pathways. Even in consanguineous families, there may exist other genetic variants that lead to the phenotype [25, 26]. Further, the observed variety in the phenotype in combination with ciliopathies and kidney disease as well as other CHDs supports this notion.

Point mutations in at least 24 different genes have been described in patients with heterotaxy and situs defects (see Table 38.1). There is a wide genetic heterogeneity including genes encoding for transcription factors, signaling molecules, ciliary

proteins, histone-modifying proteins, and others. There is no doubt that additional genes will be identified based on the technical advances [1, 3, 35]. A substantial overlap of situs genes is observed with genes associated with other CHDs such as tetralogy of Fallot and double outlet right ventricle (see Chap. 32), d-transposition of the great arteries (see Chap. 35), and atrioventricular septal defects (see Chap. 26). To date, there are only a few data pointing to an important role of sub-chromosomal changes such as copy number variation in the etiology of situs defects.

Although there is a strong genetic contribution to situs defects, the majority of situs defects are idiopathic indicating the need for better utilization of novel approaches such as whole exome/genome sequencing to find the genetic variations contributing to this severe congenital heart disease.

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Molecular Pathways and Animal Models of Defects of Situs

39

Nikolai T. Klena, George C. Gabriel, and Cecilia W. Lo

Contents

Abstract

Left-right patterning is among the least well understood of the three axes defining the body plan, and yet it is of no less importance, with left-right patterning defects causing structural birth defects with high morbidity and mortality, such as in complex congenital heart disease, biliary atresia, or intestinal malrotation. The cell signaling pathways that govern left-right asymmetry are highly conserved and involved multiple components of the transforming growth factor beta (TGFβ) superfamily of cell signaling molecules. Central to left-right patterning is the differential activation of Nodal on the left and bone morphoge-

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netic protein (BMP) signaling on the right. In addition, a plethora of other cell signaling pathways including sonic hedgehog (Shh), fibroblast growth factor (FGF), and Notch also contribute to the regulation of left-right patterning. In vertebrate embryos such as the mouse, frog, or zebrafish, the specification of left-right identity requires the left-right organizer (LRO) containing cells with motile and primary cilia. Cilia-generated flow plays an important role in the left-sided propagation of Nodal signaling. Ultimately, it is the left-sided expression of the transcription factor paired-like homeodomain 2 (Pitx2) that drives visceral organ asymmetry. Interestingly, while this overall scheme for left-right patterning is well conserved evolutionarily, are striking differences that suggests caution in broadly generalizing conclusions on the molecular pathways regulating left-right patterning.

39.1 Introduction

The developmental regulation of anteroposterior and dorsoventral body axes are well described, but the mechanisms specifying left-right asymmetry is much less understood, but no less important [1, 2]. The establishment of left-right (L-R) asymmetry in the embryo is essential for developmental patterning of normal visceral organ asymmetry, referred to as situs solitus (Fig. 39.1). This is ultimately required for proper functioning of visceral organs in the body. Laterality defects can involve either complete mirror symmetric positioning of all visceral organs, referred to as situs inversus, or the randomized left-right patterning of visceral organ situs, referred to as heterotaxy (Fig. 39.1). Individuals with situs inversus generally have no clinical presentations and often are not discovered until a clinic visit uncovers abnormal right-sided position of the heart, also known as dextrocardia. However, patients with heterotaxy can present with life-threatening birth defects, especially those associated with the cardiovascular system [4].

Patients with heterotaxy usually have complex structural heart defects with anomalous left-right atrioventricular and ventriculoarterial connections that disrupts systemic vs. pulmonary circulation. This causes high morbidity and mortality, as it prevents efficient oxygenation of blood from air in the lung. Left-right patterning also can disrupt development of the biliary duct causing biliary atresia [5]. In the absence of a functional bile duct, bile backs into the liver and causes liver cirrhosis, one of the most common causes for pediatric liver transplantation. Left-right patterning defects also can cause malrotation of the intestine, with bowel obstruction arising with formation of a volvulus where a loop of the intestine is twisted along the mesentery, a life-threatening condition that requires surgical remediation [6]. Together, these findings show disruptions in left-right patterning have important clinical ramifications, and only with more mechanistic insights into the developmental regulation of left-right patterning can we hope to improve the management of heterotaxy patient care.

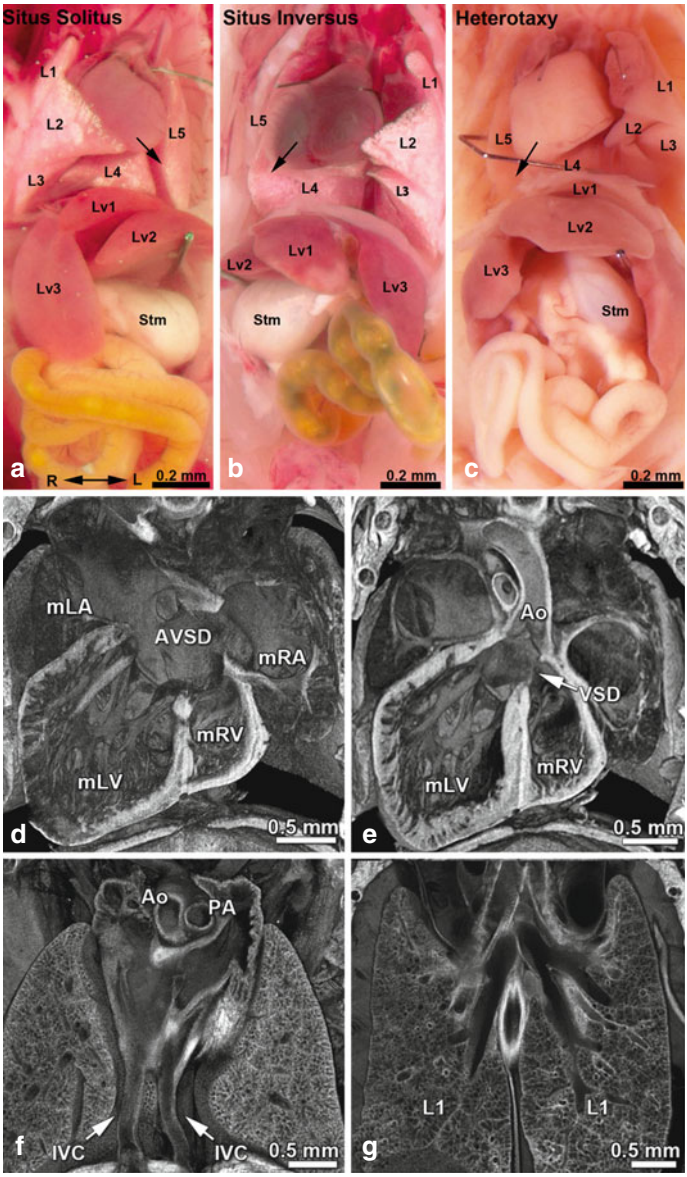

Fig. 39.1 Laterality in mutant mouse models. *Ap1b1m/m* mutants can exhibit a spectrum of laterality phenotypes spanning from normal situs solitus (**a**), to complete mirror symmetric situs inversus (**b**), or randomized visceral organ situs referred to as heterotaxy (**c**). In situs solitus, the heart apex (*arrow*) points to the left (levocardia), four lung lobes are on the right and one on the left, stomach is to the left, and the dominant liver lobe is on the right. With situs inversus, complete mirror reversal of organ situs is observed with both the heart and stomach on the right, while heterotaxy involves randomized visceral organ situs such as dextrocardia with levogastria shown in (**c**). The heterotaxy mutant in **c** exhibits complex CHD with atrioventricular septal defect (AVSD) (**d**), ventricular septal defect (VSD) (**e**), duplicated inferior vena cava (IVC) (**f**) and left pulmonary isomerism with bilateral single lung lobes (**g**). *Ao* aorta, *L1–5* lung lobes 1–5, *Lv1–3* live lobes 1–3, *mLA* morphologic left atrium, *mLV* morphologic left ventricle, *mRA* morphologic right atrium, *mRV* morphologic right ventricle, *PA* pulmonary artery, *Stm* stomach (From Li et al. [3])

39.2 Nodal and TGFβ Signaling in the Embryonic Node

Studies using different model organisms have identified multiple evolutionarily conserved signaling pathways contributing to the specification of left-right asymmetry. In many vertebrate embryos, the specification of left-right patterning involves a specialized tissue referred to as the L-R organizer, also known as the embryonic node in mice (Fig. 39.2), Kupffer's vesicle in zebrafish, the gastrocoel roof plate (GRP) in *Xenopus*, or Hensen's node in the chick. The asymmetric expression of genes in the embryonic node provides some of the earliest molecular signatures showing the evolving specification of visceral organ asymmetry (Fig. 39.3). One such gene, *Nodal*, a transforming growth factor beta (TGFβ) family member, is initially expressed bilaterally symmetric in the perinodal crown cells, but as development progresses, expression becomes elevated on the left side of the node (Fig. 39.3).

This left dominant expression of *Nodal* is propagated to the left lateral plate mesoderm (LPM) (Fig. 39.3), causing the induction of *Lefty2 (*left-right determination factor 2), another TGFβ family member, and also the transcription factor *Pitx2*,

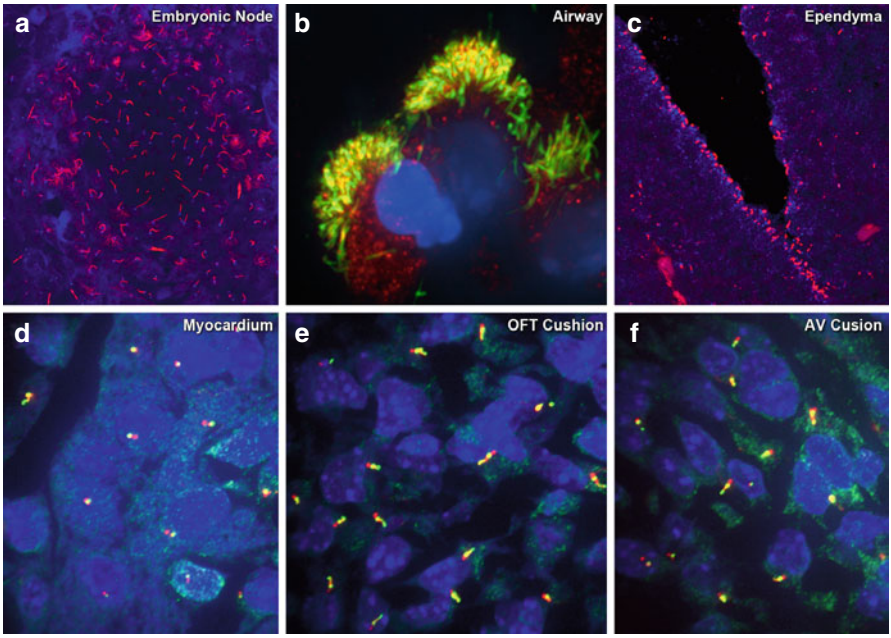

Fig. 39.2 Motile and primary cilia in different tissues of the mouse embryo. (**a**) Immunostaining with antibodies to acetylated tubulin (*red*) and γ-tubulin (*blue*) was used to visualize motile cilia in the E7.75 mouse embryonic node. (**b**–**f**) Immunostaining with antibodies to acetylated tubulin and IFT88 visualized cilia in the newborn mouse tracheal airway epithelia (**b**), in E12.5 brain ependyma (**c**), and primary cilia in the myocardium (**d**), outflow tract cushion (**e**), and atrioventricular cushion (**f**) of the E12.5 mouse embryonic heart

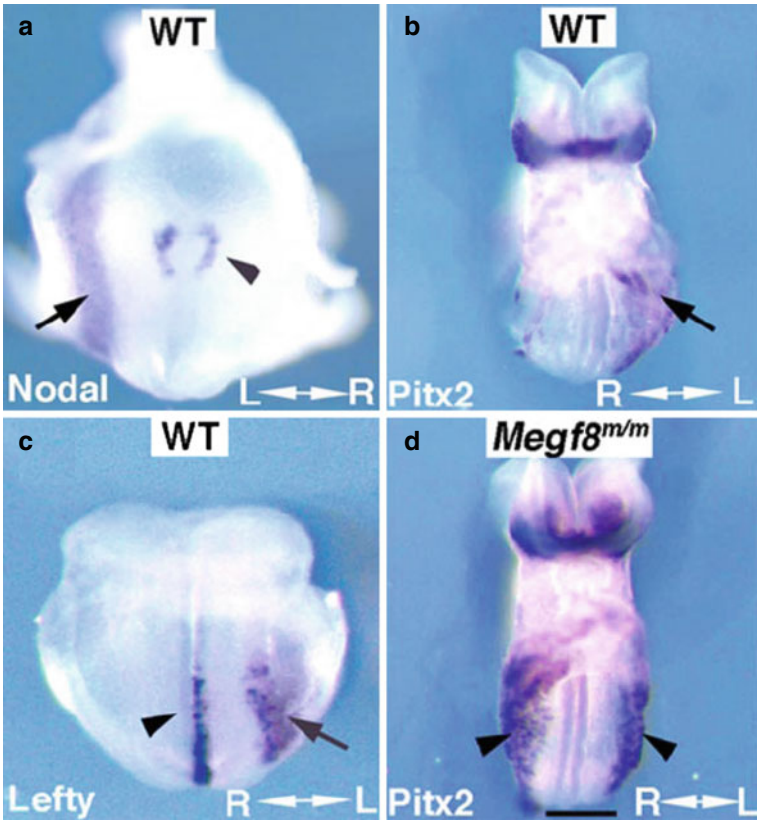

Fig. 39.3 Left-sided expression of left-determinant genes in mouse embryos. In situ hybridization analysis showed (**a**, **b**) left-sided expression of *Nodal* (**a**), Lefty (**b**), and Pitx2 (**c**) in wild-type embryos. Note Nodal expression is reduced in the right (see *arrowhead*) vs. the opposing left side of the node (**a**). Lefty panel (**b**) shows expression Lefty1 expression (*arrowhead*) on the left side of the floor plate, while Lefty2 expression (*arrow*) is seen in the left LPM. PItx2 expression was observed in the left LPM and bilaterally in the head fold (**c**). In contrast, embryo with *Megf8m/m* mutation known to cause laterality defects (**d**) shows bilateral Pitx2 expression in the right and left LPM (From Zhang et al. [7])

the master regulator of left-sided tissue morphogenesis. Lefty2 is a diffusible competitive inhibitor of Nodal, and being monomeric, it diffuses more rapidly than the functionally active dimeric Nodal, allowing it to repress Nodal signaling in the right LPM. Additionally, expression of the related family member, *Lefty 1*, along the axial midline, provides a barrier function that further ensures Nodal signaling is restricted to the embryo's right side. Ultimately, it is the Nodal-activated expression of *Pitx2* (paired-like homeodomain 2) on the embryo's left side that drives left-sided visceral organ morphogenesis.

39.3 Complex Regulation of Nodal

Nodal plays an important role not only in left-right patterning but also in specification of the anterior-posterior axis, indicating the integration of left-right patterning with anterior-posterior axis specification. The winged-helix transcription factor *FoxH1* (forkhead box H1) acts upstream of Nodal (Fig. 39.4), with *FoxH1*-deficient mice exhibiting defects including anterior-posterior patterning defects, and failure of the node to form, phenotypes recapitulated by the loss of Nodal signaling [8]. *Nodal* and *FoxH1* also show overlapping expression in the node [9], and expression of *FoxH1* can induce *Lefty2* expression asymmetrically in both the *Xenopus* and mouse embryos [9, 10]. Nodal expression at the node is also subject to regulation by Notch signaling [11]. This is indicated by the finding of several binding sites for RBP-J (recombination signal binding protein for immunoglobulin kappa J region),

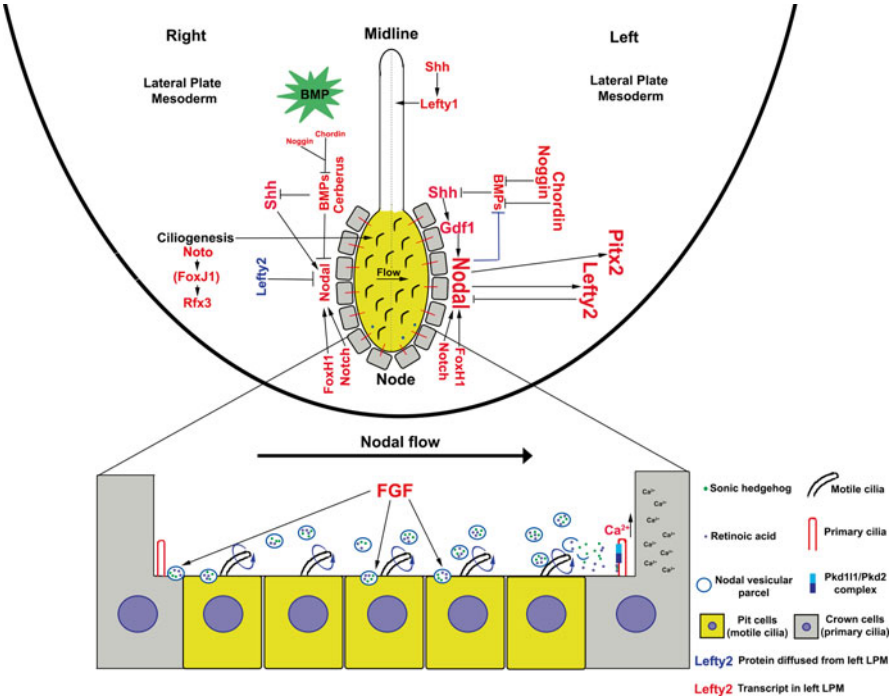

Fig. 39.4 Signaling pathways specifying left-right asymmetry. Schematic summary of cell signaling pathways contributing to the specification of laterality based on work in the mouse model. Left-right patterning requires cilia in the embryonic node. Indicated are pit cells (*yellow*) in the embryonic node with motile cilia driving unidirectional fluid flow. This causes mechanosensory transduction of primary cilia in the left perinodal crown cells and the elevation of calcium. A cascade of molecular signals help to propagate Nodal signaling on the embryo's left side, defining left identity. In contrast, Nodal signaling is repressed on the embryo's right and, together with high levels of BMP signaling, help define right identity. Unlike *Lefty2, Bmp* expression is bilaterally symmetric, but the green star serves to indicate high level of BMP signaling only on the right

the main transcription factor mediating Notch signaling, in the Nodal enhancer. In addition, mouse mutant homozygote for the Notch ligand *Dll1* or doubly mutant for *Notch1/Notch2* [11] exhibit left-right patterning defects.

Nodal is also subject to positive and negative regulation by other TGFβ family members, with GDF1 (growth differentiation factor 1) being an upstream positive regulator highly expressed in the node, acting to activate and propagate the Nodal signaling cascade on the embryo's left side. Thus, *Gdf1* knockout mouse embryos exhibit left-right patterning defects, such as situs inversus totalis or heterotaxy with congenital heart defects and right pulmonary isomerism [12]. This is associated with absence of *Lefty1* and *2* expression and failure in Nodal propagation to the left LPM.

Nodal expression mediating left-right patterning is also subject to negative regulation by various components of the TGFβ signaling pathways. In mouse embryos, *Cerberus-2 (Cerl2)*, also known as *Dand5* (DAN domain family member 5, BMP antagonist), or *Coco* in the *Xenopus* embryo, a TGFβ antagonist, is expressed on the embryo's right side, serving to repress *Nodal* expression on the right. However, beyond the 3-somite stage, Cerl2 protein shows nodal flow-dependent accumulation on the embryo's left side, while *Cerl2* transcript expression remains right-sided. This translocation of Cerl2 protein to the embryo's left side at later stages of development may serve to shut down Nodal expression on the left as the left-right axis is established [13].

39.4 Bone Morphogenetic Protein Regulation of Nodal Signaling and Right-Sided Identity

BMP (bone morphogenetic protein) signaling is also observed to play an essential role in left-right patterning, exerting both positive and negative regulation on Nodal signaling. While BMP-4 is required for left-right patterning, its expression is actually bilaterally symmetric. This apparent discordance is explained by the higher left-sided expression of the BMP antagonists, *Noggin (Nog)* and *Chordin (Chrd)*, which represses BMP signaling on the embryo's left side, allowing left-sided Nodal propagation in the LPM [14, 15]. The essential role of BMP signaling in left-right patterning is further demonstrated by the finding that deficiency in BMP type II or type I receptors, *Acvr (activin A receptor) IIb* [16] and *Acvr1* [17], respectively, causes left-right patterning defects. While these and other studies clearly show BMP signaling playing important roles in restricting Nodal activation to the right LPM, its right-sided activation may also help define right-sided identity.

39.5 Embryonic Node and Left-Right Patterning

The important role of Nodal signaling in patterning left-right visceral organ morphogenesis is unequivocal, but how the Nodal signaling cascade brings about the left-right asymmetric patterning of visceral organ morphogenesis is not well understood. The embryonic node has been the focal point of such studies. In the mouse

embryo, it is formed at the anterior tip of the primitive streak at the future posterior side of the embryo. While it is often said that breaking of left-right symmetry occurs with asymmetric activation of nodal signaling at the embryonic node, left-right symmetry is already established earlier. Thus with formation of an anterior-posterior and dorsoventral axes in the pregastrulating mouse embryo, a left-right axis is defined by default [18]. The node subsequently forms at the anterior tip of the primitive streak, playing a critical role in the differential propagation of the Nodal signaling cascade to specify left identity. This regulation involves motile cilia that exhibit clockwise rotation creating a leftward fluid flow. In addition, there are also nonmotile primary cilia in cells at the node periphery referred to as perinodal crown cells. These have been shown to have a mechanosensory function in flow sensing and may help relay calcium signaling to propagate Nodal signaling.

39.6 Motile Cilia in Left-Right Patterning

The requirement for motile cilia in left-right patterning is suggested by the finding of laterality defects in mutants with disruption of motile cilia function. Insights first came from observations of mutations in two genes encoding kinesins, *Kif3a* and *Kif3b* [19, 20]. Kinesins are molecular motors which traffic cargo along microtubule tracks and, importantly, are required for the assembly of cilia through intraflagellar transport. These kinesin mutant mice not only failed to form cilia and showed no nodal fluid flow, approximately 50 % displayed laterality defects.

In reality, the connection between motile cilia and abnormalities of the situs has long been known clinically through observations of patients with Kartagener syndrome [21], a condition in which patients can exhibit situs inversus totalis together with sinopulmonary disease and male infertility due to motile cilia dysfunction, a condition referred to as primary ciliary dyskinesia, or PCD (see Chap. 38). While the mechanism by which motile cilia defect caused laterality defects in PCD patients was unknown at the time, the requirement for motile cilia function in left-right patterning was clearly indicated by these findings.

Corroborating these clinical findings with PCD patients were observation of the *inversus viscerum (iv)* mutant mice. This mutant was later shown to harbor a mutation in the dynein gene, *Dnah (dynein, axonemal, heavy chain) 11*, a gene now demonstrated to be a common cause of PCD [22]. In *iv* mutant mice, nodal cilia are formed but are rendered immotile by the *Dnah11* deficiency, causing the disruption in left-right patterning. Such motile cilia defects underlie the sinopulmonary disease associated with PCD. Thus *Dnah11* is required for motile cilia function mediating mucus clearance in the airway and in left-right patterning in the embryonic node.

Since these early studies, many cilia-related genes have now been identified to cause PCD and laterality defects, including genes encoding other dyneins such as *DNAH5* [23, 24] and *DNAI1* [25, 26], as well as many other cilia-related genes [3]. These studies established a clear link between motile cilia defects and defects in left-right patterning. Two transcription factors, *FoxJ1* (forkhead box J1) and *Rfx3*

(regulatory factor X, 3, influences HLA class II expression), are observed to play important roles in ciliogenesis at the node, with *Foxj1* also referred to as a master regulator of ciliogenesis [27]. While *Foxj1* knockout mice and morpholino knockdown of *foxj1* show loss of cilia [28–30], ciliogenesis was not affected in a *Foxj1* mouse mutant harboring a missense mutation in the conserved DNA binding domain [3]. Cilia were observed both in the airway and in the embryonic node of this mutant, although ciliary motion was dyskinetic in the node. These findings suggest the intriguing possibility that *Foxj1* may also have a non-transcriptional role in the regulation of cilia function.

Exactly how motile cilia regulate left-right patterning is still not well understood. Currently there are two predominant models, one referred to as a "morphogen" hypothesis that suggests motile cilia play a role in transporting morphogen to the embryo's left side to specify left identity, while a second model referred to as the "two-cilia" hypothesis proposes motile cilia generate nodal flow propagates Nodal signaling via mechanosensory transduction of primary cilia in the perinodal crown cells. Below we briefly discuss evidence in support of these two models.

39.7 The "Morphogen" Hypothesis

The "morphogen" hypothesis proposes that cilia-generated flow in the node provides leftward transport of a secreted morphogen that establishes left identity [31]. This model builds on the observation of left ward transport of membrane vesicles up to 5 μm in size termed nodal vesicular parcels (NVPs) in the node. These NVPs, released from microvilli, are swept across the node by cilia-generated flow. Upon coming into contact with the rotating cilia, the NVPs undergo fragmentation, releasing smaller particles that accumulate along the left wall of the node. Significantly, these NVPs contain sonic hedgehog (SHH) and retinoic acid (RA), both known to play essential roles in left-right patterning (Fig. 39.4) [32, 33]. This left-sided concentration of morphogens from the NVPs is further proposed to trigger left-sided signal transduction mediated by sensory cilia in the perinodal crown cells, resulting in left-sided elevation of Ca^{2+}, and the propagation of *Nodal* expression, and asymmetric organ patterning.

Consistent with this NVP model, *Kif3a*-deficient embryos with no nodal cilia were observed to have significantly more NVPs [31]. Similarly, in *Dnah5* mutant embryos with paralyzed nodal cilia, we observed a similar accumulation of large vesicles, suggesting NVPs may fail to undergo fragmentation in *Dnah5* mutant embryos with paralyzed cilia (Fig. 39.5) [23]. Also of interest is our recent recovery of a mouse mutant harboring a mutation in an adaptin protein required for vesicular trafficking, *Ap1b1* (adaptor-related protein complex 1, beta 1 subunit). This mutant has laterality defects that precisely phenocopies those observed in the *Dnah5* mutants [3]. Given the known roles of AP1B1 in recycling cargo destined for the membrane, and in regulating posttranslational modifications of secreted proteins, this suggests the intriguing possibility of a possible role for *Ap1b1* in regulating NVP release at the node.

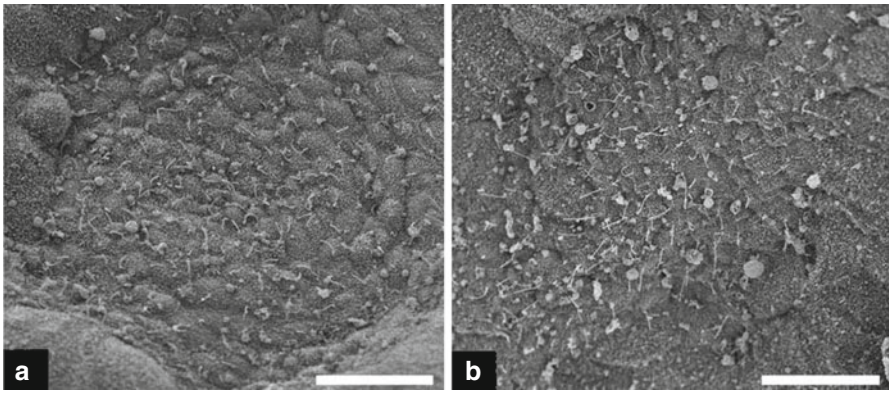

Fig. 39.5 Scanning electron microscopy (EM) images of mouse node. Scanning EM showing monociliated cells in the embryonic node of a wild-type (**a**) and Dnah5 mutant (**b**) mouse embryo. Note the markedly higher abundance of large vesicles that may correspond to an accumulation of NVPs in the *Dnah5* mutant node (From Tan et al. [23])

39.8 "Two-Cilia" Hypothesis

Another model proposed to account for the role of nodal flow in left-right patterning suggests motile and primary cilia are both involved in the specification of left-right patterning. This model proposes that primary cilia in the perinodal crown cells on the left side of the node undergo mechanosensory transduction to detect nodal flow, resulting in the elevation of Ca^{2+} to the left of the node. The notion that primary cilia may mediate flow sensing was first postulated with the finding of left-right patterning defects in polycystin-2 (*Pkd2*) mutant mice [34], an ion channel protein known to be associated with polycystic kidney disease. Pkd2 is localized in nodal cilia [35], and *Pkd2* mutant mice failed to show Ca^{2+} elevation with nodal flow, while the *iv Dnah11* mutant mice with immotile cilia displayed left-right randomized calcium elevation. Together these observations support a role for polycystin-2 in mediating Ca^{2+} signaling upstream of Nodal signaling.

As polycystin-2 is known to form a complex with polycsytin-1 encoded by *Pkd1* in the primary cilia of renal cells to initiate calcium signaling, it was surprising that *Pkd1* mutants were found not to have any left-right patterning defects [36]. However, subsequent studies showed *Pkd1* is actually not expressed in the node [36]. Instead, node expression was observed for a *Pkd1* paralogue, *Pkd1l1*, and *Pkd1l1* deficiency disrupted the propagation of Nodal signaling and resulted in left-right patterning defects [37]. PKD1L1 (polycystic kidney disease 1 like 1) was shown to bind PKD2, an interaction required for ciliary localization of both proteins [38]. Together these observations provide further evidence in support of the essential role of calcium signaling and the two-cilia hypothesis in left-right patterning.

39.9 Specification of Visceral Organ Asymmetry

The ultimate driver of left-right patterning of visceral organs is the left-sided expression of *Pitx2*. Loss of function in genes downstream of *Pitx2* in progenitor cells of the heart, lungs, liver, brain, and other tissues can cause regionalized laterality defects [39], confirming the importance of *Pitx2* in visceral organ patterning. It is presumed the downstream response to *Pitx2* expression help orchestrate patterning of visceral organ left-right asymmetry, but precisely how this is translated to visceral organ asymmetry is not well understood. *Pitx2* has been shown to regulate the left-right asymmetry of the heart through patterning myocardium derived from the second heart field [40]. A conditional knockout of *Pitx2* in the secondary heart field perturbed outflow tract expansion and caused various outflow tract anomalies [40]. Interestingly, a nodal-independent pathway for heart looping has been described in zebrafish. This involves actin polymerization and requires myosin II activity [41], with Nodal signaling shown to initiate actomyosin polymerization that drives dextral heart looping. However, actomyosin dynamics was shown to be sufficient in driving dextral looping even with loss of Nodal signaling. Conversely, Nodal signaling alone could not overcome the dextral heart looping defects caused by loss of actomyosin activity. These findings suggest orientation of actin polymerization, and the resulting tissue tension may drive heart looping, a mechanism similar to that proposed for cellular flow in establishing tissue polarity in the *Drosophila* wing [42].

Asymmetry of the developing gut is also regulated by *Pitx2* in conjunction with expression of the transcription factors *Isl1* (Islet1 or ISL LIM homeobox 1) on the left vs. *Tbx (T-box) 18* on the right side of the dorsal mesentery (DM) [43]. This transcriptional cascade results in left-sided condensation of cells in the DM, forming a trapezoidal shape that causes a leftward tilt that drives stereotypical looping of the primitive gut tube. This involves downstream activation of *Daam2* (dishevelled-associated activator of morphogenesis 2), a GTPase binding protein regulating the actin cytoskeleton, mediated through Pitx2 modulation of noncanonical Wnt signaling components Gpc3 (glypican 3) and Fzd (frizzled class receptor) [44]. Binding of activated Daam2 to α-catenin and N-cadherin modulates cell adhesion to promote cell condensation in the left DM, driving orderly L-R asymmetric looping of the gut [44].

Pitx2 also plays an important role in left-right asymmetric lung lobation. Typically there are three lung lobes on the right and two on the left in the mouse and humans. However, it should be noted the pattern of mouse lung lobation is often referred to as four on the left and one on the right, as the left lower lung lobe is actually connected to the right bronchus. With disruption of left-right patterning, lung lobation may exhibit left isomerism with a single or two lung lobes observed bilaterally or right isomerism with bilateral three or four lung lobes. Interestingly, knockout of all three isoforms of *Pitx2 (a, b, c)* causes right lung isomerism [45–47]. Deficiency in *FGF8*, which acts upstream of *Nodal*, also causes right pulmonary isomerism [48]. Mutation in *Mmp21*, an uncharacterized matrix metalloproteinase, is observed to heterotaxy with fully penetrant right pulmonary isomerism [3], indicating a role for the extracellular matrix in specification of lung asymmetry. The spleen is found

along the curvature of the stomach on the left side of the abdomen, but perturbations in Nodal signaling and left-specific asymmetry pathways can result in polysplenia with several smaller, punctate spleens, asplenia, or hypoplastic spleen [9].

An area much less studied is lateralization of the brain. In zebrafish, Nodal signaling has been shown to regulate the asymmetry of the epithalamus [49], a major division of the diencephalon that functions in photoreception and communication. Interestingly, morpholino knockdown of the *nodal*-related gene *southpaw* in zebrafish causes diencephalic and pancreatic left-right patterning defects in addition to disruption of cardiac looping [50], demonstrating coordination in the patterning of visceral organ and brain left-right asymmetry. Additionally, a clinical study using magnetic resonance imaging (MRI) showed individuals with situs inversus have reversed frontal and occipital petalia, while left hemisphere dominance for language and handedness remained unchanged [51]. Overall, there is a paucity of information on lateralization of the brain, making it difficult to ascertain its potential importance in neurobehavioral and neurocognitive function.

39.10 Conserved Yet Divergent Mechanisms Regulating Left-Right Patterning

The developmental pathways regulating left-right patterning are largely conserved in evolution, but there are some notable differences observed. Thus, the essential role of motile cilia-driven flow in left-right patterning has been well demonstrated in most vertebrates such as the mouse, zebrafish, or *Xenopus*, but unexpectedly, motile cilia do not appear to play a role in left-right patterning in the chick or pig embryos [52]. Furthermore, the "two-cilia hypothesis" does not appear to be applicable to zebrafish or Medaka fish, as only motile cilia are observed in Kupffer's vesicle [38].

The overall molecular mechanism governing L-R asymmetry with Nodal signaling is largely conserved, but some species specific differences have been noted downstream of *Nodal*. For example, the asymmetric expression of *activin-BB*, *activin receptor IIA*, and *Shh* in Hensen's node of the chick is not observed in the mouse, *Xenopus*, or zebrafish. In the chick embryo, *Shh* expression is asymmetrically expressed in the left LPM, a pattern not observed in the mouse or other vertebrate species. In chick embryos, *Shh* signaling is observed to mediate L-R asymmetry by inducing the Cer-like protein Caronte (Car) [53]. Car then activates *Nodal* expression by antagonizing Notch repression mediated by BMP signaling. This complex molecular cascade involving Shh, Caronte, BMP, and FGF8 appears to be unique to the chick embryo.

Also notable is the finding in *Xenopus*, of early specification of left-right asymmetries mediated by differential expression of H^+-/K^+-dependent ATPase transcript and protein at the two-cell stage [54]. Ablation of H^+- and K^+-dependent ATPase causes heterotaxy not only in *Xenopus* embryos but also in the zebrafish and chick embryos [55]. Its loss disrupts the normal pattern of asymmetric Shh expression in Hensen's node of the chick [54]. In *Xenopus* embryos, the asymmetric expression of

the H^+-/K^+-dependent ATPase together with gap junction-mediated cell-cell communication is proposed to specify asymmetric expression of the neurotransmitter serotonin in the 32-cell stage embryo. Its importance in left-right patterning is indicated by the finding of heterotaxy with loss of serotonin receptors R3 and R4 [56]. To date, the role of serotonin in the regulation of left-right patterning has not been studied in other species.

Conclusion

Left-right patterning is regulated by a conserved pathway involving a complex cascade of positive and negative regulation of Nodal signaling. This involves cross regulation by a plethora of other cell signaling pathways including Shh, Bmp, and Fgf. This culminates in the left-sided expression of *Pitx2* specifying left identity, while BMP signaling contributes to right identity. Both motile and primary cilia play important roles in laterality specification. This likely involves the downstream modulation of calcium signaling. The requirement for motile cilia in left-right patterning is not universal nor the mechanosensory transduction by primary cilia. As yet, precisely how *Pitx2* expression translates to a pattern of visceral organ asymmetry is not well understood, but regulation of cytoskeletal organization and cell migration may contribute to the patterning of tissue asymmetries.

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Part XI

Semilunar Valve and Aortic Arch Anomalies

Clinical Presentation and Therapy of Semilunar Valve and Aortic Arch Anomalies

40

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Contents

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40.1 Aortic Stenosis and Insufficiency

40.1.1 Pathologic Physiology

There are three types of congenital aortic stenosis. The most common is aortic valve stenosis and this comprises approximately 5 % of congenital heart defects. The aortic valve cusps or leaflets are congenitally malformed. They may be thickened and the raphe may be fused. Usually these valves are bicuspid.

One to two percent of the general population has a bicuspid aortic valve that may or may not be stenotic or insufficient. However, over time these valves may develop stenosis or insufficiency. In addition, even in the absence of stenosis or insufficiency, they can be associated with ascending aortic aneurysm formation.

The second type of aortic stenosis is supravalvar aortic stenosis. This involves a narrowing of the ascending aorta just above the sino-tubular junction. The third type of aortic stenosis is subvalvar aortic stenosis. In this condition, there is a fibromuscular ridge or tunnel that causes obstruction a short distance below the valve.

Aortic insufficiency frequently occurs in conjunction with aortic valve stenosis especially if the valve is bicuspid (Fig. 40.1). However, aortic insufficiency can occur because of a bicuspid aortic valve even in the absence of aortic stenosis. Aortic insufficiency frequently is a complication of balloon valvuloplasty or surgical aortic valvotomy.

40.1.2 Clinical Presentation

The clinical presentation of aortic valve stenosis depends upon the severity of the obstruction. The measure of severity of the obstruction is based upon the pressure drop across the valve during systole. In trivial aortic stenosis, the pressure difference is 0–25 mmHg; in mild stenosis it is 26–50 mmHg. In moderate stenosis, the pressure difference is 51–79 mmHg, while it is >80 mmHg in severe stenosis.

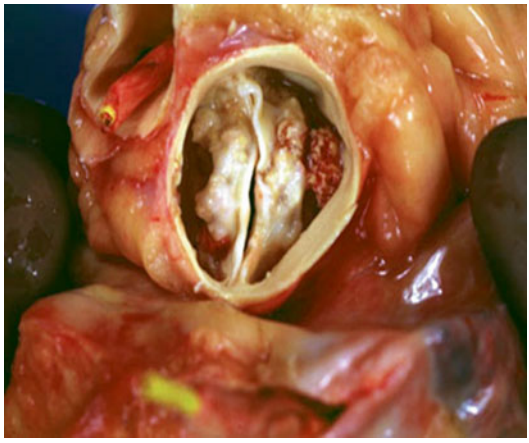

Fig. 40.1 Pathologic specimen of a bicuspid stenotic calcified aortic valve

Most patients with aortic stenosis are asymptomatic and are diagnosed because of the presence of a cardiac murmur. Rarely, syncope is the presenting sign of aortic stenosis. Some infants with severe aortic stenosis can present with low cardiac output, severely reduced ejection fraction, shock, and/or congestive heart failure.

Aortic insufficiency usually presents with a murmur. Severe or progressive aortic insufficiency may present with fatigue, shortness of breath, exercise intolerance, orthopnea, and/or paroxysmal nocturnal dyspnea.

40.1.3 Physical Examination

The apical impulse may be increased for patients with moderate or severe stenosis. There may be a thrill palpable at the base of the heart and over the carotid arteries. The first heart sound is normal and the second heart sound is normal. With aortic valve stenosis (but not with supravalvar or subvalvar stenosis) an ejection click will be heard immediately after the first heart sound.

The murmur of aortic stenosis is systolic and crescendo decrescendo or "diamond shaped." It is best heard along the left sternal border and radiates to the right upper sternal border. Some patients with aortic stenosis have associated aortic insufficiency, and, if that is the case, a decrescendo diastolic murmur will be audible.

Aortic insufficiency is characterized by a decrescendo murmur that begins immediately after S2.

40.1.4 Echocardiography and Cardiac Catheterization

Using echo/Doppler techniques, it is possible, noninvasively, to estimate the transaortic pressure gradient. Using echocardiography, one can assess left ventricular wall thickness, which also is a reflection of the severity of the stenosis.

Echocardiography and Doppler can confirm the presence of aortic insufficiency and is useful in determining the severity of the leakage. Using these techniques, one can determine the regurgitant fraction, the left ventricular systolic and diastolic dimensions, and the left ventricular ejection fraction.

40.1.5 Treatment

Treatment of aortic valve stenosis involves manipulating the valve to reduce the stenosis. This can be accomplished by transvenous balloon dilation of the valve, open surgical valvotomy, or surgical valve replacement. Valve replacement includes several possibilities. The stenotic valve could be replaced with a homograft valve, a porcine valve, or a mechanical valve.

An additional option is the Ross procedure. In this operation the patient's pulmonary valve is removed and sewn into the aorta in place of the stenotic aortic valve. A homograft valve is then used in the pulmonary position.

There are several approaches to the patient with significant aortic insufficiency. The options for valve replacement are the same as those discussed above for aortic stenosis. In addition, the surgeon can, in select cases, repair the valve.

Since patients with bicuspid aortic valve or aortic stenosis can develop an ascending aneurysm, repair of the aneurysm will be necessary if it is large.

40.1.6 Outcome

Aortic stenosis is a lifelong issue despite treatment. After balloon dilation or surgical valvotomy, there is a 40 % chance over the next 25 years that additional surgery will be necessary, usually to treat aortic regurgitation. If aortic valve replacement is done using a mechanical prosthesis, the patient will require lifelong anticoagulation therapy. If a tissue valve is used, it eventually will have to be replaced. In general the half-life for a tissue valve is 10–15 years.

Primary aortic valve insufficiency can be treated, in some cases, by repair of the leaking aortic valve. However, in most cases valve replacement will be necessary. The periprocedural mortality for these procedures is less than 5 %.

40.2 Coarctation of the Aorta

40.2.1 Pathologic Physiology

Coarctation of the aorta consists of a ledge-shaped protrusion of tissue into the aorta immediately opposite the insertion of the ductus or ligamentum arteriosum (Fig. 40.2). Frequently, there is an associated bicuspid aortic valve.

In some patients the coarctation can compromise the orifice of the left subclavian artery. In this case there may not be a blood pressure discrepancy between the left arm and the leg. Rather, the discrepancy will be between the right arm and leg. Also if there is aberrant origin of the right subclavian artery from the descending aorta distal to the coarctation of the aorta, then there may not be a discrepancy in the blood pressure or pulses between the right arm and the leg. Thus, it is important to measure the blood pressure in both arms and one leg or one could fail to make the correct diagnosis.

40.2.2 Clinical Presentation

The clinical presentation of coarctation of the aorta depends upon the severity of the obstruction and the associated anomalies. Very severe coarctation of the aorta may become apparent at 2–5 days of life when the ductus arteriosus closes and congestive heart failure occurs.

If the coarctation is not severe enough to cause heart failure in infancy, the patient usually remains asymptomatic. The presence of the defect may be heralded by the

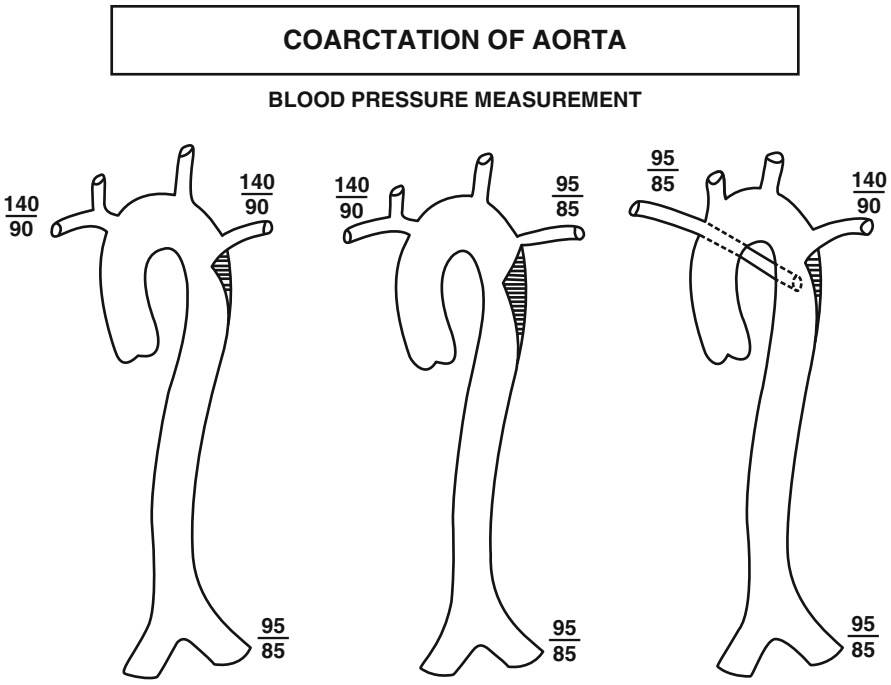

Fig. 40.2 Diagrammatic representation of anatomic variants of coarctation of the aorta. The *left-sided* figure is the most common form. The *middle figure* illustrates associated stenosis of the left subclavian artery and the *right-sided* figure demonstrates associated anomalous origin of the right subclavian artery. The blood pressure measurements illustrate how the anatomic variants will affect the blood pressure measurements (Reproduced or adapted from Driscoll, David, *Fundamentals of Pediatric Cardiology*, Lippincott Williams & Wilkins, 2006, with permission of the author and publisher)

presence of a heart murmur or systemic hypertension. Unfortunately, in some instances the presence of a coarctation is not appreciated until adolescence. Rarely, with previously undetected coarctation of the aorta, a cerebral vascular accident may be the presenting event.

40.2.3 Physical Examination

Infants with severe coarctation of the aorta may have the usual signs of congestive heart failure including tachycardia, poor perfusion, grunting, tachypnea, and hepatomegaly. Low amplitude or absent pulses in the lower extremities as compared to the upper extremities is the sine quo non of coarctation of the aorta.

Since many patients with coarctation of the aorta have an associated bicuspid aortic valve, an aortic ejection click frequently is heard shortly after the first heart sound.

Turbulent blood flow through the area of coarctation produces a systolic ejection murmur that is best heard at the cardiac apex and below the left scapula. Because

many patients with coarctation of the aorta have subtle or important abnormalities of the mitral valve, a diastolic murmur may be heard at the apex of the heart.

The formation of collateral vessels can affect the physical findings. Parascapular collateral vessels can produce a continuous murmur in the region of the scapula, and, in some patients, these vessels can be palpated. As collateral vessels form, the pressure gradient across the coarctation can decrease making the arm-to-leg blood pressure gradient an unreliable measure of severity of the coarctation.

40.2.4 Echocardiographic and Cardiac Catheterization Issues

The presence of coarctation of the aorta is ascertained by clinical examination. Although the vast majority of coarctations are located opposite the aortic insertion of the ductus or ligamentum arteriosus, rarely the lesion may be located more distally in the aorta. Thus, echocardiography or some other imaging form is necessary to localize the area of coarctation and to establish the length of the narrow segment. In addition, associated lesions such as aortic stenosis, VSD, or mitral valve disease can be identified and quantified. The severity of the coarctation can be estimated by the degree of left ventricular hypertrophy and, in some cases, by measuring the cross-sectional area or the diameter of the coarctation site.

Newer MRI methods are quite good at imaging coarctation of the aorta and assessing its severity. MRI allows detailed imaging of the entire aorta in multiple planes and views. It also allows identification of collateral vessels.

Cardiac catheterization is no longer necessary for the diagnosis of coarctation of the aorta. However, in some cases, it is employed to treat coarctation of the aorta using balloon dilation or stent implantation.

40.2.5 Treatment

The treatment of coarctation of the aorta is to remove or eliminate the obstruction. This can be done surgically or by balloon dilation with or without stent implantation.

40.2.6 Outcome

The preprocedure mortality is less than 5 % for the management of coarctation of the aorta. However, long-term problems persist. Recurrent or persistent coarctation occurs in a significant number of patients and can be as high as 30 % if operation or balloon dilation is necessary in the first 6 weeks of life. Since 50–80 % of patients with coarctation of the aorta have associated bicuspid aortic valve, eventual treatment of aortic stenosis or insufficiency may be necessary. Also, even for patients with repaired coarctation of the aorta, persistent hypertension may be present.

40.3 Pulmonary Valve Stenosis and Insufficiency

40.3.1 Pathologic Physiology

Pulmonary valve stenosis (with intact ventricular septum) usually occurs sporadically.

Isolated pulmonary valve insufficiency is rare. It can accompany idiopathic dilation of the pulmonary artery and pulmonary vascular obstructive disease. Most case of pulmonary insufficiency results from surgical or balloon procedures to treat pulmonary valve stenosis. Pulmonary insufficiency also can be associated with other conditions such as repaired tetralogy of Fallot.

40.3.2 Clinical Presentation

Most patients with pulmonary stenosis or pulmonary insufficiency are asymptomatic and present with a cardiac murmur. The exception to this is the infant with severe pulmonary stenosis (the so-called critical pulmonary stenosis) who presents with cyanosis because of right-to-left shunting at the atrial level.

Pulmonary insufficiency as a result of operation or balloon procedures to relieve right ventricular outflow tract obstruction was, at one time, thought to have minimal significant long-term consequences. It is now becoming clear that, in some patients, long-standing pulmonary valve insufficiency and right ventricular volume overload may be associated with right ventricular failure and tricuspid regurgitation. In this situation, patients may develop fatigue, exercise intolerance, and/or arrhythmias.

40.3.3 Physical Examination

With the exception of infants with critical pulmonary stenosis, patients with pulmonary valve stenosis are acyanotic. Careful palpation at the lower left sternal border will reveal an increased right ventricular impulse. The first sound is normal and is followed by a click. The click is best heard at the upper left sternal border. The second heart sound may be normal, widely split, or single depending on the severity of the pulmonary stenosis. In very mild cases, S2 can be normally split. As the severity increases, S2 becomes more widely split. With severe stenosis, the second heart sound becomes single (it is still widely split but closure of the pulmonary valve becomes inaudible).

The murmur of pulmonary stenosis is an ejection murmur (crescendo decrescendo or “diamond shaped”), and the peak intensity of the murmur occurs later as the severity of the stenosis increases. If ventricular failure occurs, a prominent fourth sound may be heard.

Pulmonary insufficiency is characterized by a mid- to high-frequency decrescendo murmur that begins immediately after S2. In contrast to the murmur of aortic insufficiency, the murmur is louder when the patient is supine than when sitting.

With significant pulmonary insufficiency, the right ventricular impulse, felt at the lower left sternal border in the xiphoid area may be prominent.

40.3.4 Echocardiographic and Cardiac Catheterization Issues

The presence and severity of pulmonary stenosis can be assessed using echocardiography. The valve morphology can be determined (i.e., bicuspid or tricuspid; thickness and dysplasia as well as the mobility of the cusps). Using the maximum instantaneous Doppler gradient, the RV to PA pressure gradient can be estimated. Right ventricular pressure can be estimated using the velocity of the tricuspid regurgitation jet. The presence of pulmonary regurgitation can be confirmed by echocardiography, and the severity can be ascertained by observing the degree of right ventricular and atrial enlargement, the degree of right ventricular function, and the degree of tricuspid regurgitation.

40.3.5 Treatment

The currently accepted best method of treatment of significant pulmonary valve stenosis is balloon pulmonary valvuloplasty. Most people agree that valvuloplasty should be performed if the transpulmonary gradient exceeds 50 mmHg or if there is evidence of right ventricular hypertrophy on the electrocardiogram. In infants, the gradient is less useful, and if the patient has critical pulmonary stenosis (evidenced by poor right ventricular function and/or right-to-left shunting a blood at the atrial level), valvotomy is indicted regardless of the gradient.

The timing and optimum treatment for pulmonary insufficiency is less well defined than that for pulmonary stenosis. It is generally thought that operation to restore a competent valve in the pulmonary position should be done if there is progressive right ventricular enlargement and/or tricuspid regurgitation especially in the setting of ventricular arrhythmias and/or progressive exercise intolerance. Unfortunately, the treatment for pulmonary insufficiency is valve replacement, and none of the current implantable valves last for an extended time. Also, it is unclear whether the patient is best served by insertion of a mechanical or tissue valve.

40.3.6 Outcome

The outcome of balloon dilation or surgical valvotomy for pulmonary valve stenosis is excellent with 25-year survival not different from that of the general population. Pulmonary valve insufficiency is well tolerated long term. However, some patients may develop progressive right ventricular dilation and reduced function secondary to chronic pulmonary insufficiency and require late valve replacement.

40.4 Aberrant Origin of the Right Subclavian Artery

Normally the first branch of the aorta (subsequent to the coronary arteries) is the right innominate artery which gives rise to the subclavian and the right carotid arteries. However, the right subclavian artery can arise directly from the aorta distal to the origin of the left subclavian artery (Fig. 40.2). In this instance, the right subclavian artery travels behind the esophagus to reach the right arm. This anomaly is of no clinical significance and, contrary to some belief, does *not* result in a vascular ring. It can, however, be associated with a 22q11.1 microdeletion or duplication.

40.5 Interrupted Aortic Arch

Interrupted aortic arch (IAA) implies discontinuity between the ascending aorta and the descending aorta. There are three types of IAA. The most common is type B which occurs between the left carotid artery and the left subclavian artery. It comprises 47–85 % of cases. Type A occurs distal to the left subclavian artery and comprises 11–47 % of cases. Type C occurs between the right and left carotid artery and is the least common type.

IAA occurs between 19 and 66 per million live births. It is very unusual for IAA to occur as an isolated anomaly. Type A frequently is associated with aorticopulmonary window with an intact ventricular septum. Type B frequently is associated with conotruncal defects. IAA frequently is associated with 22q11 microdeletion. Ten to twenty percent of cases of truncus arteriosus are associated with IAA.

With IAA perfusion of the descending aorta is dependent upon the presence of a patent ductus arterious. Hence, newborns with IAA need to be maintained on a prostaglandin infusion until continuity of the ascending and descending aorta can be established surgically.

Human Genetics of Semilunar Valve and Aortic Arch Anomalies

41

Matina Prapa and Siew Yen Ho

Contents

Abstract

Lesions of the semilunar valve and the aortic arch can occur either in isolation or as part of well-described clinical syndromes. The polygenic cause of calcific aortic valve disease will be discussed including the key role of *NOTCH1* mutations. In addition, the complex trait of bicuspid aortic valve disease will be outlined, both in sporadic/familial cases and in the context of associated syndromes, such as Alagille, Williams–Beuren, and Kabuki syndromes. Aortic arch abnormalities particularly coarctation of the aorta and interrupted aortic arch, including their association with syndromes such as Turner and 22q11 deletion, respectively, are also discussed. Finally, the genetic basis of congenital pulmonary valve stenosis is summarized, with particular note to Ras-/mitogen-activated protein kinase (Ras/MAPK) pathway syndromes and other less common associations, such as Holt–Oram syndrome.

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41.1 Introduction

Congenital malformations of the arterial valves and of the aortic arch can occur separately, or in isolation, or as part of well-defined clinical syndromes, many of which now have established genetic causes. The process of semilunar valvulogenesis has also been well described in animal models with a number of key pathways involved in endocardial cushion formation and subsequent migration of neural crest cells to the distal outflow tract contributing to aorticopulmonary septation [1].

Table 41.1 Summary of genotype–phenotype correlations in syndromic and nonsyndromic forms of arterial valve and aortic arch abnormalities

Phenotype	Gene	Associated syndrome	Extracardiac features	Causative gene
Calcific aortic stenosis	NOTCH1	no	no	no
Supravalvar aortic stenosis	ELN	Williams–Beuren	Elfin facies, mental deficiency, overfriendly personality, short stature, renal artery stenosis	ELN
Bicuspid aortic valve	NOTCH1 NKX2-5 SMAD6	Williams–Beuren	As above	ELN
		Alagille	Jaundice in early infancy, characteristic facies, butterfly vertebrae, ocular anomalies	JAG1
		Kabuki	Distinctive facies, mental retardation, skeletal abnormalities, recurrent otitis media in infancy	MLL2
Aortic coarctation	NKX2-5 SMAD6	Turner	Ovarian failure, short stature, dysmorphic stigmata, neck webbing	
		Kabuki	As above	MLL2
Interrupted aortic arch	–	22q11 deletion	Hypocalcemia, immunodeficiency, facial dysmorphia, learning difficulties	
		CHARGE	Coloboma, heart disease, atresia choanae, retarded growth and development and/or CNS anomalies, genital hypoplasia, and ear anomalies and/or deafness	CHD7
Pulmonary stenosis	GATA4	Noonan	Renal and hematological abnormalities, developmental delay, short stature, characteristic facies	PTPN11
		NFNS	Overlap of neurofibromatosis and Noonan syndrome features	NF1
		Holt–Oram	Abnormalities of upper extremities, thumb anomalies	TBX5

Abbreviations: *CNS* central nervous system, *NFNS* neurofibromatosis–Noonan syndrome

The following sections will focus on our current knowledge of genes implicated in semilunar valve and aortic arch abnormalities including a summary (see Table 41.1) of genotype–phenotype correlations in both syndromic and nonsyndromic forms of the above lesions.

41.2 Aortic Valve Stenosis

Calcific aortic valve disease traditionally has been regarded as a degenerative process involving passive wear and tear of the valve leaflets. On the contrary, current evidence points to a continuum disease process similar to that of atherosclerosis with active cell proliferation and chronic inflammation resulting in leaflet calcification [2].

Familial predisposition to aortic valve sclerosis has been shown by the Hypertension Genetic Epidemiology Network (HyperGEN) Study Group reporting a sibling recurrence risk ratio of 2.3 with several associated chromosomal regions identified by genome-wide linkage analyses [3]. The above results suggested a polygenic nature of aortic sclerosis with a number of susceptibility genes having potentially differential effects on valve calcification. A different study in Western France focused on the more advanced disease trait of calcified aortic valve stenosis requiring surgical replacement to identify clusters of large families segregating aortic stenosis [4].

Congenital malformation of the aortic valve is an additional major risk factor for aortic stenosis [5]. In 2005, Garg et al. performed a genome-wide association study in two kindreds affected by congenital heart disease (CHD) and aortic valve calcification [6]. The first pedigree was a five-generation European-American family in which nine affected members had aortic valve disease (six had bicuspid aortic valve and seven had calcific aortic stenosis). Other cardiac malformations included ventricular septal defect, tetralogy of Fallot, and mitral stenosis. A genome-wide scan in all available family members revealed linkage to a single locus on chromosome 9q34-35 including *NOTCH1* gene with a nonsense mutation in affected individuals. Additional screening of a smaller kindred of Hispanic origin revealed a frameshift *NOTCH1* mutation in three affected members with bicuspid aortic valve (BAV) [6]. *NOTCH1* gene encodes a transmembrane protein regulating cell fate decisions involved in the development of the atrioventricular canal, ventricular myocardium, and cardiac outflow tract [7]. *NOTCH1* signaling pathway also represses Runx2 (runt-related transcription factor 2), a critical transcription factor involved in osteoblast differentiation, which is upregulated in human calcified aortic valves [2]. Thus, mutations in *NOTCH1* may have a key role in aortic stenosis, resulting in an early developmental defect and a later derepression of calcium deposition in the aortic valve.

Following these findings, Mohamed et al. investigated the role of *NOTCH1* gene mutations in 48 German patients with sporadic BAV [8]. A mutation analysis was performed on all 34 coding exons and adjacent intronic and 5′ and 3′ untranslated sequences. Two BAV-causing mutations were identified (p.Thr596Met and p.Pro1797His); both patients had calcification of the aortic valve and concomitant aneurysms of the ascending aorta. These mutations could not be identified in 327 healthy controls.

41.3 Supravalvar Aortic Stenosis

Congenital supravalvar aortic stenosis (SVAS) belongs to a different disease spectrum of diffuse arteriopathy characterized by medial and/or intimal thickening of the aortic wall with narrowing of its lumen [9]. SVAS is commonly a feature of Williams–Beuren syndrome (WBS) with an estimated prevalence of approximately 70 % but can also occur in nonsyndromic forms [10]. Both WBS and nonsyndromic SVAS are caused by disruptions in the elastin (*ELN*) gene with reduced ELN expression found in vascular smooth muscle cells (VSMCs) of affected patients [11]. Reductions in elastin, a key structural protein of the extracellular matrix, lead to elastic fragmentation with increased collagen and VSMC hypertrophy in the media of SVAS patients [9].

41.4 Aortic Coarctation and Bicuspid Aortic Valve

Aortic coarctation accounts for 5–7 % of CHD lesions [12, 13]. More than 50 % of patients with coarctation of the aorta are also found to have a BAV [12, 13] (Fig. 41.1). The latter is the commonest congenital heart lesion affecting 0.5–2 % of the general population and is a more complex trait than initially thought, with coexistence of defective valvulogenesis and an additive effect on structural properties of the aortic wall [14, 15].

Aortic coarctation and BAV disease can occur in isolation or as part of genetic syndromes, with their underlying genetic basis under ongoing investigation. Both defects frequently can be found in females with Turner syndrome (45X0)

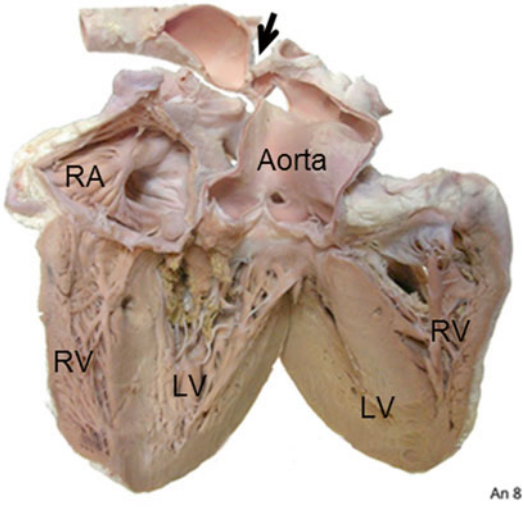

Fig. 41.1 The left ventricle (*LV*) has been opened longitudinally through the outflow tract and the aortic valve. There is a bicuspid aortic valve with dilatation of the ascending aorta and coarctation (*arrow*) where the arch narrows discretely. *RA* right atrium, *RV* right ventricle (Leon Gerlis Museum, Royal Brompton Hospital, London, UK)

characterized by the presence of webbed neck, short stature, and gonadal dysgenesis [16]. Cardiovascular abnormalities are found in up to 45 % of Turner patients, with BAV and aortic coarctation in 30 % and 12 %, respectively [16, 17]. Bileaflet aortic valve can also be a component part of familial thoracic aortic aneurysm or hypoplastic left heart syndrome [18, 19]. Genes located on the short arm of chromosome X are considered key to aortic valve and arch development with haploinsufficiency for Xp associated with higher prevalence of BAV and coarctation in Turner syndrome [17]. A maldevelopment of neural crest cells, which give rise to the outflow tract of the heart, the aortic arch system, and the cervicocephalic arteries, may also underlie development of these lesions in non-syndromic cases [20].

The presence of BAV can comprise a feature of a number of additional syndromes, such as Alagille, Williams–Beuren, and Kabuki syndromes. Alagille syndrome (AGS) consists of jaundice in early infancy, characteristic facies, butterfly vertebrae, ocular anomalies, and a wide spectrum of cardiovascular anomalies, the most common of which being stenosis or hypoplasia of the branch pulmonary arteries. Mutations in the jagged 1 (*JAG1*) gene, encoding a NOTCH receptor ligand, are present in up to 75 % of individuals with AGS and may also be found in patients with only a few characteristics of the syndrome [21, 22]. In 2002, McElhinney et al. reviewed the cardiac phenotype of 200 individuals with AGS or a *JAG1* mutation [23]. Two of the probands had BAV disease as a primary cardiovascular anomaly and another two as a secondary anomaly, with *JAG1* mutations present in three of the BAV patients. Coarctation of the aorta was also present in four subjects with *JAG1* mutations found in two of these. Isolated reports of systemic vascular abnormalities in AGS patients exist in the literature, including intracranial aneurysms as well as coarctation of the thoracic and abdominal aorta, with presence of *JAG1* mutations in the latter [24, 25]. Expression of *JAG1* has been previously shown to contribute to angiogenesis, both in *in vitro* models as well as in animal studies, with disruption of the mouse *JAG1* gene leading to lethal diffuse hemorrhages [26, 27].

Notably, the presence of a bileaflet aortic valve has also been -reported in 11.6 % of patients with a clinical diagnosis of WBS [28]. In 2003, Sugayama et al. used fluorescence in situ hybridization (FISH) methods to detect microdeletions in the elastin gene in 20 WBS patients [29]. All FISH-positive patients (85 %) had cardiovascular malformations, out of which 18 % had combined BAV and SVAS. Another syndromic association of both BAV disease and coarctation of the aorta is that of Kabuki syndrome, characterized by distinctive facies, mental retardation, skeletal abnormalities, and recurrent otitis media in infancy. Congenital heart lesions are present in 31–58 % of affected patients with common occurrence of coarctation of the aorta [30, 31]. A recent study undertaking exome sequencing in a discovery cohort of ten unrelated patients with Kabuki syndrome led to identification of *MLL2* (myeloid/lymphoid or mixed-lineage leukemia 2) gene loss-of-function mutations in 90 % of cases, with further targeted sequencing identifying additional mutations in 60 % of a replication cohort [32]. Notably, four out of the ten initially studied

subjects had coarctation of the aorta, including three patients with a concomitant BAV. MLL2 (also known as *KMT2D*) encodes a histone methyltransferase, lysine (K)-specific methyltransferase 2D, regulating the transcription of a diverse group of genes with murine studies of *MLL2* loss-of-function phenotype exhibiting increased apoptosis and retarded development [33, 34].

The cardiac homeobox gene *NKX2-5* has a major role in cardiac development with mutations in this gene associated with a range of cardiac malformations [35–37]. Targeted sequencing of *NKX2-5* in 608 subjects with conotruncal anomalies, left-sided lesions, atrial septal defect, and Ebstein's anomaly resulted in identification of 12 distinct mutations in 3 % of the studied population, including a single (out of 59) patient with coarctation of the aorta [38]. Moreover, previous murine studies have exhibited that heterozygous *NKX2-5* ablation results in an eightfold increase in the prevalence of stenotic BAV [35]. Following these findings, Majumdar et al. performed a targeted mutational analysis of the protein coding sequence of *NKX2-5* gene in 19 patients with BAV and ascending aortic aneurysm [39]. DNA was extracted from peripheral blood leukocytes and diseased aortic tissue. Three subjects with normal aortic valves and isolated ascending aortic aneurysm were used as controls. Sequence analysis of leukocyte DNA demonstrated a single known polymorphic alteration in the 3′ untranslated region adjacent to exon 2 in four BAV and two control subjects. The same polymorphism was detected following DNA sequencing from aortic tissue samples, in six BAV and one control subject. Interestingly, in four of the BAV patients (and none of the controls), the mutation was only present in the DNA extracted from the aortic tissue suggesting somatically acquired mutation and clonal expansion during aortogenesis.

Bone morphogenetic protein (*BMP*) signaling also has a pivotal role in cardiac morphogenesis with mice genetically disrupted for *BMP* exhibiting hypoplastic cardiac cushion formation [40, 41]. SMAD family member 6 (*SMAD6*) is an important intracellular inhibitor of the BMP pathway, with its genetic disruption in mice resulting in heart valve defects and aortic ossification [42]. Non-synonymous mutations in *SMAD6* gene have been recently found in 3 out of 436 individuals with a wide range of CHD lesions, out of which two had a BAV: a 30-year-old male with aortic stenosis, coarctation, and calcification of the aorta and an infant with moderate aortic stenosis [43]. In a different study undertaking exome sequencing of 362 parent–offspring trios with severe CHD, a significantly higher burden of protein-altering *de novo* mutations in genes with high heart expression (HHE) was found in CHD patients compared to controls [44]. Out of the above studied population, 10 % had coarctation of the aorta, 11 % had BAV, and 4 % had non-BAV aortic stenosis. One of the most interesting genes highlighted in this study is H3K4me, an epigenetic mark involved in activation of key developmental genes. Mutations in genes involved in the H3K4me pathway accounted for 27 % of pathogenic mutations in the HHE gene set with some examples of the above mutated genes in patients with left ventricular outflow obstructive lesions including *MLL2* (frameshift mutation), *KDM5A* encoding lysine (K)-specific demethylase 5A (missense mutation), and *USP44* encoding ubiquitin-specific peptidase 44 (missense mutation).

41.5 Interrupted Aortic Arch

Interrupted aortic arch (IAA) accounts for approximately 0.2–1.4 % of all congenital heart defects and is commonly associated with other cardiac lesions, such as ventricular septal defect, patent arterial duct, BAV, truncus arteriosus, and complete transposition of the great arteries [45, 46]. IAA can occur in the context of coloboma, heart disease, atresia choanae, retarded growth and development and/or CNS anomalies, genital hypoplasia, and ear anomalies and/or deafness (CHARGE) syndrome, with a frequency of 3/299 in a study population carrying pathogenic *CHD7* mutations for CHARGE syndrome [47]. However, its commonest association is with 22q11 deletion syndrome with 50 % of IAA patients testing positive for a deletion [48].

The 22q11 deletion syndrome has a wide spectrum of clinical features, including the phenotypes of DiGeorge and velocardiofacial syndrome (DGS/VCFS) [49]. The syndrome is characterized by cardiovascular defects, hypocalcemia, immunodeficiency, facial dysmorphia, and learning difficulties. Common associated cardiac malformations include tetralogy of Fallot, IAA, and truncus arteriosus [50]. Most DGS/VCFS patients are hemizygous for a 1.5–3.0 Mb 22q11.2 deletion encompassing up to 40 genes, including TBX (T-box) 1 with development of conotruncal defects in heterozygous mice for a null *TBX1* mutation [51, 52]. Interestingly, the incidence of 22q11 deletions varies significantly between different types of IAA, with approximately half of type B patients (interruption between the left carotid and left subclavian artery) and none of the type A patients (interruption distal to the left subclavian artery) having a deletion, suggestive of differential underlying pathologies [48, 53].

41.6 Pulmonary Stenosis

Congenital pulmonary stenosis can occur in isolation (Fig. 41.2) and in combination with other congenital heart lesions, such as tetralogy of Fallot, or as part of genetic syndromes with cardiac involvement. A characteristic example of the latter is Noonan syndrome, the second commonest syndromic cause of congenital heart disease (CHD) after trisomy 21 [54]. Noonan syndrome is an autosomal dominant trait affecting multiple systems, including renal and hematological abnormalities, developmental delay, short stature, and characteristic facial features. Pulmonary stenosis is found in up to 60 % of affected patients with other associated heart lesions including hypertrophic cardiomyopathy, ventricular and atrial septal defect, aortic and mitral valve lesions, and aortic coarctation [55].

Noonan syndrome has been linked to *PTPN11* gene mutations encoding for SHP-2, a protein tyrosine phosphatase with signaling roles in semilunar valvulogenesis [56]. The prevalence of *PTN11* mutations in Noonan syndrome is approximately 50 % with affected patients having a significantly higher frequency of pulmonary stenosis and a lower frequency of hypertrophic cardiomyopathy [57]. *PTPN11* gene belongs to the Ras-/mitogen-activated protein kinase (MAPK)

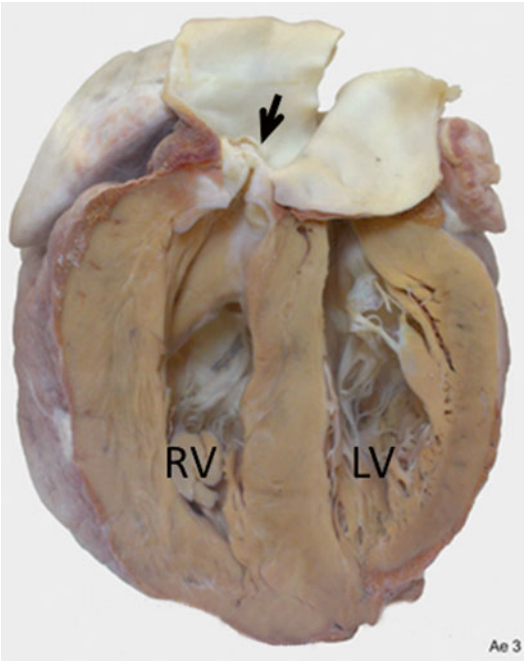

Fig. 41.2 The heart has been cut longitudinally through the right ventricular outflow tract. There is a thickend dome-shaped pulmonary valve (*arrow*) causing pulmonary stenosis. Flow is through a small orifice. Consequently there is remarkable hypertrophy of the right ventricular wall. *RV* right ventricle, *LV* left ventricle (Leon Gerlis Museum, Royal Brompton Hospital, London, UK)

signaling cascade with a number of additional integral genes implicated in Noonan syndrome [55]. The Ras/MAPK pathway is a well-recognized signaling pathway which via a series of phosphorylation events in the cytoplasm results in entry of activated ERK (extracellular signal-regulated kinase) in the nucleus and appropriate response to the initial stimulus. Noonan syndrome-causing mutations in respective genes usually enhance signaling via the Ras/MAPK pathway, with mutations in son of sevenless homolog 1 (*SOS1*) gene accounting for approximately 10 % of cases and mutations in v-raf-1 murine leukemia viral oncogene homolog 1 (*RAF1*) for another 10 % of cases [55]. Phenotypic correlations include smaller incidence of atrial septal defect in patients harboring *SOS1* mutations and higher incidence of hypertrophic cardiomyopathy in those with *RAF1* mutations [55].

In a Dutch genotype–phenotype study undertaken in 33 patients with a clinical diagnosis of Noonan syndrome who tested negative for *PTPN11* mutations, 42 % of cases had mutations in genes involved in RASopathies with a higher prevalence of heart defects (79 % versus 56 %) and pulmonary stenosis (69 % versus 39 %) compared to mutation-negative patients [58]. The genetically heterogeneous Noonan syndrome overlaps clinically with a number of other Ras/MAPK pathway syndromes, such as cardio-facio-cutaneous (CFC), multiple lentigines (also known as LEOPARD), and Costello syndrome. The frequency of pulmonary stenosis in the setting of Costello syndrome (HRAS) appears to be relatively rare (20 %) and non-progressive in nature in comparison with CFC syndrome (BRAF) and Noonan syndrome with multiple lentigines (*PTPN11*) with a frequency of approximately 40 % based on a recent systematic literature review [59]. In another RASopathy,

neurofibromatosis type 1 (NF1), pulmonary stenosis has an estimated frequency of 1.1 % with a 20 times higher prevalence in patients with NF1–Noonan syndrome (NFNS), the majority of which only have NF1 gene mutations. Importantly, a novel genotype–phenotype correlation has been reported, with non-truncating NF1 gene mutations associated with the presence of pulmonary stenosis in both NF1 and the related NFNS [60].

Pulmonary stenosis can also occur in other less prevalent familial and syndromic forms. An example is that of Holt–Oram syndrome, a rare heart–hand syndrome characterized by mutations in the *TBX5* transcription factor. Associated heart defects commonly include atrial septal defect and conduction abnormalities, with the less typical finding of pulmonary stenosis recently reported in a five-generation family carrying a novel intragenic *TBX5* duplication segregating with the disease phenotype [61]. Interestingly, *TBX5* is known to interact with transcription factor *GATA4* (GATA binding protein 4) with an increasing number of familial studies reporting *GATA4* mutations segregating with atrial septal defect and pulmonary stenosis [62–64].

Conclusion

Although there is increasing evidence of genetic involvement in outflow malformations that include the arterial valves as well as the great arteries, other factors especially hemodynamics must also be considered in the light of genetic susceptibility in this region of the heart. In both isolated and in syndromic forms, there may be common causative genes affecting both the valvar and the arterial parts of the outflow. Coordinated clinical hemodynamic/imaging and biological/genetic studies will help distinguish cases in which genetic factors play the dominant role and lead to further advances in clinical management.

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Molecular Pathways and Animal Models of Semilunar Valve and Aortic Arch Anomalies

42

Amy-Leigh Johnson and Simon D. Bamforth

Contents

Abstract

The great arteries of the vertebrate carry blood from the heart to the systemic circulation and are derived from the pharyngeal arch arteries. In higher vertebrates, the pharyngeal arch arteries are a symmetrical series of blood vessels that rapidly remodel during development to become the asymmetric aortic arch arteries carrying oxygenated blood from the left ventricle via the outflow tract. At the base of the aorta, as well as the pulmonary trunk, are the arterial, or semilunar, valves. These valves each have three leaflets and prevent the backflow of blood into the heart. During development, the process of aortic arch and valve formation may go wrong, resulting in cardiovascular defects, and these may, at least in part, be caused by genetic mutations. In this chapter, we will review models harbouring genetic mutations that result in cardiovascular defects affecting the great arteries and the arterial valves.

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42.1 Introduction

The great arteries of the human heart are derived from the pharyngeal arch arteries (PAAs) [1, 2]. The PAAs form symmetrically and sequentially during early embryogenesis in a cranial to caudal sequence and are rapidly remodelled over a ~3-week period in embryonic development to form the asymmetric great arteries seen in the adult, namely, the aorta, carotid and subclavian arteries. Initially, the PAAs are derived from endothelial cell precursors and form the five pairs of vessels known as PAA 1–4 and 6. The first two pairs of PAA (1 and 2) degenerate early and contribute to the vascular plexus within the forming jaw. The asymmetric remodelling process that will result in the formation of the mature aorta, carotid and subclavian arteries involves PAA 3, 4 and 6. The third PAAs, on both the right and the left, persist as the stem of the internal carotid arteries and part of the common carotid arteries. The fourth PAA persists on the right as the proximal region of the subclavian artery, whilst on the left it contributes to the section of the mature aortic arch between the origins of the left common carotid artery and the left subclavian artery. The right sixth PAA regresses, whilst the left sixth PAA forms the arterial duct (ductus arteriosus).

Each PAA is housed within a pharyngeal arch, a series of transient structures important for the development of the jaw, ears, cartilages of the throat and glands [3]. The pharyngeal arches are bordered on the outside by ectoderm and the inside by endoderm, and within is a mesodermal core surrounded by neural crest-derived mesenchyme. Neural crest cells (NCC) will differentiate into the smooth muscle cells that surround the arch arteries, as well as migrating into the outflow tract to septate the single vessel into the separate aorta and pulmonary trunk. Each tissue plays a role in creating the great arteries of the heart, and consequently, many molecular pathways are important for the establishment and patterning of the PAAs into the great arteries found in the adult.

The semilunar valves, also known as the arterial valves, are found at the base of the aorta and pulmonary trunk, each comprising of three leaflets, and prevent the backflow of blood into the heart following ventricular systole. Whereas it is the extrapericardial components of the arterial trunks that are derived from the PAAs, the intrapericardial arterial trunks are derived from the distal part of the outflow tract, subsequent to growth from the second heart field, with the arterial valves formed in the intermediate component [4]. When the three leaflets of the aortic valve fail to form correctly, only two leaflets are seen giving rise to the condition known as bicuspid aortic valve (BAV). This is the commonest form of congenital heart disease and results in regurgitation of blood from the aorta back into the ventricle. Although this may be a mild phenotype, and is often asymptomatic at birth and in childhood, it is thought to predispose an affected individual to more severe heart disease in later life [5].

Animal models are useful to investigate the roles of individual genes in the process of arterial valve and PAA formation and remodelling. Cardiovascular development in the mouse closely mimics that seen in the human, albeit at a more rapid pace (total gestation time in the mouse is approximately 19 days). Moreover, current scientific knowledge and tools mean that genetic modification in mice is a relatively straightforward process. The chicken is also a useful animal model for PAA

development as it has many similarities to mammals although the development of the aortic arch arteries varies somewhat; the chicken develops a right-sided aortic arch, with two brachiocephalic arteries and an arterial duct on each side. The chicken embryo is directly accessible through a window made in the eggshell, allowing for the application of substances or genetic material to modify development. The zebrafish is also utilised as an animal model for the formation of the PAAs due to its ability to be genetically manipulated, but the more evolutionary primitive body plan means that the PAAs mostly remain as symmetric and paired blood vessels and do not remodel as in mammals.

In this chapter, we will review several molecular pathways that are known to play a role in the formation and development of the great arteries and arterial valves, predominantly focusing on the mouse model and the genetic or chemical manipulation of this model that results in a number of congenital defects seen in infants, including interruption of the aortic arch (IAA), aberrant right subclavian artery (A-RSA), coarctation of the aorta, bicuspid aortic valve (BAV) and aortic and pulmonary stenosis.

42.2 Aortic Arch Anomalies

A review of the published literature on transgenic mouse models presenting with cardiovascular defects including the aortic arch arteries reveals that many genes from different molecular pathways can result in similar heart defects (Table 42.1).

42.3 T-Box 1

The gene thought to be responsible for the cardiovascular defects seen in 22q11 deletion syndrome (22q11DS, also known as DiGeorge, velocardiofacial, or Shprintzen syndrome) is T-box 1 (*TBX1*) [6–8]. Although clinically rare, 50 % of all cases of IAA occur in this syndrome [9]. Transgenic mice homozygous null for

Table 42.1 Genes when mutated in mice give rise to defects affecting the development of the pharyngeal arch arteries which subsequently result in defects such as interruption of the aortic arch and anomalous right subclavian artery

Class	Genes
Transcription factor	*Cited2, Eya1, Foxc1, Foxc2, Gata3, Gata6, Gbx2, Hand2, Pbx1, Prdm1, Prdm3, Prrx1;Prrx2, Tbx1, Tcfap2a, Zic3*
Signalling molecule	*Notch, Rara; Rarb, Scrib, Sema3c*
Growth factor	*Fgf8, Tgfb2, Vegfa*
Protein binding	*Bmp4, Chrd, Crkl, Edn1, Ednra, Flna, Ltbp1L, Vangl2*
Receptor	*Acvr1, Bmpr2, Folr1, Pdgfra, Plxnd1, Tgfbr2*
Enzyme	*Aldh1a2, Chd7, Ece1*

Other congenital cardiovascular malformations are also seen in some of these mutants (e.g. common arterial trunk – see Chap. 48)

Tbx1 die in the perinatal period with common arterial trunk (CAT). However, mice lacking one copy of *Tbx1* (i.e. heterozygous, *Tbx1+/−*) are generally healthy and fertile, but, importantly, a proportion do display fourth PAA defects such as IAA (which is lethal) and A-RSA (which may be tolerated) (Fig. 42.1). This heterozygous phenotype makes the *Tbx1+/−* mouse a relatively good clinical model for 22q11DS, and transgenic embryos have been produced to investigate the tissue-specific requirements in PAA morphogenesis in the *Tbx1* heterozygous state. Hypoplasia of the fourth PAA was achieved by removing *Tbx1* from the second heart field, mesoderm, ectoderm and endoderm using forkhead box G1/Cre-recombinase (*Foxg1Cre*) transgenic mice [11]. As conditional deletion from the mesoderm using mesoderm posterior 1 homolog (*Mesp1*)-*Cre* transgenic mice does not result in PAA defects, this seems to exclude the role of Tbx1 in the mesoderm for fourth PAA morphogenesis [11]. Additional evidence for this is provided from pharyngeal epithelial deletion using fibroblast growth factor (Fgf) 15-*Cre* and

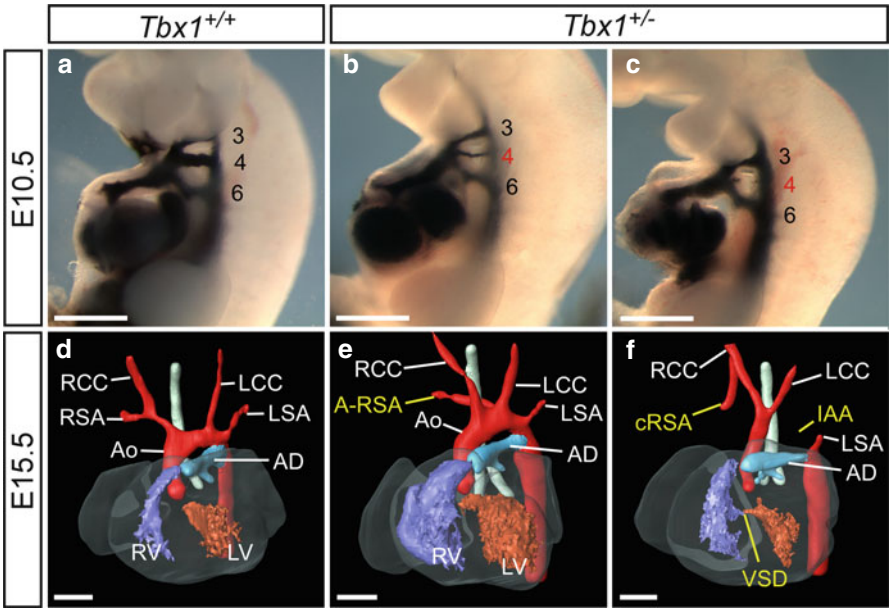

Fig. 42.1 Cardiovascular defects in *Tbx1*-heterozygous (*Tbx1+/−*) mouse embryos and foetuses. (**a–c**) Injection of India ink into the heart of E10.5 embryos. (**a**) In the wild-type embryo (*Tbx1+/+*), three bilaterally symmetrical pharyngeal arch arteries (PAA) are present (numbered *3*, *4* and *6*) which are fully patent to ink. (**b**, **c**) In *Tbx1+/−* embryos, the fourth pharyngeal arch arteries (PAA) may either be hypoplastic (**b**) or non-patent to ink (**c**) revealing that this PAA has failed to form properly. (**d–f**) 3-D reconstructions from MRI datasets [10] of E15.5 foetuses. (**d**) By E15.5, the heart and its associated great vessels have developed into the mature configuration. (**e, f**) The hearts of *Tbx1+/−* foetuses present with great artery defects related to the failure of the fourth PAA to form correctly: A-RSA (**e**) and IAA (**f**) which, in this case, is accompanied by cervical origin of the RSA (cRSA) and a VSD. Abbreviations: *Ao* aorta, *AD* arterial duct, *IAA* interrupted aortic arch, *LCC* left common carotid; *LSA* left subclavian artery, *LV* left ventricle, *RCC* right common carotid, *RSA* right subclavian artery, *RV* right ventricle. Scale, 500 μm

activating enhancer binding protein 2 α (*AP-2α*)-*IRESCre*, a targeting cassette containing the Cre-recombinase coding sequence and an internal ribosome entry site (IRES), which also results in fourth PAA defects [11].

In the zebrafish, mutant alleles of *tbx1* have been generated through the DNA damaging agent ENU (N-ethyl-N-nitrosourea) [12, 13]. The two separately identified alleles cause a complete loss of function of *tbx1*. These *tbx1* mutant zebrafish have small otic vesicles and show defects in the segmentation of the posterior pharyngeal arches. Whereas in mice or humans, the PAAs become asymmetrically remodelled, in zebrafish, the four posterior PAAs persist into adulthood and only the two anterior PAAs become remodelled [14]. In all *tbx1*-mutant larvae, however, the PAAs are highly reduced in number, with only one or two PAAs formed which are capable of carrying blood. Similarly to the mouse model of *Tbx1* loss, the neural crest defects are secondary to endodermal defects [12]. A genetic interaction between *tbx1* and *vegfa* has been demonstrated in the zebrafish through co-injection of morpholinos to knock down each gene simultaneously [15].

Direct targets of *Tbx1* have been identified, either through microarray analysis or genetic interaction studies *in vivo*. For example, microarray analyses comparing *Tbx1* null embryo tissue with wild-type controls have generated many potential downstream targets of *Tbx1* [16, 17], although not all will directly affect PAA development. Studies *in vivo* have shown that Tbx1 is in epistasis, or able to genetically interact, with a number of other genes and pathways, including a bone morphogenetic protein (*Bmp*)-*Smad* pathway [18], chromodomain helicase DNA-binding protein 7 *(Chd7*) [19], V-Crk avian sarcoma virus CT10 oncogene homolog-like (*Crkl*) [20], *Fgf8* [21], *Fgf-Six homeobox 1 (Six1*) signalling [22], gastrulation brain homeobox 2 (*Gbx2*) [23], hes family bHLH transcription factor 1 (*Hes1*) [24], paired-like homeodomain 2 (*Pitx2*) [25], PR domain containing 1 (*Prdm1*) [26], SMAD family member (*Smad*) 7 [27] and wingless-type MMTV integration site family, member 5A (*Wnt5a*) signalling [28].

42.4 Fibroblast Growth Factor 8

As mentioned above, *Fgf8* is in epistasis with *Tbx1*. *Fgf8* is an important signalling factor in PAA development, and the *Fgf8* gene is expressed in the ectoderm and endoderm of the pharyngeal arch region. Mice null for *Fgf8* die early in embryogenesis [29], but mice expressing a *Fgf8* hypomorphic allele present with IAA, RAA (right aortic arch) and A-RSA, as well as other intracardiac and outflow tract defects such as CAT and double-outlet right ventricle (DORV) [30, 31]. Mice doubly heterozygous for *Tbx1* and *Fgf8* have a higher penetrance of fourth PAA defects than *Tbx1* heterozygotes, i.e. IAA-B, RAA, A-RSA and cervical origin of the aortic arch [21]. The PAA defects were attributed to the loss or hypoplasia of the fourth PAA as a result of neural crest cell disruption, suggesting that *Fgf8* is essential in neural crest cell survival [30]. Interestingly, an expanded expression pattern of heart and neural crest derivatives expressed 2 (*Hand2*) in *Fgf8* hypomorphs was observed, which could suggest a complex regulatory loop between these two genes [30].

Tissue-specific removal of *Fgf8* from the pharyngeal epithelia (ectoderm and endoderm) has revealed that ectodermal expression of the gene is invaluable in PAA development [32]. Removal of *Fgf8* from the pharyngeal ectoderm using *AP-2α-IRESCre* resulted in 95 % penetrance of fourth PAA malformations at E18.5, of which 30 % were IAA. Other defects included abnormalities of the subclavian arteries, circumflex RAA with or without a right-sided arterial duct and malformations of the coronary arteries. Overall, the levels of PAA malformation mirror that of hypomorphic mutants, suggesting that the pharyngeal ectoderm is the important expression domain of *Fgf8* for fourth PAA development. Early differentiation and migration of endothelial cells were unaffected in these mutant embryos; however, these cells failed to organise correctly into vascular tubes. Additionally, removal of *Fgf8* from the ectoderm and endoderm using homeobox A3 (*Hoxa3*)-*IRESCre* revealed a comparable number of PAA malformations to those seen in the ectoderm-specific deletion. Thymus and parathyroid malformations, in addition to bicuspid aortic valve (BAV), were uniquely identified in these mutants, suggesting that endodermal *Fgf8* expression is key in gland and valve development. Apoptosis of neural crest cells was identified within the third pharyngeal arch mesenchyme and that of the forming fourth and sixth pharyngeal arches. It appears that the ectodermal expression of *Fgf8* is crucial for the formation of the fourth pharyngeal arch; however, the defects observed in *Fgf8*;*Hoxa3-IRESCre* mutants are perhaps the result of a synergistic effect of *Fgf8* removal from both the ectoderm and endoderm simultaneously. A signalling pathway between the neural crest-derived mesenchyme and endothelial cells is apparently required for correct formation of the PAA, at a time prior to differentiation of neural crest cells into vascular smooth muscle cells [32].

42.5 Gastrulation Brain Homeobox 2

Another gene that has been linked to *Tbx1*, as a potential downstream target, is the transcription factor gastrulation brain homeobox 2 (*Gbx2*) [16]. Mice null for *Gbx2* present with a range of cardiovascular malformations including IAA and A-RSA, as well as a small number of outflow tract defects, such as overriding aorta and DORV [23, 33]. Reminiscent of the *Tbx1*-null phenotype, these malformations appear to be related to disrupted neural crest cell migration at E8.0–9.5 of mouse embryonic development. However, as *Gbx2* is not expressed in neural crest cells, it was determined that *Gbx2* expression within the pharyngeal epithelia is responsible for the activation of signalling pathways required in neural crest cell migration into the pharyngeal arches [23]. Conditional deletion of *Gbx2* from the pharyngeal ectoderm, using *AP-2α-IRESCre*, resulted in a comparable level of fourth PAA defects as seen in *Gbx2−/−* embryos, when examined at E10.5 by ink injection. This suggests that ectodermal expression of *Gbx2* is key to the neural crest cell migration defects observed in *Gbx2−/−* embryos. A fusion of the neural crest cell streams as they entered the pharyngeal arches was also observed. These defects were found to be the result of disruption of the Slit homolog (Slit)/roundabout axon guidance receptor homolog (Robo) pathway, involved in migration of the neural crest cells to the pharyngeal arches [23].

Additionally, there is evidence to support a potential *in vivo* genetic interaction between *Gbx2* and *Tbx1*[23]. Doubly heterozygous mutant embryos (i.e. *Tbx1+/−;Gbx2+/−*) show a statistically significant increase in fourth PAA malformation at E10.5 than that observed in *Tbx1*-heterozygous mutant embryos (i.e. *Tbx1+/−;Gbx2+/+*). In addition, higher numbers of embryos showed bilateral defects instead of unilateral defects. Furthermore, reduced expression of *Slit2* was also observed in *Tbx1+/−* mutant embryos, and those with a pharyngeal ectoderm-specific deletion, to a greater extent than that seen in *Gbx2* mutants. This suggests that *Tbx1* may also be capable of regulating *Slit2*, independent of *Gbx2*.

A further genetic interaction has been proposed between *Gbx2* and *Fgf8*. The expression patterns of both *Gbx2* and *Fgf8* overlap, suggesting that the two genes may be interlinked, although there is no change in the expression levels of *Fgf8* in *Gbx2* mutant embryos [33]. However, *Gbx2* can be induced by ectopic FGF8 expression, and *Gbx2* expression in E8.5 *Fgf8* hypomorphs was found to be reduced in the region overlying the future fourth PAA. This, therefore, suggests that *Gbx2* is a downstream effector of *Fgf8* signalling (Fig. 42.2). This notion was confirmed by changes in the number of PAA defects in complex mutant embryos. Double heterozygous embryos (i.e. *Gbx2+/−;Fgf8+/−*) showed an increase in the incidence of A-RSA and RAA, whilst no defects were observed in either of the single heterozygotes. Generating embryos homozygous null for *Gbx2* and heterozygous for *Fgf8* (i.e. *Gbx2−/−;Fgf8+/−*) resulted in a significant increase in the number of PAA

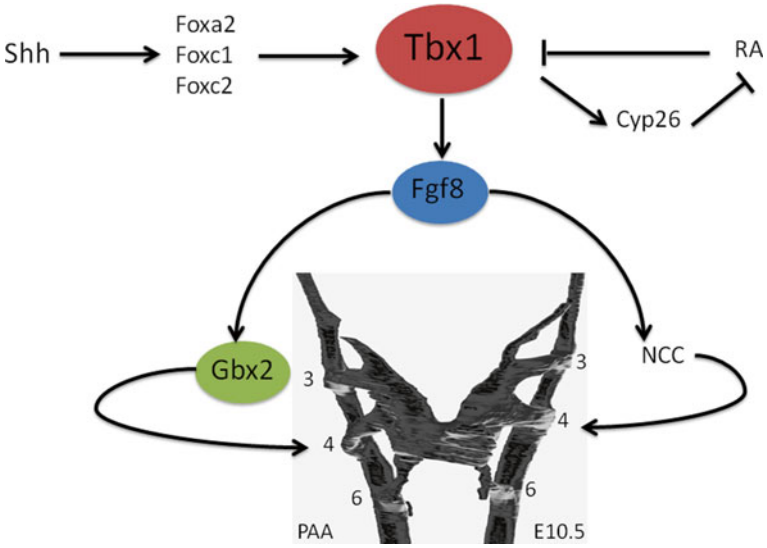

Fig. 42.2 Schematic model of the genes discussed in this chapter and their involvement in regulating *Tbx1* and the development of the fourth pharyngeal arch arteries (PAA). Abbreviations: *Cyp26* cytochrome P450, family 26 protein, *Fgf8* fibroblast growth factor 8, *Fox* forkhead box protein, *Gbx2* gastrulation brain homeobox protein 2, *NCC* neural crest cells, *RA* right atrium, *Shh* sonic hedgehog, *Tbx1* T-box protein 1 (Figure adapted from Refs. [33, 34])

malformations, compared to those seen in *Gbx2−/−* embryos. It is proposed that *Tbx1* is upstream of *Fgf8*, which then activates *Gbx2* and results in correct PAA development. It is also postulated that *Fgf8* is capable of acting independently, through a parallel pathway which controls neural crest cell survival, in forming the PAA, as neural crest cell apoptosis is observed in *Fgf8* mutants, but not in *Gbx2*-null embryos [32, 33].

42.6 Transforming Growth Factor-β Signalling

Transforming growth factor (TGF)-β signalling is crucial for regulating basic cell responses, including proliferation, migration and apoptosis and has been shown to be important for embryonic development in mouse models. The TGFβ signalling pathway is initiated by the binding of TGFβ ligand to the cell surface TGFβ type II receptor (TGFBR2), which activates the TGFβ type I receptor, TGFBR1 (also known as ALK5), and phosphorylates SMAD2 and SMAD3 proteins, allowing association with SMAD4 and translocation to the nucleus to regulate downstream gene expression.

Transgenic mice null for the TGFβ2 ligand (*Tgfb2*) die perinatally with cardiovascular defects, including IAA and A-RSA, as well as VSD, DORV and CAT [35, 36]. Aberrant apoptosis within the fourth PAA was observed, without any defects in neural crest cell migration, which may contribute to the IAA phenotype as well as causing problems with its innervation [36, 37]. The TGFβ receptor *Tgfbr2* is essential for embryogenesis, as mice lacking this gene die around mid-gestation [38, 39]. The conditional deletion of *Tgfbr2* from the neural crest results in IAA [40]. Disruption of the *Ltbp1* gene, which encodes for the long form of latent TGF-β-binding protein 1 and is required for correct storage of TGFβ ligands in the extracellular matrix, also results in embryos with IAA [41].

In a non-transgenic system, pregnant mice administered all-*trans* retinoic acid-produced foetuses with severe cardiovascular defects including IAA and A-RSA, as well as transposition of the great arteries (TGA) and DORV [42]. More information on TGA can be found in Chap. 36.

42.7 Coarctation

Coarctation of the aorta is defined as a narrowing of a section of the aorta that will restrict the flow of blood from the heart. This defect has been recognised in mouse models with heart and great vessel defects, but rarely as an isolated phenotype. It is much more commonly seen within the context of complex cardiovascular malformations.

Aortic coarctation is present within the spectrum of cardiovascular phenotypes seen in 22q11DS patients, and coarctation has also been seen in mouse models genetically connected to *Tbx1*. The expression of *Tbx1* is induced and sustained by Sonic hedgehog (Shh) in the pharyngeal endoderm [43]. However, the forkhead

transcription factors *Foxa2*, *Foxc1* and *Foxc2* have been shown to recognise and bind to cis-regulatory elements in the *Tbx1* promoter and to directly promote the expression of Tbx1 [44, 45]. It is proposed that Tbx1 is a direct transcriptional target of Fox proteins, which play an intermediary role in the regulation of Tbx1 by Shh (Fig. 42.2). Mice null for *Foxc1* have coarctation and aortic and pulmonary valve dysplasia, as well as IAA-B and patent arterial duct [46], and mice null for *Foxc2* have IAA-A and IAA-B [46, 47]. The different descriptions for IAA reflect the location of the interruption: IAA type A is found when the segment of the aortic arch distal to the left subclavian artery is interrupted, IAA type B occurs when the segment of the aortic arch between the left common carotid and left subclavian artery is missing, and type C is when the aortic arch between the right and left common carotid arteries is absent. Mice doubly heterozygous for *Foxc1* and *Foxc2* (i.e. *Foxc1+/−;Foxc2+/−*) also present with coarctation and aortic and pulmonary valve dysplasia and also have IAA-A and IAA-B [46], whereas individual heterozygotes do not.

Folr1 is a folate-binding protein gene, and mice null for *Folr1* die at mid-embryogenesis from extensive cell death. However, folic acid supplementation of pregnant females greatly reduces this early embryonic lethality and allows for *Folr1*-null mice to be born and bred [48]. A small proportion of these *Folr1*-null foetuses had coarctation of the aortic arch or IAA, as well as a right-sided aortic arch and A-RSA. In these foetuses, coarctation was found proximal to the arterial duct.

Coarctation has also been described in transgenic mice doubly homozygous for the retinoic acid receptors alpha and beta (*Rara−/−; Rarb−/−*), and additionally these mice present with CAT, right-sided aortic arch and A-RSA [49]. Also, mice null for *Vangl2*, a membrane protein involved in the regulation of planar cell polarity, and the gene mutated in the loop-tail mouse, die from complex cardiovascular defects that include preductal coarctation as well as IAA, DORV, VSD and right-sided aortic arch [50].

42.8 Bicuspid Aortic Valve

The arterial (semilunar) valves form the three leaflets of the aorta and pulmonary trunk that prevent the backflow of blood to the heart. When only two leaflets of the aortic valves are formed, this is known as bicuspid aortic valve (BAV). Although BAV is frequently seen in patients, it has rarely been described in transgenic mouse models with cardiovascular malformation. This is either because the mouse is resilient to forming this phenotype, or more likely, its presentation is under-reported due to the technical difficulties in identifying it.

The transcription factor GATA binding protein (*Gata*) 5 is required for the development of the heart and endoderm in zebrafish [51]. In the mouse, *Gata5* is expressed in the developing heart [52], and the Gata family (*Gata4*, *Gata5* and *Gata6*) may function in endoderm induction in the mouse [53]. Mouse embryos null for *Gata5* present with BAV [54], and *GATA5* has been suggested to be a candidate modifier gene for congenital heart disease [55].

BAV and coarctation are seen in Turner syndrome patients with a specific deletion of the short arm of the X chromosome [56]. One of the genes in the deleted region, which will therefore be predicted to be haploinsufficient, is *USP9X*, a ubiquitinase involved in TGFβ-Smad signalling and which is expressed in the heart. Genes within the TGFβ superfamily of structurally related signalling proteins may therefore be important for the correct formation of the arterial valves. A mouse model of deficient signalling via the activin A receptor, type I (*Acvr1*) presents with BAV [57]. Activins are dimeric growth and differentiation factors which belong to the TGFβ superfamily. Moreover, transgenic mice lacking the bone morphogenetic receptor *Bmpr2* present with absent arterial valves, as well as CAT, IAA and VSD [58]. The ligands of this receptor are BMPs, which are also members of the TGFβ superfamily. Mice deficient in *Adamts5* (a disintegrin-like and metalloprotease domain with thrombospondin-type 1 motifs) display myxomatous valve disease where the arterial valves are enlarged [59]. This is linked to an accumulation within the valves of uncleaved versican, a large chondroitin sulfate proteoglycan. When mice null for *Adamts5* are complexed with *Smad2* heterozygosity (i.e. *Adamts5−/−;Smad2+/−*), a highly penetrant BAV phenotype occurs, concomitant with insufficient cleavage of versican as well as Smad2 phosphorylation [60]. This suggests that a reduction in TGFβ signalling exacerbates the *Adamts5* valve phenotype.

As described in Chap. 48, mice null for the homeodomain transcription factor *Pbx1* have CAT, but the other Pbx family members, *Pbx2* and *Pbx3*, when deleted have normal cardiovascular development [61, 62]. However, complex alleles of these three genes (homozygous and heterozygous) reveal their importance in the development of the arterial valves. Mice triply heterozygous for all three Pbx alleles (i.e. *Pbx1+/−;Pbx2+/−;Pbx3+/−*) display BAV [61], as do *Pbx1+/−;Pbx2−/−;Pbx3+/−* embryos who also present with a tetralogy of Fallot-type phenotype.

Conditional deletions of *Fgf8* have revealed that this gene is implicated in correct development of the arterial valves. BAV has been described in transgenic mice with a mesoderm-specific deletion of *Fgf8* [63] and when *Fgf8* was deleted from the pharyngeal epithelia [32].

In mice null for endothelial nitric oxide synthase (*Nos3*), a bicuspid valve is observed where it is the non-adjacent and right coronary leaflets that form the conjoined leaflet [64]. BAV also occurs in transgenic mice where Rho kinase signalling is disrupted specifically in neural crest cells [65].

Although the majority of great artery and valve defects have been described as a result of a genetic manipulation, it is sometimes possible to discover a phenotype within a wild-type animal model. This is the case with BAV seen in the Syrian hamster [66]. In this model, a significant proportion of hamster embryos present with a fusion of the leaflets associated with the coronary arteries to produce a bicuspid valve [67].

42.9 Aortic and Pulmonary Stenosis

Stenosis of the aortic and/or pulmonary outflow tract has been described in several transgenic mouse models. These include mutations in *Acvr1* [57], *Hand2* [68], hes-related family bHLH transcription factor with YRPW motif 2 (*Hey2*) [69], msh

homeobox 1 and 2 (*Msx1;Msx2*) [70], *Prdm1* [26] and transcription factor AP-2 α (*Tcfap2a*) [71]. In the majority of cases, the stenosis is seen in combination with other cardiovascular defects such as CAT, IAA, A-RSA, DORV and VSD. Most of these genes are expressed in the neural crest and the second heart field, underlying the importance of these cells in the formation of the vessels of the outflow tract.

Conclusion

As discussed in this chapter, there are many genes that appear to play a role in the formation and development of the arterial valves and the great arteries and when mutated or mis-expressed in animal models will result in blood vessel and valve defects. That different genes coding for a variety of transcription factors, growth factors and other types of molecules can give rise to similar defects to each other when mutated and illustrates the complexity of genetic networks and signalling pathways that control and orchestrate a very complex morphogenetic pathway that results in a functioning cardiovascular system. Also of interest is the apparent sensitivity of the fourth PAA to developmental defects and raises questions as to why this vessel in particular is so vulnerable to genetic or environmental insult with devastating clinical implications when affecting the left fourth PAA in particular. Various explanations have been postulated including its particular morphogenetic characteristics, blood flow during remodelling and gene expression.

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Part XII

Coronary Artery Anomalies

Clinical Presentation and Therapy of Coronary Artery Anomalies

43

David J. Driscoll

Contents

43.1 Normal Coronary Artery Anatomy

There are two major coronary arteries. The left main coronary artery divides into the left anterior descending and the circumflex coronary arteries. Branches of the left anterior descending coronary artery include the left conus, septal, and diagonal arteries. Branches of the circumflex coronary artery may include the sinus node artery, Kugel's artery, marginal arteries, and the left atrial circumflex artery (Fig. 43.1). Branches of the right coronary artery include the conal branch, the sinus node artery, an atrial branch, the right ventricular muscle branches (including the acute marginal branch), the posterior descending coronary artery, the atrioventricular node artery,

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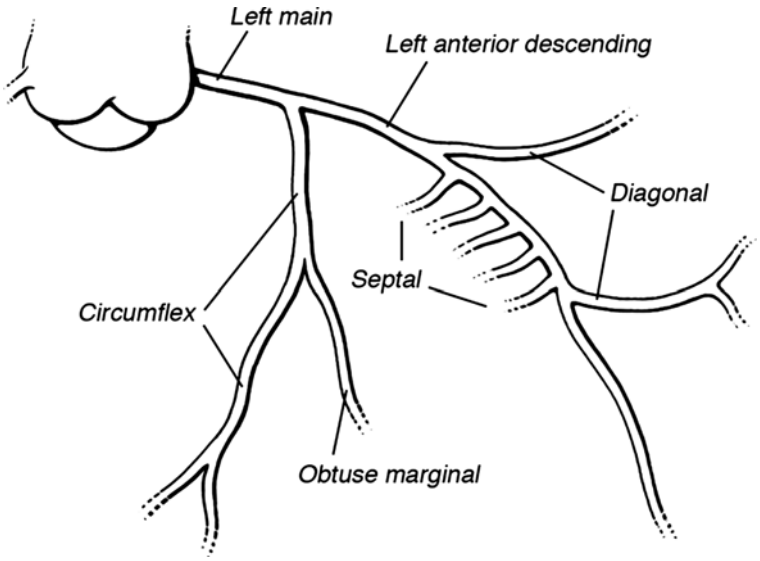

Fig. 43.1 Normal left coronary artery system

and septal branches (Fig. 43.2). The "dominant coronary artery" is the one giving rise to the posterior descending coronary artery. It originates from the right coronary artery in 80 % of people.

43.2 Anomalous Origin of the Left Coronary Artery from the Pulmonary Artery

A patient with anomalous origin of the left coronary artery from the pulmonary artery (ALCAPA) may present with signs and symptoms of myocardial infarction and congestive heart failure in infancy, or the condition may be unassociated with myocardial infarction or symptoms of heart disease until detected serendipitously in adulthood or at autopsy.

The ideal treatment of ALCAPA is to detect the presence of the anomaly before myocardial infarction occurs and to establish a coronary system that prevents myocardial infarction. All cases in infancy, however, come to medical attention only after myocardial ischemia and infarction have occurred. Attempts to establish a two-coronary-artery system for patients with ALCAPA are indicated when the condition is discovered.

43.3 Anomalous Origin of the Left Coronary Artery from the Right Sinus of Valsalva

Anomalous origin of the left coronary artery from the right sinus of Valsalva is a rare but important malformation because it is associated with a significant risk of sudden death (Fig. 43.3). Sudden death presumably is due to myocardial

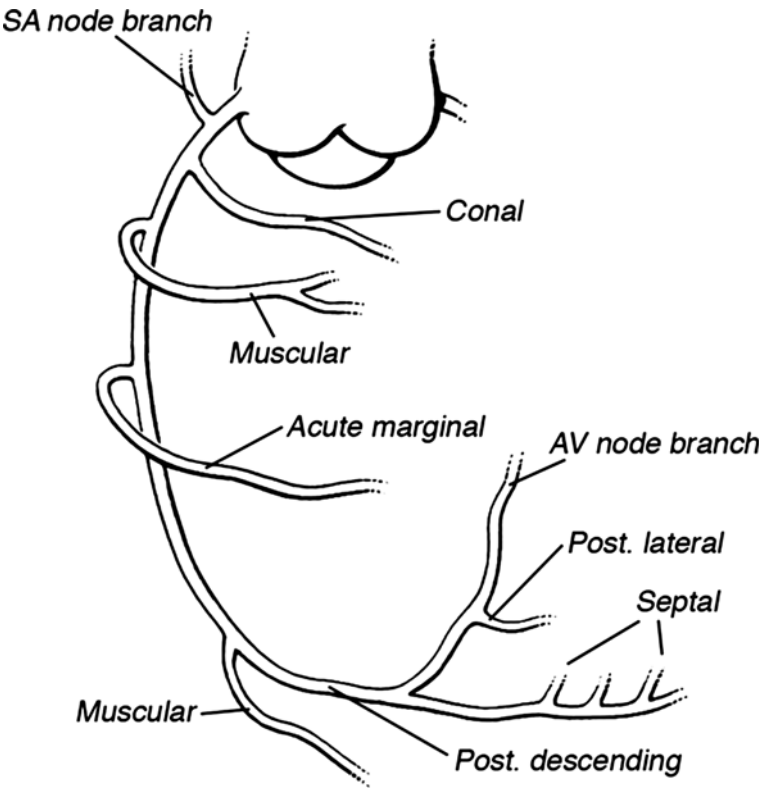

Fig. 43.2 Normal right coronary artery system

ischemia as a result of compression of the left coronary artery between the aorta and the pulmonary artery, an elliptical rather that a circular os of the left coronary artery, and compromise of the lumen of the left coronary artery due to acute angulation near its origin. Patients usually are asymptomatic until sudden death occurs, although some patients may have symptoms of angina or coronary insufficiency. Symptoms may include syncope or light-headedness associated with exercise.

43.4 Anomalous Origin of the Right Coronary Artery from the Left Sinus of Valsalva

It is unclear whether or not anomalous origin of the right coronary artery from the left sinus of Valsalva is associated with sudden death. Thus, the indications for operative repair of this lesion are less clear than when the left coronary artery arises anomalously from the right sinus of Valsalva. If the patient is symptomatic or there is evidence of myocardial ischemia, most clinicians would recommend repair.

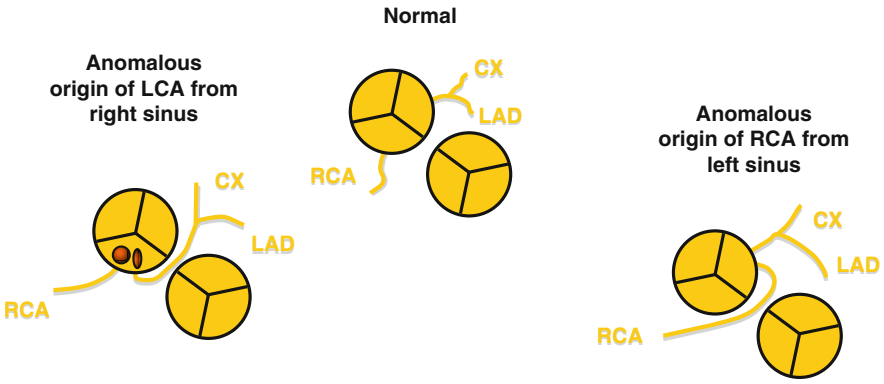

Fig. 43.3 Origin of left coronary artery from the right aortic sinus of Valsalva (*left panel*). Origin of the right coronary artery from the left sinus of Valsalva (*right panel*). Normal coronary origin (*center panel*). Abbreviations: *CX* circumflex coronary artery, *LAD* left anterior descending coronary artery, *RCA* right coronary artery (Reproduced or adapted from Driscoll, David, *Fundamentals of Pediatric Cardiology*, Lippincott Williams & Wilkins, 2006, with permission of the author and publisher)

43.5 Single Coronary Artery

Single coronary artery occurs in approximately two of every 1000 patients. It is associated with transposition of the great arteries, coronary artery fistula, and bicuspid aortic valve.

43.6 Coronary Artery Fistula

Coronary artery fistula constitutes 0.2–0.4 % of congenital cardiac defects. Fistulae originate equally from the right and left coronary arteries. Usually, the fistula connects to the right ventricular cavity. The right atrium is the second most common terminus, and two thirds of fistulae draining into the right atrium originate from the right coronary artery. A fistula also can terminate in the pulmonary artery, left atrium, left ventricle, superior vena cava, coronary sinus, or a persistent left superior vena cava. The involved coronary artery usually is dilated, and the chamber in which the fistula terminates may be enlarged.

43.7 Coronary Artery Patterns Associated with Congenital Heart Defects

43.7.1 Tetralogy of Fallot

Although only 4–5 % of patients with tetralogy of Fallot have associated coronary artery anomalies, these abnormalities must be identified so that damage to essential coronary arteries is avoided during repair of tetralogy of Fallot. Origin of the left

anterior descending coronary artery from the right coronary artery occurs in 4 % of patients with tetralogy of Fallot. Single coronary artery is the second most common coronary anomaly associated with tetralogy of Fallot.

43.7.2 d-Transposition of the Great Arteries

Usually, the right coronary artery arises from the posterior aortic sinus, and the left coronary artery arises from the left coronary sinus and divides into a circumflex and an anterior descending coronary artery. The right aortic sinus of Valsalva is the noncoronary cusp.

Alternatively, the right coronary artery may arise from the posterior aortic sinus and gives rise to the circumflex coronary artery, which passes posterior to the pulmonary artery. The anterior descending coronary artery may arise from the left coronary sinus, and the right aortic sinus of Valsalva is the noncoronary sinus.

43.7.3 l-Transposition of the Great Arteries

The aorta is anterior and to the left of the pulmonary artery in l-transposition of the great arteries (also known as corrected transposition of the great arteries or ventricular inversion). One aortic sinus of Valsalva is oriented anteriorly (anterior sinus of Valsalva), one posterior and rightward (right sinus of Valsalva), and one posterior and leftward (left sinus of Valsalva). The right coronary artery originates from the right aortic sinus of Valsalva and divides into an anterior descending branch that follows the course of the interventricular sulcus. The right coronary artery continues to follow a course in the right atrioventricular sulcus. The left coronary artery originates in the left aortic sinus of Valsalva and follows the course of the circumflex coronary artery in the left atrioventricular sulcus. The left (circumflex) coronary artery produces a marginal branch and continues as the posterior descending coronary artery.

Human Genetics of Coronary Artery Anomalies

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Beatriz Picazo and José M. Pérez-Pomares

Contents

Abstract

The genetics of human congenital coronary vascular anomalies (CCVAs) remains largely under-researched. This indeed is surprising, as coronary vascular defects represent a significant proportion of human congenital heart disease (CHD) and frequently are associated with malformation of different cardiac structures and diseases. In this brief chapter, we will attempt to summarize our current knowledge of the topic and propose a rationale for the development of novel approaches to the genetics of CCVAs.

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44.1 Introduction to Congenital Coronary Vascular Defects: An Isolated Condition?

Like the majority of CHDs, CCVAs do not seem to segregate in Mendelian ratios. However, some familial cases have been reported [1, 2] evidence that has been overlooked historically by the application of standard CHD diagnostic methods [3]. Regardless of our perception of the heritability of CCVAs, these should not be considered as isolated conditions only, as they strongly correlate with the malformation of other cardiac structures such as heart valves, cardiac chambers, and the myocardium. Furthermore, CCVAs can form part of the phenotype of well-characterized, complex CHDs such as transposition of the great arteries, tetralogy of Fallot, and double outlet right ventricle [4]. However, in these cases, it is difficult to assess whether coronary vascular anomalies are a direct consequence of the genetic defect or secondary to the disrupted mechanics of embryonic development. The tight relationship between CCVAs and other pathological cardiac entities is, nonetheless, emphasized by the classification proposed by the International Pediatric and Congenital Cardiac Code (IPCCC, www.ipccc.net) that groups in the same category developmentally related abnormalities such as arteriovenous fistulae, defects coronary artery patterning, and anomalies of the arterial duct and the pericardium (for an elaboration of these developmental concepts as related to the pathogenesis of CCVAs, please refer to Chap. 45).

44.2 Congenital Coronary Anomalies in the Context of Adult Cardiac Disease

Congenital coronary vascular anomalies have been found in cases of ventricular fibrillation, cardiomyopathy, myocardial infarction, and sudden death, among other severe cardiac conditions [5]. Sudden death can be regarded as a showcase for the clinical significance of CCVAs, since 11–19 % of sudden deaths in American athletes were reported to involve coronary malformations [6]. Accordingly, attempts to classify forms of CCVAs have frequently been based on the severity of the clinical outcome of the malformation [7] rather than on comprehensive, anatomical, and/or developmental criteria [8]. This has resulted in a biased, incomplete understanding of CCVAs which precluded the identification of the embryological origin of these defects, as well as the establishment of a causal association between defined genes and morphogenetic mechanisms.

Congenital anomalies of the heart are important, but also it is true that today many CHD patients survive to adulthood [9]. However, a negative counterpart of this positive clinical outcome is that the potential genetic substrates for congenital heart defects are progressively increasing their chances of transmission to the next generation. This unambiguously points to the necessity of planning new diagnostic criteria for CCVAs in the near future.

44.3 Candidate Genes for Human Coronary Blood Vessel Congenital Defects

Given the clinical relevance of CCVAs, it is surprising to discover that our knowledge of the genetics of these anomalies is extremely poor. Association of multiple gene variants with coronary atherosclerosis (simply referred to as "coronary disease") is common in the literature [10], but the number of systematic studies of the genetics of CCVAs is very low. In humans, several genome-wide association studies (GWAS) have identified specific regions (e.g., 12q24 locus) which are related to distinct CHD forms, namely, tetralogy of Fallot and Noonan and LEOPARD syndromes. Deletions at this locus have also been associated with atherosclerotic coronary disease, but no specific reference to CCVAs was made in this study [11]. Other investigators have identified a role for copy number variations (CNVs) in several forms of CHD but not coronary artery congenital anomalies [12].

These results might not seem encouraging to those interested in the genetics of CCVAs; however, we would like to suggest a strategy to reevaluate data from previously existing genetic screenings. This strategy is based on the known association of CCVAs with increased risk of atherosclerosis and deficient myocardial perfusion and myocardial infarction [13]. Knowledge of embryonic coronary blood vessel development can be applied to the identification of genetic variants in regions close to genes important for coronary vascular morphogenesis. We should therefore focus on studies aimed at the identification of genetic factors for coronary atherosclerotic disease (see [14]), and search for genes related to cellular and molecular mechanisms involved in blood vessel development. Following this rationale, two good candidates are to be found associated with variations at regions 11q22.3 [15] and 6q23.2 [16], close to *PDGF* (platelet-derived growth factor) and *TCF21* (transcription factor 21), which are key genes in epicardial and coronary blood vessel embryonic development, respectively [17, 18]. Schunkert and colleagues [16] also identified variations at 13q34, close to the *COL4A1* (collagen type IV alpha 1) gene, reported to be required for vascular formation in the embryo [19]. Even more interesting is the case of variations identified at 10q11, close to the *CXCL12/SDF1* (chemokine (C-X-C motif) ligand 12) gene [20], since the chemokine encoded by this gene is necessary for the proper septation of the cardiac outflow tract [21], anomalous development of which can lead to coronary vascular defects [22]. In accordance with our proposed strategy, it also seems logical to reconsider previously described single nucleotide polymorphisms (SNPs) in the vascular endothelial growth factor (VEGF) promoter (C2578A, G1154A, C634G) [23, 24] as potential genetic substrates for CCVAs. This is supported by multiple studies highlighting the relevance of VEGF during blood vessel formation in general [25] and coronary vasculature morphogenesis in particular [26].

44.4 Does Coronary Blood Vessel Embryonic Morphogenesis Influence Adult Coronary Disease?

Besides the adult persistence of coronary blood vessel congenital anomalies, coronary disease is, in large part, regarded as a synonym for coronary blood vessel atherosclerotic disease (CAD). Generally considered as an "acquired" disease, CAD is a prevalent condition that could, at least in part, have a congenital substrate. This hypothesis is supported by the finding of early atherosclerotic traits like intimal thickening and fatty streaks in fetuses and newborns [27], including coronary arteries [28]. It is not known whether factors related to the peculiarities of coronary artery embryonic development may impact the initiation of atherosclerotic disease in fetuses, newborns, and children and therefore the full progression of the disease in the adult. It is possible that sustained hyperlipidemia elicits an autonomous endothelial response to pathologic metabolic stimuli [27], but intrinsic differences in distinct populations of endothelial cells should also be considered. In this regard, it has been shown that arteries from different anatomical positions show a differential resistance to hypercholesterolemia-induced fatty streaks [29], and it is thus tempting to speculate with embryonic coronary vessel endothelial heterogeneity as a factor involved in the early susceptibility of these structures to atherosclerosis.

Conclusions

Systematic studies will be required to improve our knowledge of the genetics of coronary congenital anomalies. These studies should use criteria related to the embryonic development of coronary vasculature to filter data from homozygosity mapping, transcriptome and array comparative genomic hybridization (array CGH) for linkage analyses, GWAS, and CNV approaches and identify potential candidates for CCVAs. This could be relevant especially if applied to samples from familial cases of CCVAs or selected cases of atherosclerotic coronary artery disease in which plaque formation relates to morphologically anomalous coronary blood vessels.

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Molecular Pathways and Animal Models of Coronary Artery Anomalies

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Abstract

The coronary vascular system is a sophisticated, highly patterned anatomical entity, and therefore a wide range of congenital malformations of the coronary vasculature has been described. Despite the clinical interest of congenital coronary artery anomalies (CCA), very few attempts have been made to relate specific embryonic developmental mechanisms to the congenital anomalies of these blood vessels. This is so because developmental data about the morphogenesis of the coronary vascular system is derived from complex studies carried out in animals (mostly transgenic mice) and may not be noted by the clinicians who take the care of these patients. We will try to offer embryological

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explanations for a variety of CCA based on the analysis of multiple animal models for the study of cardiac embryogenesis, and suggest to the reader developmental mechanistic explanations for the pathogenesis of these anomalies.

45.1 Introduction to Embryogenesis of the Coronary Vasculature

45.1.1 The Basic Elements of the Coronary Vascular System

A wide range of congenital coronary artery anomalies (CCA) has been described [1, 2]. To properly understand the origin of CCA, it is important to be aware of some critical developmental and anatomical features of this vascular system. As evidenced by a simple phylogenetic analysis of the vertebrate clade, the coronary vasculature forms by the coalescence of two anatomical domains or elements. The first, proximal one, is represented by aortic arch efferent vessels in fish or the aortic root in most tetrapods (including the coronary ostia). The second, distal element, includes the characteristic inverted crown-shaped network of blood vessels lying over the cardiac atrioventricular and ventricular surfaces. The connection of these two domains guarantees the provision of oxygenated blood to the working myocardium from the closest possible source. This connection is, however, a late event in avian and mammalian coronary vascular development, and therefore any mechanistic analysis of CCA has to take into account that these two components of the adult coronary vascular system form separately.

45.1.2 Coronary Arteries and Veins

The embryonic formation of coronary arteries and veins (the two main blood vessel types of the coronary vascular system) deserves attention of its own. Recently, a number of investigators have reported the origin of the arterial and venous endothelium from a variety of sources, suggesting that coronary endothelium is a developmental mosaic [3–6] (see Chap. 6). Since the endothelium is the tissue scaffolding that guides blood vessel development in all vertebrates, it is obvious that the origin of embryonic coronary endothelium is key point to understand coronary blood vessel development.

The findings quoted above (for specific details, please refer to Chap. 6) explain why arteries and veins, as reported by several authors over three decades of research on coronary embryonic development [7, 8], form as two separate vascular plexuses which merge shortly before birth. The early uncoupling of coronary arterial and venous vascular networks during coronary development adds an additional layer of complexity to the identification of the cellular and molecular mechanisms accounting for CCA.

45.2 A Brief Classification of CCA

A simple, summarized description of the nature of these congenital anomalies is necessary for the understanding of the following sections.

CCA often are classified, in accordance to their clinical impact, into "major" and "minor" anomalies [9]. This classification may be practical to the clinician, but does not provide information as to the specific developmental and/or genetic origin of the known spectrum of CCA, nor allows for the establishment of potential correlations between different cardiac and coronary blood vessel anomalies. We favor a simple classification which groups CCA following an embryological rationale. This classification has three main categories:

(a) *Anomalies of coronary artery connection to the great arteries* (*CCA Group 1*)
These include (1) the anomalous connection to the pulmonary root; (2) anomalous connection to the non-coronary (posterior/dorsal) sinus of Valsalva; (3) absence of the right, left, or both coronary arteries; (4) and alteration of the position, form, and size (width) of coronary *ostia*.

(b) *Anomalies of coronary anatomy* (*CCA Group 2*)
These include coronary aneurysms and abnormal ramification of coronary blood vessels.

(c) *Anomalies of myocardial-coronary interaction (CCA Group 3)*
These include (1) the abnormal intramural or subendocardial course of coronary arteries and (2) fistulae (abnormal connection of coronary arteries to the ventricular or atrial cavities, sinus venosus, superior vena cava, aorta, or pulmonary artery).

In the following section, we will present and discuss animal models for, at least, part of the CCA groups listed above (graphically summarized in Fig. 45.1).

45.3 Developmental Substrates of Congenital Coronary Anomalies

In principle, any gene required for proper blood vessel development could be a genetic substrate for CAA. However, animal models for these genes (mostly transgenic/mutant mice) often die before coronary blood vessels form, as they often display deficient yolk sac vascularization [10]. Therefore, in this chapter, we will focus on mice, a species displaying defects in coronary blood vessels similar to those observed in humans.

45.3.1 Defective Outflow Tract Septation and Rotation

The majority of the known forms of congenital heart disease affect the outflow tract (arterial pole of the heart), and some of them include coronary anomalies as part of their phenotype. Several cellular mechanisms coordinate outflow tract transformation into the mature aorta and pulmonary arteries and valves. The first one is the

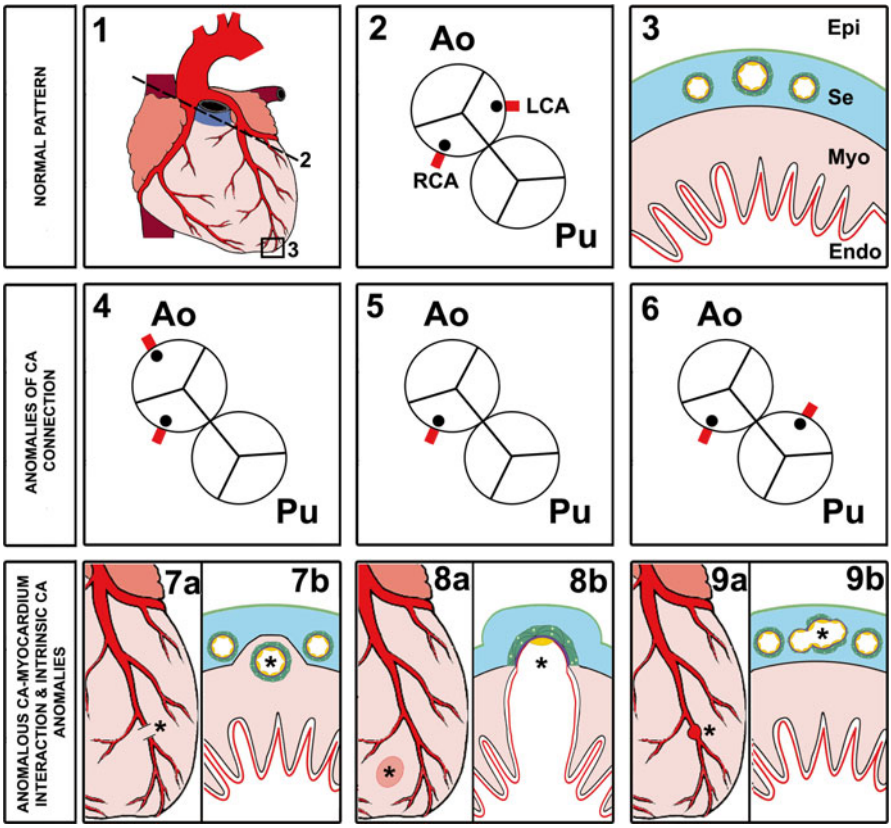

Fig. 45.1 Normal and abnormal coronary anatomy. Cartoons illustrate normal (*1–3*) coronary anatomy and relevant cases of coronary congenital anomalies (*4–9*). *Asterisks* indicate the specific location of myocardial bridges (*7b*), coronary fistulae (*8b*), and coronary aneurysms (9b)

septation of the embryonic outflow tract of the heart (common truncus arteriosus), predominantly driven by cardiac neural crest cells (NCC) [11, 12]. Persistent truncus arteriosus (PTA) is a severe congenital heart disease that results from restricted or absent outflow tract septation [13]. This anomaly also affects the structure of arterial semilunar valves and the relative position of the coronary *ostia*. PTA is characteristic of null mice for genes relevant to NCC development like *Pax3* (paired box 3) [14] or *Pbx1* (pre-B-cell leukemia homeobox 1) [15].

Abnormal NCC migration defects also arise as a result of mutations of genes in the NOTCH signaling pathway, which has been shown to be required for the proper development of outflow tract, endocardial cushions, and arterial valve primordia [16]. This study emphasizes the importance of tissue interaction during outflow tract development, proving that NCC and secondary heart field progenitor interaction is necessary for endocardial-derived valvuloseptal mesenchyme formation via NOTCH signaling. Accordingly, mutant mice for the NOTCH target gene *Hes1* (hes family bHLH transcription factor 1) display outflow defects [17].

The best known mouse model for outflow tract anomalies is that of the *Tbx1* (T-box 1) knockout, a candidate gene for DiGeorge or 22q11.2 deletion syndrome. In *Tbx1* null mice with PTA both right and left coronary ostia preferentially locate at the right or ventral aortic sinus of Valsalva and present a proximal left coronary artery stem that courses abnormally ventrally rather than laterally [18]. These authors suggest it is the loss of the subpulmonary myocardium, hypothesized to be refractory to blood vessels, that may play a role in this coronary defect.

Proper rotation of the outflow tract is as important as correct outflow tract septation, and both phenomena are developmentally linked by the neural crest [19] and the genetic specification of outflow tract myocardial subpopulations [19, 20]. For example, the loss of the extracellular matrix component PERLECAN (*Hspg2* encoding heparan sulfate proteoglycan 2) first affects NCC migration and then outflow tract rotation, resulting in transposition of the great arteries [21] and in multiple defects in left and right coronary stem course [22]. These latter anomalies suggest that it is the location of the aortic root that determines the path followed by coronary blood vessels at the base of the heart and this can be quite variable [1, 23]. As a result of outflow tract malrotation, coronary ostia frequently appear at abnormal anatomical positions [24].

In summary, we suggest that the disruption of outflow tract septation and rotation, especially when combined with mispatterning of the unique antivascular properties of the subpulmonary myocardium [18] and the provascular properties of aortic myocardium (via Vegf-C encoding vascular endothelial growth factor C, [25]), is sufficient to explain an important part of group 1 CAA.

45.3.2 Anomalous Epicardial Development

It has been proposed that the epicardium and embryonic coronary blood vessels are a developmental continuum [26]. The intricate relationship existing between these two structures can explain some CAA (groups 1–3) through the alteration of four basic developmental epicardial mechanisms:

45.3.2.1 Epicardial Formation and EMT

The primitive epicardial epithelium forms after epicardial progenitor cells are transferred from the proepicardium to the myocardial surface [27, 28]. Abnormal primitive epicardium formation is known to affect ventricular myocardium growth and coronary vascular development [29]. Integrins are currently the only type of adhesion molecule that have been shown to be involved in this process [30]. The α4-integrin null mouse displays severe ventricular myocardial thinning and coronary phenotype [31]. Soon after the primitive epicardium forms, epicardial epithelial-to-mesenchymal transition (EMT) is initiated. Epicardial EMT is a finely regulated process which supplies the heart with highly invasive mesenchymal epicardially derived cells (EPDCs) [26]. Multiple molecules participate in triggering EMT. To date, TGFβ (transforming growth factor beta) 1–2 appears to be the strongest epicardial EMT inducer [32–34], as shown by the epicardial deletion of the

TGFβ type I receptor ALK5 (TGFBR1), which disrupts epicardial EMT and coronary blood vessel development [35].

Epicardial transcription factors Wt1 and Tbx18, together with canonical WNT effectors such as β-CATENIN and retinoic acid, also participate as effectors of the EMT process by modulating cell adhesion and motility [36, 37]. Potentially, genetic defects in any of the genes encoding for all these molecules (or participating in their biosynthesis) could give rise to CCAs, but due to the importance of primitive epicardial formation and EMT, individuals carrying such a defect are likely to die during gestation. Still, experimental studies using the chick embryo as experimental animal model reveal that a simple delay in epicardial formation and EMT activation is sufficient to generate abnormal coronary patterning and arterioventricular connections or fistulae (CAA group 3) [38, 39], anomalies which are associated with survival to adulthood.

45.3.2.2 Epicardial and Myocardial Instructive Signaling

The epicardium does not only contribute cells to the developing coronary vascular system, but it also acts as a signaling center by secreting various morphogens. These epicardial-secreted molecules include retinoic acid, FGF (fibroblast growth factor) 9,16,20, and IGF2 (insulin-like growth factor 2) [26, 40]. Retinoic acid seems to also act in an autocrine fashion supporting the secretory activity of the epicardium [41] which in turn affects coronary development. Although the characterization of this secretome has been carried out with the main goal of identifying epicardial-secreted molecules regulating myocardial compact layer growth, many of these molecules are likely to affect coronary blood vessel development. As a matter of fact, the thin myocardium that characterizes mice mutant epicardial molecules frequently carries aberrant coronary blood vessels [31, 36, 42], reflecting abnormal ventricular patterning and ramification of coronary vasculature (group 2 CCA).

The epicardium also is sensitive to widely distributed molecules like erythropoietin (EPO) [43] and, accordingly, erythropoietin receptor knockouts have severe coronary dysmorphogenesis [44]. Interfering with embryonic epicardial transcriptional regulation, as shown by the molecular characterization of *Wt1* (Wilms tumor 1) and *Tbx18* mouse mutants [36, 45], reveals anomalies in the expression of secreted molecules such as VEGF-A and ANGIOPOIETIN-1, growth factor receptors (e.g., PDGFRα,β), or mediators like smoothened and patched (components of the hedgehog signal transduction machinery), all of which are known to be involved in coronary blood vessel development [5, 46–48].

The developing myocardium also contributes to coronary development by providing a material substrate for blood vessel formation, as evidenced by the altered coronary patterning found in mice defective for myocardially expressed *Vcam-1* (vascular cell adhesion molecule 1) and *Vangl-2* (VANGL planar cell polarity protein 2). Of these two genes, *Vcam-1* encodes for a cell adhesion molecule which can act as ligand for α4 INTEGRIN-containing cell receptors [49], while VANGL-2 is a molecule involved in the planar cell polarity signaling pathway [50]. Genetic deletion of cofactors involved in the transcriptional regulation of the myocardium like FOG-2 (zinc finger protein, FOG family member 2) also gives rise to anomalous coronary development [51].

45.3.2.3 Determination and Differentiation of Coronary Progenitor Cells

Misspecification of coronary cell types and/or poor deployment of mesenchymal EPDCs can alter early coronary vascular formation. Knockout embryos for the gap junction *connexin 43* gene, which is expressed by epicardial progenitor (pro-epicardial) cells, have severe defects in coronary vascular patterning [52]. Accordingly, *Wt1-null* mice present decreased coronary endothelial and ectopic smooth muscle differentiation, eventually impairing coronary formation at midgestation [36]. Abrogation of NOTCH1 signaling (a well-known cell fate determinant) in the epicardium severely impacts coronary morphogenesis [42, 53].

Some other genes such as *TCF21* (transcription factor 21) have been reported to be involved in the early specification of fibroblasts from the epicardium, as shown by the epicardial EMT and coronary morphogenesis defects displayed by *TCF21* mutants [54]. It is of particular interest to consider here that epicardial progenitor (pro-epicardial) cells seem to carefully balance their developmental fate as a response to local BMP2 (bone morphogenic protein 2) and FGF2 levels [55], suggesting that genetic defects in any component of these signaling pathways may result in defects in coronary development.

45.3.2.4 Muscularization and Stabilization of Coronary Artery Anatomical Pattern

The muscular fate of EPDCs is modulated by the subepicardial microenvironment and requires activation of SRF-dependent pool of characteristic smooth muscle genes [56] and p160 RHO-KINASE activation [57]. Both VEGF-A and retinoic acid have been shown to act synergistically to favor early endothelial differentiation from EPDCs at the expenses of smooth muscle cell differentiation [58]. This study proposes, for the first time, a mechanism that explains the physiological delay in the muscularization of developing blood vessels, which is supposed to allow for the extensive remodeling of the primitive coronary vasculature before its maturation and stabilization is initiated. Therefore, premature muscularization of the developing coronary blood vessels may alter the ramification and complexity of adult coronary vasculature, whereas a delay in the formation of the vascular medial wall could result in the formation of aneurism-like defects (Group 2 CCA).

45.3.3 Outgrowth of Endocardial Cells

Defective endocardial incorporation to the coronary vascular system from both sinus venosus-derived and ventricular endocardium can impair coronary vascular patterning. In order to establish a functional vascular circuitry, independent coronary vascular plexuses have to connect growing through the ventricular chamber wall. Altered transmural vascular growth gives rise to abnormal coronary arteriovenous shunts, arterioventricular connections (fistulae), defects that are compatible with postnatal and adult life [2].

It is logical to think that *VEGF* is a good candidate gene to explain these developmental alterations, because epicardial-to-endocardial VEGF gradients (sensitive to the transmural changes in oxygen tension) are known to determine compact myocardium colonization by embryonic blood vessels [5], and developing vascular structures are known to be extremely sensitive to VEGF doses [59].

Conclusion

As a summary for this chapter, a list of selected genes involved in CAA is provided in Table 45.1. Please note that the selection of these molecules is based on their known involvement in coronary morphogenesis in mice. The functional properties of the molecules encoded by these genes have been experimentally tested by germ line (systemic mutants) or conditional (tissue specific) gene deletion, but determining whether these genes also participate in human coronary development will require further, systematic genetic analysis of human CCAs.

Table 45.1 Genes required for coronary vascular development

Genes deleted or altered	Phenotype	References
Neuropilin overexpression	Hypoplastic ventricular myocardium; aberrant coronary vasculature	[60]
VCAM k.o.	Hypoplastic ventricular myocardium, poor epicardial integrity, and abnormal coronary development	[49]
a-4 integrin k.o.	Poor epicardial integrity; abnormal coronary development	[31]
Erythropoietin receptor k.o.	Hypoplastic ventricular myocardium; epicardial detachment and underdeveloped subepicardium; abnormal coronary morphogenesis	[44]
WT1 k.o.	Hypoplastic ventricular myocardium, poor epicardial integrity reduced EPDCs, and abnormal coronary morphogenesis	[36, 61]
FOG-2 k.o.	Hypoplastic ventricular myocardium; abnormal coronary vessels	[51]
Connexin 43 k.o.	Abnormal coronary patterning and presence of conal “pouches”	[52]
RXRa epicardial deletion	Thin myocardium; abnormal epicardial epithelial-to-mesenchymal transformation; defects in coronary vascular formation	[62]
KCNJ8 k.o.	Defects in coronary vascular development	[63]
Tgf-b type I receptor Alk5 epicardial deletion	Thin myocardium and defective coronary muscularization	[35]
A_{2A} *receptor* k.o.	Defects in coronary artery formation	[64]
Vangl2 k.o.	Disrupted the organization of the cardiomyocytes and formation of the coronary vessel	[50]
*PDGFR*beta k.o.	Thin myocardium; fail to form coronary vessels on the ventral heart surface	[47, 65]

Table 45.1 (continued)

Genes deleted or altered	Phenotype	References
Tgfbr2 smooth muscle cell deletion	Anomalous epicardium, myocardium, and coronary smooth muscle cell formation	[66]
Notch k.o.	Disrupts coronary artery differentiation, reduces myocardium wall thickness and myocyte proliferation; smooth muscle differentiation	[42, 53]
Tgfbr3 k.o.	Poor coronary vessel development	[67]
Nephrin k.o.	Abnormal epicardial cell morphology and a reduced number of coronary vessels	[68]
FAK smooth muscle cell deletion	Defective coronary smooth muscle cell formation	[69]
Hand2	Defective epicardialization and failure to form coronary arteries	[70]
Tcf21 k.o.	Hemorrhaging in the pericardial cavity and thin myocardium	[54]
COUP-TFII endocardial and myocardial deletion	Atrioventricular septal defects; thin-walled myocardium; abnormal coronary morphogenesis	[71]
Tbx18 k.o.	Perturbation of EPDCs; altered coronary development	[45]
VEGF-C k.o.	Inhibited dorsal and lateral coronary growth	[25]
Tbx5 pro-epicardial deletion	Epicardial and coronary blood vessel formation defects	[72]

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Part XIII

Truncus Arteriosus

Clinical Presentation and Therapy of Truncus Arteriosus

46

David J. Driscoll

Contents

46.1 Introduction

Truncus arteriosus (TA, also known as common arterial trunk) consists of a ventricular septal defect and only one great artery ("the truncus") arising from the heart (Fig. 46.1). This great artery is positioned above the ventricular septal defect and gives rise to the coronary arteries, the pulmonary arteries, and the aortic arch. TA has been classified as three types. In type 1 the right and left pulmonary arteries arise from a main pulmonary artery that arises from the aorta. In type 2 the right and left pulmonary arteries arise from separate orifice but close together. In type 3 the pulmonary arteries arise separately and distant from each other. Significant associated anomalies include truncal valve insufficiency, stenosis, and interrupted aortic arch. Truncus arteriosus constitutes approximately 0.7 % of all congenital heart defects. It results from abnormal conotruncal septation. There is a relatively

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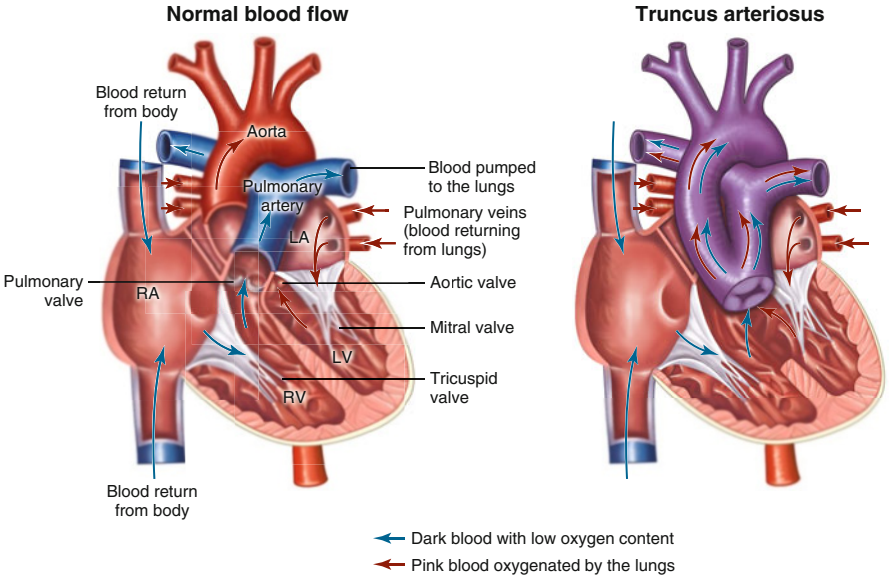

Fig. 46.1 Diagrammatic representation of normal circulation (*right panel*) and truncus arteriosus (*left panel*). Note that in truncus arteriosus, there is only one semilunar valve and this is referred to as the "truncal valve." It can be tricuspid or quadricuspid (as is shown in the diagram). Abbreviations: *RA* right atria, *LA* left atria, *RV* right ventricle, *LV* left atrium (Reproduced or adapted from Driscoll D (2006) Fundamentals of pediatric cardiology. Lippincott Williams & Wilkins, with permission of the author and publisher)

common association between microdeletion 22q11.2 and truncus arteriosus especially if there is interruption of the aortic arch.

46.2 Pathologic Physiology

Cyanosis occurs because of a right-to-left shunt at the level of the ventricular septal defect and is dependent upon the volume of pulmonary blood flow. The relative volume of pulmonary and systemic blood flow depends on the relative resistance to flow into the pulmonary vascular bed and into the systemic vascular bed. The resistance to flow into the pulmonary vascular bed also will be effected by the presence, absence, and severity of pulmonary arterial stenosis.

46.3 Clinical Presentation

Infants with truncus arteriosus may present with cyanosis or severe congestive heart failure.

46.4 Physical Examination

These patients have a prominent right ventricular impulse at the lower left sternal border. Usually, there is a systolic ejection murmur at the left sternal border. There may be an apical aortic ejection click and increased pulse pressure. If there is truncal valve insufficiency, there will be a decrescendo diastolic murmur. If there is associated interruption of the aortic arch, the femoral pulses may be decreased or absent.

46.5 Echocardiography and Cardiac Catheterization

The diagnosis is made by echocardiography. Cardiac catheterization is necessary for cases in which knowledge of the pulmonary vascular resistance is necessary to determine operability.

46.6 Treatment

In infancy, as pulmonary arteriolar resistance decreases, significant congestive heart failure develops. The initial management consists of medical treatment of the congestive heart failure (diuretics and, perhaps, afterload-reducing agents). Surgery to correct truncus arteriosus is necessary and should be performed before age 3 months. Surgical correction involves closure of the ventricular septal defect, separation of the pulmonary arteries from the truncus, and establishing continuity between the right ventricle and pulmonary arteries with a conduit. Early surgical correction is necessary to prevent the development of pulmonary vascular obstructive disease and to treat the congestive heart failure.

46.7 Outcome

Operative mortality depends on the associated problems in a patient with truncus arteriosus. In the absence of interrupted aortic arch and/or truncal valve stenosis/insufficiency, the surgical mortality is in the range of 5–10 %. Since the repair includes placement of a conduit from the right ventricle to the pulmonary artery, this will have to be replaced as the patient outgrows it or it becomes stenotic or insufficient. For patients with interrupted aortic arch, reoperation or balloon dilatation may be necessary because of the recurrent narrowing of the aorta as the patient grows.

Human Genetics of Truncus Arteriosus

47

Hiroyuki Yamagishi

Contents

Abstract

Human genetic studies have revealed that truncus arteriosus is highly associated with 22q11.2 deletion syndrome. Other congenital malformation syndromes and mutations in genes encoding NKX and GATA transcription factors have also been reported as its etiology.

47.1 Introduction

Truncus arteriosus (TA) is an uncommon congenital heart defect (CHD) that results from complete failure of aorticopulmonary septum formation. Embryologically, ablation of cardiac neural crest cells (CNCC) leads to failure of partitioning the

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embryonic truncus arteriosus and disrupts conotruncal development by interfering with addition of the myocardium derived from the second heart field (SHF) (Fig. 47.1), resulting in TA [1].

The etiology of most patients with TA is usually unknown, and it is considered to be heterogenous and multifactorial in nature. Approximately 60 % of TA occurs as an isolated cardiovascular malformation, while the remainder has additional extracardiac anomalies and is frequently syndromic [2]. A diabetic mother has an increased risk of developing TA compared with the infant of a nondiabetic mother [2, 3]. A typical pattern of malformation was reported in embryos exposed to retinoic acid involving conotruncal and aortic arch anomalies including TA [2, 3].

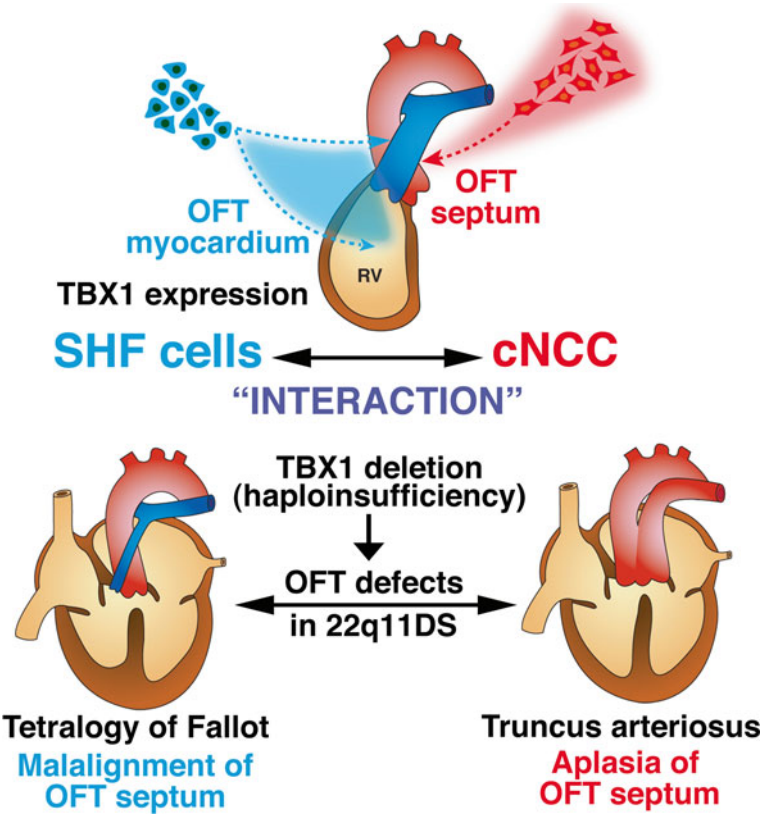

Fig. 47.1 Cellular and molecular basis for normal and abnormal outflow tract development of the heart. The interaction of progenitor cells derived from the second heart field (SHF cells) and the cardiac neural crest (*CNCC*) that give rise to the outflow tract (*OFT*) myocardium and septum, respectively, plays a key role in the development of the OFT. TBX1, a major genetic determinant of the 22q11.2 deletion syndrome (22q11DS), is exclusively expressed in the SHF cells. TBX1 deletion in 22q11DS may affect not only the SHF cells but also the interaction of the SHF cells and CNCC and result in OFT defects ranging from tetralogy of Fallot, which is characterized by malalignment of the OFT septum, to truncus arteriosus, which results from aplasia of the OFT septum

Several genes encoding transcription factors and signaling proteins highlight the importance of the CNCC and SHF and their interaction [4]. Mice deficient for genes encoding these proteins result in TA. Syndromes with chromosome 22q11.2 microdeletion served as an entry to understanding the genetic basis for TA, and a gene encoding the transcription factor T-box 1 (TBX1) has been proposed as a major genetic determinant [5, 6]. To date, mutations of genes encoding NKX or GATA transcription factors have been reported in patients with TA without 22q11.2 deletion.

47.2 Genetics

TA has no striking gender difference in frequency. Several reports describe families with one proband with TA and several relatives with CHD although most cases of TA occur sporadically. A recurrence risk for siblings of probands with TA is reported as 1.2 % in a combined study [7] and 6.6 % in a consecutive study of 49 families [8]. In some families, the affected relatives have concordant defects, namely, conotruncal or cardiac outflow tract defects, whereas in other families, the defects are morphologically discordant, suggesting that the recurrence risk is highly variable and dependent on the specific etiology in each family. Syndromes and genes associated with TA are summarized in Table 47.1.

47.2.1 Syndromes and Chromosomal Anomalies

Approximately 40 % of patients with TA are syndromic. TA accounts for approximately 10 % of cardiovascular malformations associated with the 22q11.2 deletion syndrome. It was also reported that 2–3 % of TA patients have chromosomal anomalies other than 22q11.2 deletion [2].

Table 47.1 Genetic etiology for truncus arteriosus (TA)

Genetic causes	Locus	Frequency
Syndrome		
22q11.2 deletion syndrome (22q11DS) DiGeorge syndrome Velo-cardio-facial syndrome Conotruncal anomaly face syndrome	22q11.2 (10p14-13)	Common[a]
Single gene		
TBX1 (transcription factor)	22q11.2	Rare
NKX2-5 (transcription factor)	5q35.1	Rare
NKX2-6 (transcription factor)	8p21.2	Rare
GATA4 (transcription factor)	8p23.1	Rare
GATA6 (transcription factor)	18q11.2	Rare

CHD congenital heart disease

[a]TA accounts for about 10 % of CHD in 22q11DS; 22q11DS accounts for about 35 % of patients with TA

47.2.2 22q11.2 Deletion Syndrome (22q11DS)

Monoallelic microdeletion of chromosome 22q11.2 results in the most common human genetic deletion syndrome with an incidence of 1 in 4000–5000 live births [5, 6, 9]. Recognition that DiGeorge syndrome (DGS), velo-cardio-facial syndrome (VCFS), and conotruncal anomaly face syndrome (CAFS) have overlapping clinical presentations and share 22q11.2 deletion revealed a common etiology of these clinical entities.

The clinical findings associated with 22q11DS are highly variable. Approximately 80 % of patients with 22q11DS are born with CHD. The types of CHD are variable, but characterized as conotruncal and aortic arch defects including tetralogy of Fallot (TOF) (~30 %) (see Chap. 32), interrupted aortic arch (IAA) type B (~15 %), ventricular septal defect (VSD) (~15 %), TA (~10 %), and others (~5 %) (Fig. 47.1). 22q11.2 deletion is the second most common genetic cause of CHD and is present in ~60 % of patients with IAA type B, ~35 % of patients with TA, and ~15 % of patients with TOF (~55 % of patients with TOF plus pulmonary atresia and major aortopulmonary collateral arteries) [10, 11].

As for the type of TA associated with 22q11.2 deletion, Momma et al. reported that three had type A1 and two had type A3 with no other in their five consecutive patient types, according to the Van Praagh classification [12], while Goldmuntz et al. reported all four types in their largest series of consecutive patients with TA and 22q11.2 deletion [10]. They also found that patients with the additional finding of an aortic arch anomaly (such as a right aortic arch or aberrant subclavian artery) were more likely to have a 22q11.2 deletion than those with a normal left aortic arch.

A genetic test for 22q11.2 deletion would be recommended to screen all infants who are newly diagnosed with TA because the syndromic feature might be difficult to identify in the neonatal period and the finding of the deletion could facilitate the clinician to carefully detect the associated extracardiac features. It is also important to provide accurate recurrence risk and comprehensive genetic counseling for the family.

47.2.3 Other Syndromes

CHARGE syndrome is an acronym characterized by a pattern of congenital anomalies including coloboma of the eye (C), heart anomaly (H), atresia of choanal (A), retardation of mental and somatic development (R), genital anomalies (G), and ear abnormalities and/or deafness (E). Mutations of CHD7 on 8q12.1-q12.2 are found in approximately 60 % of patients with CHARGE syndrome [13]. A mutation of *SEMA3E* on 7q21.11 encoding semaphorin 3E was also reported [14]. Approximately 30–40 % of CHD associated with CHARGE syndrome represents outflow tract defects including TA.

VACTERL association is an acronym for the nonrandom association of vertebral defects (V), anal atresia (A), cardiac malformations (C), tracheoesophageal fistula

(T) with esophageal atresia (E), radial or renal dysplasia (R), and limb anomalies (L). A mutation of *HOXD13* (homeobox D13) on 2q31.1 was reported in a patient with VACTERL association [15]. Up to 75 % of patients with VACTERL association have CHDs. VSD, atrial septal defects (ASD), and TOF are commonly seen, whereas TA and transposition of the great arteries (TGA) are less common.

DGS is highly associated with TA and the common 22q11.2 deletion as mentioned above, but some cases have no detectable molecular defect of this region. Relatively rare cases of DGS can be caused by heterozygous deletion of 10p14-13 (DGS2 locus) [16]. We reported a unique patient with a *de novo* deletion beginning in the intron between exons 5 and 6 of *CDC* (cell division cycle) *45L* and deleting exons 1–3 of *UFD* (ubiquitin fusion degradation) *1L* on 22q11.2 critical region and typical features of the DGS including TA [17]. The *CDC45L* gene is situated immediately telomeric of UFD1L and is transcribed in the opposite direction through a common cis-acting element [18].

Association between TA and the following rare syndromes has also been cited in Online Mendelian Inheritance in Man (OMIM): cleft-limb-heart malformation [19]; fibuloulnar aplasia/hypoplasia with renal abnormalities [20]; microcephaly, congenital heart disease, unilateral renal agenesis, and hyposegmented lungs [21]; and renal-hepatic-pancreatic dysplasia 2 implicated with mutation of *NEK8* (nimA-related kinase 8) on 17q11.2 [22].

47.2.4 Gene Mutations

47.2.4.1 TBX1

Because of the high incidence and association with CHD, including TA, 22q11DS has attracted attention as a model for investigating the genetic and developmental basis for TA. Although extensive gene searches have been successful in identifying more than 30 genes in the critical 22q11.2 locus, direct sequencing has failed to detect a responsible gene [5, 6, 23]. Elegant efforts to model 22q11DS in mice by creating orthologous chromosomal deletions were successful in reproducing aortic arch defects of 22q11DS, leading to identification of a T-box transcription factor, TBX1, as a major genetic determinant of CHD associated with 22q11DS [24, 25].

We and other groups found that Tbx1 was expressed in the SHF, but not in the CNCC [26–28]. This finding was surprising because CHD associated with 22q11DS had been considered primarily to be a result of developmental defects of CNCC [5, 6, 9, 23]. Our Cre-mediated murine transgenic system suggested that Tbx1-expressing descendents representing a subset of cells derived from the SHF contribute predominantly to the pulmonary infundibulum [29]. Developmental defects of this subset of cardiac progenitor cells may cause hypoplasia of the pulmonary infundibulum, resulting in TOF. A more severe decrease in number or absence of this subset of cells may affect development and/or migration of CNCC, resulting in TA. This hypothetical model is supported by the observation that outflow tract defects ranging from TOF to TA are highly associated with 22q11DS (Fig. 47.1).

There is a report of *TBX1* mutations in three unrelated patients with CHD without the common 22q11.2 deletion [30]. Yagi et al. identified mutations in the *TBX1* gene in heterozygous state in three patients with phenotypes related to the 22q11.2 deletion syndrome, including the characteristic conotruncal anomaly face.

47.2.4.2 NKX Transcription Factors

Homeobox-containing genes regulate tissue-specific gene expression and play roles in differentiation and patterning of tissues during development [31]. *NKX2-5*/*CSX* and *NKX2-6* were identified as vertebrate homologs of a Drosophila "tinman," a homeobox-containing gene essential for development of the heart-like dorsal vessel [32, 33].

Schott et al. reported that heterozygous mutations of *NKX2-5* caused familial ASD with atrioventricular block [34]. This was the first report clearly showing that a single gene mutation could cause a nonsyndromic CHD. McElhinney et al. examined the *NKX2-5* gene in 474 patients with CHD and identified a heterozygous mutation in 1 (4 %) of 22 patients with TA [35]. A number of subsequent studies have indicated that mutations in the homeodomain of *NKX2-5* are likely to cause ASD, whereas mutations located outside the homeodomain may be associated with outflow tract defects including TA [36, 37].

By mutation analyses for *NKX2-6*, Heathcote et al. identified homozygosity for a mutation (F151L) in the *NKX2-6* gene in affected members of a large consanguineous Kuwaiti family with TA [38]. Ta-Shma et al. identified a homozygous truncating mutation in the *NKX2-6* gene in three sibs, born of consanguineous Palestinian parents with TA [39]. These findings indicate that mutations of *NKX2-5* or *NKX2-6* are the genetic cause of a minority of cases of TA.

47.2.4.3 GATA Transcription Factors

GATA transcription factors are characterized as zinc finger proteins and play roles in the differentiation and survival of many cell types, including cardiomyocytes [40]. Among six GATA transcription factors, GATA4, GATA5, and GATA6 are expressed in the precardiac mesoderm and are thought to play essential roles during development of the heart.

Garg et al. reported that mutations in *GATA4* caused ASD/VSD, probably as a result of disruptions to interactions with TBX5 [41]. A few reports have demonstrated an association between *GATA4* mutations and outflow tract defects [42]. We identified a novel *GATA4* sequence variants in a patient with TA [43]. This variant (p.Thr330Arg) was located in a common basic region of the GATA transcription factors and resulted in disruption of the synergistic activity between *GATA4* and *NKX2-5* or *GATA6*. It has been reported that a reciprocal regulation and a synergistic activity between GATA4 and NKX2-5 are essential for the expression of several cardiac-specific genes [44, 45]. Other studies have indicated that GATA4 plays a role in cardiovascular development in collaboration with GATA6 [46, 47] Taken together, a decrease in the synergistic and/or collaborative activity of GATA4 with NKX2-5 and/or GATA6 may be implicated in TA.

We also identified two different *GATA6* mutations in two probands with nonsyndromic TA [48]. Our subsequent biological analyses revealed that *SEMA3C* and

plexin (*PLXN*) A2 were directly regulated by GATA6 and that both GATA6 mutant proteins failed to transactivate these genes. During development, SEMA3C and PLXNA2 mediate signal transduction essential for neurovascular guidance as a ligand and a receptor, respectively. Our data implicate mutations in *GATA6* as genetic causes of TA, as a result of the disruption of the direct regulation of semaphorin-plexin signaling. In fact, a mutation in the *PLXND1* which encodes another receptor for *SEMA3C* was reported to be associated with nonsyndromic TA [49]. Other reports [43, 50, 51] came out on mutations in *GATA6* that are associated with CHD including atrioventricular septal defects (AVSD), TOF, and ASD. It is of note that the *GATA6* mutations identified in humans are predominantly associated with outflow tract defects, whereas *GATA4* mutations are commonly associated with ASD/VSD, although there are some phenotypic overlaps likely to result from a redundant role of both genes.

It was reported that pancreatic agenesis and congenital heart defects (PACHD) could also be caused by heterozygous mutation in the *GATA6* gene [52]. In the examination of 27 individuals with pancreatic agenesis by Allen et al., CHDs were present in 14 (93 %) of the 15 patients with mutations in the *GATA6* gene, whereas affected individuals in whom no *GATA6* mutation was detected rarely had extrapancreatic features. In the report by Yorifuji et al., the dominantly inherited *GATA6* mutation resulted in a variable degree of pancreatic hypoplasia, and similar intrafamilial variability was observed with regard to the types of CHD present in affected individuals [53]. Recently, a patient with PACHD associated with a *GATA6* mutation who manifested a severe form of TA was reported [54].

Conclusion

Although human genetic studies have revealed frequent association of 22q11.2 microdeletion with TA, the etiology of most TA is still unknown. Recently, single gene mutations associated with TA are beginning to emerge. Through the use of next generation sequencing technique, we can now elucidate more genetic causes associated with TA, some of which might provide targets for prevention or genetic intervention of this CHD.

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Molecular Pathways and Animal Models of Truncus Arteriosus

48

Amy-Leigh Johnson and Simon D. Bamforth

Contents

Abstract

In normal cardiovascular development in birds and mammals, the outflow tract of the heart is divided into two distinct channels to separate the oxygenated systemic blood flow from the deoxygenated pulmonary circulation. When the process of outflow tract septation fails, a single common outflow vessel persists resulting in a serious clinical condition known as persistent truncus arteriosus or common arterial trunk. In this chapter we will review molecular pathways and the cells that are known to play a role in the formation and development of the outflow tract and the genetic manipulation of these models that can result in common arterial trunk.

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48.1 Introduction

The outflow tract (OFT) of the mammalian heart is formed at the arterial pole of the primary heart tube concomitant with additional contributions of cells from the second heart field. The newly formed tract interposes between the developing right ventricle and the aortic sac, the latter manifold giving rise to the pharyngeal arch arteries (PAA). Usually described as the "conotruncus", it is now recognised that it is preferable to consider the outflow tract as possessing distal, intermediate, and proximal components [1, 2]. When first formed, the OFT possesses a solitary lumen, which is then divided into the aortic and pulmonary pathways by fusion of the major outflow cushions, thus separating the intermediate and proximal components, and by the growth of a protrusion from the dorsal wall of the aortic sac, which separates the intrapericardial parts of the aortic and pulmonary trunks. Both the major cushions and the protrusion are populated by cells migrating from the neural crest (NCC). Fusion between the cushions themselves, and then between the distal end of the fused cushions and the protrusion, is needed to produce complete separation of the aortic and pulmonary pathways. It is failure of fusion of the major outflow cushions that results in the persistence of a common outflow vessel, described as a common arterial trunk (CAT), but also frequently known as persistent truncus arteriosus. The intrapericardial arterial trunks themselves, however, can be well separated in some examples of CAT, indicating that growth of the protrusion from the dorsal wall of the aortic sac, which produces the aortopulmonary septum, is independent of fusion of the outflow cushions. It is the failure of fusion of the cushions that provides the phenotypic feature of CAT, rather than failure of distal aortopulmonary septation. A review of the published literature on transgenic mouse models presenting with CAT demonstrates that many genes from different molecular pathways can result in the phenotypic insult (Table 48.1).

48.2 T-Box 1

Malformations of the great arteries are a major feature in 22q11 deletion syndrome (22q11DS, also known as DiGeorge, velocardiofacial or Shprintzen syndrome). The transcription factor T-Box 1 (*TBX1*) is thought to be the main causative gene for

Table 48.1 Genes when mutated in mice, either globally or conditionally, that result in common arterial trunk (persistent truncus arteriosus). Other congenital cardiovascular malformations are also seen in some of these mutants (e.g. IAA, DORV, VSD, A-RSA)

Class	Genes
Transcription factor	*Cited2*, *Foxp1*, *Gata3*, *Gata4*; *Gat6*, *Gata6*, *Jun*, *Msx1*; *Msx2*, *Pax3*, *Pbx1*, *Prdm1*, *Prdm3*, *Sox4*, *Tbx1*, *Tcfap2a*
Signalling molecule	*Rara*; *Rarb*, *Rara*; *Rarc*, *Rxra*, *Sema3c*, *Shh*
Growth factor	*Fgf8*, *Vegfa*
Protein binding	*Bmp4*, *Chrd*, *Edn1*, *Ednra*, *Flna*, *Ltbp1L*, *Pinch1*
Receptor	*Acvr1*, *Bmpr2*, *Pdgfra*, *Plxnd1*, *Smo*, *Tgfbr1*, *Tgfbr2*
Enzyme	*Aldh1a2*, *Ece1*

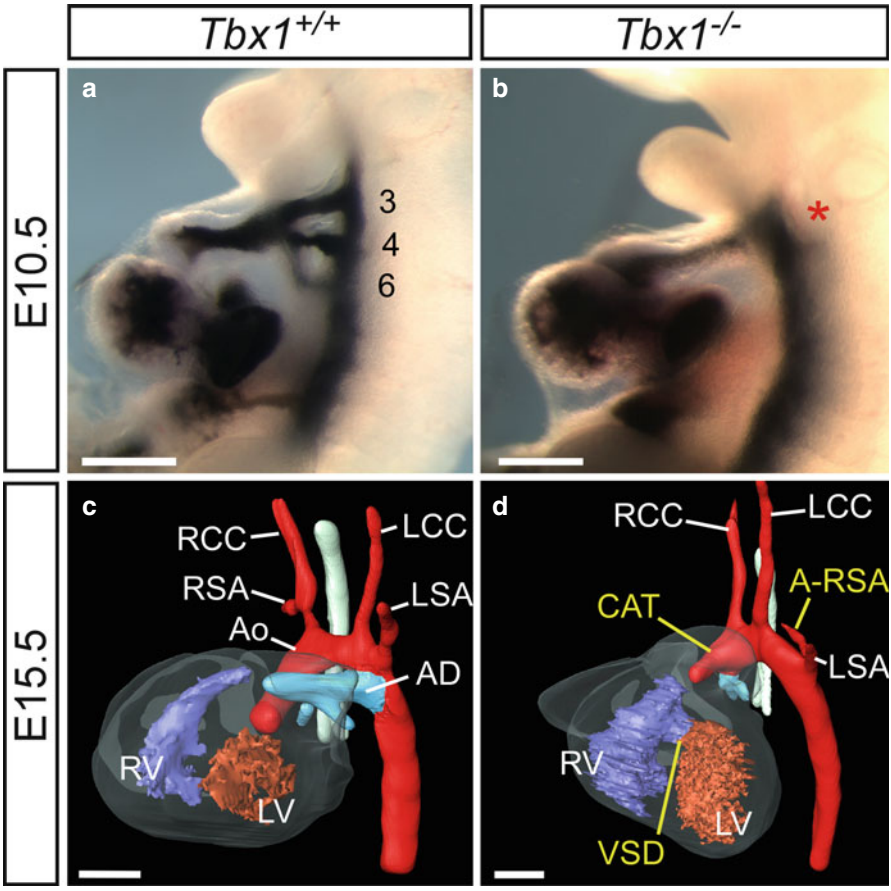

Fig. 48.1 Cardiovascular defects in *Tbx1*-null (*Tbx1*$^{-/-}$) mouse embryos and foetuses. (**a**, **b**) Injection of India ink into the heart of E10.5 embryos. (**a**) In the wild-type embryo (*Tbx1*$^{+/+}$) three bilaterally symmetrical PAA are present (numbered *3*, *4* and *6*) which are fully patent to ink. (**b**) In the *Tbx1*$^{-/-}$ embryo a single outflow vessel is present (*asterisk*) revealing that the PAA have failed to form properly. (**c**, **d**) 3-D reconstructions from MRI datasets [6] of E15.5 foetuses. (**c**) By E15.5 the heart and its associated great vessels have developed into the mature configuration. (**d**) The heart of a *Tbx1*$^{-/-}$ foetus presents with great artery defects related to the failure of the PAA to form correctly: CAT and A-RSA as well a VSD. The heart in this foetus is abnormally rotated to the left. Abbreviations: *Ao* aorta, *AD* arterial duct, *LCC* left common carotid, *LSA* left subclavian artery, *LV* left ventricle, *RCC* right common carotid, *RSA* right subclavian artery, *RV* right ventricle. Scale, 500 μm

the 22q11DS phenotype [3–5] and several animal models, including mouse and zebrafish, with mutant *Tbx1* alleles have been created, allowing this gene to be studied in great depth to understand its role in the formation and patterning of the cardiovascular system, including the pharyngeal arch arteries (see Chap. 42).

Transgenic mice null for *Tbx1* have a severe phenotype, and they die in the perinatal period with cardiovascular defects including CAT (Fig. 48.1). Although this phenotype is assumed to be caused by a failure of neural crest cells to properly

septate the outflow tract, Tbx1 protein is not expressed within the neural crest itself. It is therefore believed that loss of *Tbx1* affects neural crest cell migration [7–9].

Genetic modification of mice can be used to conditionally inactivate a gene in a temporal and spatial manner. When this is performed with *Tbx1*, the timing of expression within the developing embryo, and the consequences this has on cardiovascular development, can be ascertained. For example, when *Tbx1* is inactivated between E7.5 and E8.5 of embryonic development, PAA defects occur such as interruption of the aortic arch (IAA) and aberrant right subclavian artery (A-RSA), but later deletion between E8.5 and E9.5 affects outflow tract septation, resulting in CAT [10]. Regulation of *Tbx1* gene dosage is also critical. *Tbx1* mRNA dosage levels can be controlled through a series of mutant *Tbx1* alleles in transgenic mice [11]. These hypomorphic alleles reveal that a critical threshold of *Tbx1* mRNA is required for correct formation of the PAA. A reduction in *Tbx1* mRNA levels to ~20 % of wild type was sufficient to introduce the CAT phenotype. The overexpression of *Tbx1* in transgenic mice also results in cardiovascular abnormalities, including CAT and IAA [12].

As the *Tbx1*-null phenotype affects a wide range of tissues, including many of the embryonic structures derived from the pharyngeal arches and second heart field, including the outflow tract, interventricular septum, PAA, thymus, parathyroids, ears and craniofacial development (reviewed in [13, 14]), it is possible that each expression domain may contribute to different aspects of the phenotype. Therefore, conditional deletions of *Tbx1* have been used to investigate its tissue-specific requirements in the context of *Tbx1*-null embryos. Conditional deletion of *Tbx1* within the pharyngeal endoderm, ectoderm and mesoderm in addition to the SHF through the use of *Nkx2-5Cre* resulted in full recapitulation of the CAT and ventricular septal defect (VSD) phenotypes observed in $Tbx1^{-/-}$ mutants [15]. A reduced and disorganised smooth muscle cell layer surrounding the outflow tract was also observed, possibly due to the reduced proliferative activity in the second heart field [15]. Mesoderm posterior 1 homolog/Cre-recombinase (*Mesp1Cre*)-mediated deletion of *Tbx1*, which removed the gene from all mesodermal derivatives, including the endothelium, also resulted in 100 % penetrance of CAT, VSD and severe PAA malformations [9]. In addition, the segmentation of the pharynx is disrupted, resulting in the loss of the caudal pharyngeal arches, as is observed in $Tbx1^{-/-}$ mutant mice. Further defects affecting the migration of the NCCs into the pharyngeal arches, disruptions of the neural crest-derived cranial ganglia and reduced proliferation of the neural crest-derived pharyngeal arch mesenchyme were also observed. Reactivation of *Tbx1* within the mesoderm of mutant embryos rescued the outflow tract and VSD phenotypes, but not the 4th PAA defects, suggesting other tissues are required in this aspect of the phenotype [9], most likely the epithelium.

Other animal models have been employed to study the role of *Tbx1* in cardiovascular development, and Tbx1 is expressed throughout the pharyngeal regions of the frog [16], chicken [17] and zebrafish [18]. In the chicken, blocking cytochrome P450, family 26, subfamily A, polypeptide 1 (*Cyp26a1*), a gene required for retinoic acid inactivation during embryogenesis, results in outflow tract defects, i.e. CAT and double-outlet right ventricle (DORV), and PAA defects similar to that seen in the mouse model of *Tbx1* loss [17]. *Tbx1* levels were downregulated, and the

retinoic acid-synthesising enzyme retinaldehyde dehydrogenase 2 (*Raldh2*) was upregulated. Consistent with a role for sonic hedgehog (Shh) in regulation of Tbx1, chick embryos exposed to Shh-soaked beads responded by upregulating Tbx1 expression in the pharyngeal arches [19].

48.2.1 Fibroblast Growth Factors

Mice expressing a hypomorphic allele of fibroblast growth factor 8 (*Fgf8*) present with outflow tract defects such as CAT and DORV [20, 21]. The *Fgf8* receptors, *FGFR1* and *FGFR3*, are expressed within neural crest cells and are thought to be required in neural crest cell migration. Plating neural crest cells in the presence of Fgf8 *in vitro* increased their migratory capacity, while inhibition of FGFR1 both *in vitro* and *in vivo* reduced migration [22], indicating that this pathway may be required in neural crest cell migration and survival within the pharyngeal arches. A reduction in Fgf8 signalling may therefore result in a decreased number of neural crest cells entering the outflow tract, thereby causing DORV, while a more severe reduction in the number of cells of this type would result in CAT [22].

48.2.2 Transforming Growth Factors

The signalling molecule transforming growth factor (TGF)-β is vital for regulating many basic cell responses and has been shown to be important for the development of the great arteries in mouse models (see Chap. 36). The TGFβ signalling pathway involves the binding of the TGFβ ligand to the cell surface TGFβ type II receptor (TGFBR2), which subsequently activates the TGFβ type I receptor, TGFBR1 (also known as ALK5). This results in the phosphorylation of SMAD family member protein (SMAD) 2 and SMAD3 proteins, binding to SMAD4 and translocation to the nucleus to regulate downstream gene expression. Transgenic mice null for the TGFβ2 ligand (*Tgfb2*) die perinatally with cardiovascular defects, which includes CAT [23, 24]. The TGFβ receptors *Tgfbr1* and *Tgfbr2* are essential for embryogenesis as mice lacking these genes die around mid-gestation [25, 26]. Conditional alleles, however, allow for this early embryonic lethality to be avoided and the role of these genes in cardiovascular development dissected. When the *Tgfbr1* and *Tgfbr2* receptors are deleted from the neural crest using wingless-type MMTV integration site family, member 1 (*Wnt1*)-*Cre* transgenic mice, the embryos present with CAT [27–29]. This suggests that TGFβ signalling is required for neural crest cells to control septation of the aorta and pulmonary trunk. CAT is also seen in embryos when the long form of latent TGFβ binding protein 1 gene (*Ltbp1*) is disrupted [30].

The Pbx (pre-B-cell leukaemia homeobox) family of homeodomain transcription factors, *Pbx1*, *Pbx2* and *Pbx3*, plays an important role in cardiovascular development. Mice null for *Pbx1* have CAT, although *Pbx2*- and *Pbx3*-null mice do not have any cardiovascular defects [31, 32].

48.2.3 Neural Crest Cells

The importance of NCC per se in the septation of the outflow tract, rather than the genes expressed within them, has been demonstrated through the ablation of NCC in the chick [33, 34]. The accessibility of this experimental model allowed for the manual removal, or ablation, of discrete segments of the embryo comprising the cardiac NCC (cNCC) that would result in cardiovascular defects. For example, it was found that the ablation of the cNCC, either bilaterally or unilaterally, resulted in high proportions of embryos with CAT and VSD. In addition, smaller deletions – ablating only a single somite region unilaterally – also uncovered further, less severe OFT defects such as DORV and overriding aorta. It was also found that bilateral deletion of a region encompassing just two somites (somites 1–2 or 2–3) resulted in 100 % penetrance of CAT and VSD, while unilateral deletion of the same regions caused DORV in 40 % of cases.

NCC ablation studies have also been achieved in the mouse using genetic modification allowing the specific expression of toxins (e.g. thymidine kinase and diphtheria toxin) to kill the NCC. The first study investigating NCC ablation by inducing toxicity in those cells of the neural crest lineage utilised NCC-specific expression of the herpes simplex virus type 1 thymidine kinase (TK) suicide gene, which relies on the administration of the prodrug ganciclovir (GCV) in order to induce toxicity [35]. TK expression causes susceptibility to GCV, which prevents the incorporation of guanosine residues into elongating DNA, which makes the cells incapable of DNA synthesis. They therefore undergo apoptosis, resulting in cell death but only in those cells having expressed TK and also receiving GCV. Administration of GCV daily from E7.5 to E9.5 resulted in no embryos with normal cardiovascular development. The majority of the embryos (75 %) had CAT, with the remainder presenting with DORV or overriding aorta. In addition, all embryos also displayed a PAA defect. A second study of NCC ablation in the mouse used the diphtheria toxin fragment A (DTA) to induce toxicity in NCCs by interfering with the RNA translation machinery and causing the cells to apoptose [36]. All affected mutant mice presented with CAT.

Conclusion

Correct cardiovascular development relies on a complex morphogenetic pathway to ensure that all the required tissue components are formed and remodelled precisely. When this process fails, either due to genetic or environmental consequences, cardiovascular malformations arise. Septation of the OFT is a vital component of heart development to separate the systemic and pulmonary circulations, and a failure in this process results in a common arterial trunk. As described in this chapter, and demonstrated by animal models, it is clear that NCC play a crucial role in OFT septation. The genes that are expressed within NCC are also clearly important, but as are other genes, such as *Tbx1*, which are not expressed in NCC. Tbx1 is expressed within the tissue layers of the pharyngeal arches, and it is through this structure that the NCC must pass to reach the outflow tract. This illustrates the requirement of other tissue types that are vital for controlling NCC migration to allow OFT septation to occur properly.

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Part XIV

Tricuspid Atresia and Univentricular Heart

Clinical Presentation and Therapy of Tricuspid Atresia and Univentricular Heart

49

David J. Driscoll

Contents

49.1 Introduction

Although the terms "single ventricle" and "univentricular heart" frequently are used to describe a variety of complex congenital heart defects, in fact, all hearts have two ventricles although one of the two may be too small to be functional. A better term for these hearts would be "functional single ventricle." Within the spectrum of "functional single ventricle," one could include tricuspid atresia, double inlet left ventricle, common ventricle, mitral atresia with ventricular septal defect, double outlet right ventricle with straddling atrioventricular valve, and hypoplastic left ventricle among others. The principles outlined for the management of tricuspid atresia can be applied to the management of other forms of functional single ventricle.

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49.2 Pathologic Physiology

In tricuspid atresia, there is no direct communication between the right atrium and the right ventricle. The initial survival of infants with tricuspid atresia depends upon the presence of an interatrial communication to allow egress of blood from the right atrium to the left atrium. Tricuspid atresia occurs in combination with normally related or transposed great arteries and with or without pulmonary stenosis or atresia. The clinical presentation, physiologic manifestations, and treatment for all forms of functional single ventricle depend, to a large extent, on the relationship of the great arteries (normal or transposed) and the presence or absence of pulmonary stenosis or atresia and the size of an interatrial communication.

49.3 Clinical Presentation

Infants with tricuspid atresia and functional single ventricle usually present with cyanosis and frequently have a murmur. However, the degree of cyanosis will be dependent upon the volume of pulmonary blood flow which, in turn, is related to the degree of pulmonary and/or subpulmonary obstruction. The greater the obstruction, the more severe will be the cyanosis. With unimpeded pulmonary blood flow, the patient can be quite pink but present with signs and symptoms of congestive heart failure.

49.4 Physical Examination

The cardiac precordial impulse may be overactive. The presence of a systolic ejection murmur can result from associated pulmonary or subpulmonary stenosis. A mid-diastolic apical cardiac murmur may be present, particularly if there is increased pulmonary blood flow and congestive heart failure. For patients with tricuspid atresia, the electrocardiogram is quite helpful in suggesting this diagnosis. It is characterized by left axis deviation.

49.5 Echocardiography and Cardiac Catheterization

The diagnosis of tricuspid atresia and other forms of functional single ventricle and delineation of most of the associated anomalies is made by echocardiography. Because all forms of single ventricle can be associated with pulmonary stenosis and pulmonary atresia, it is important to establish the sources and reliability of the pulmonary blood flow. If these are not readily apparent by echocardiography, cardiac catheterization and angiography may be necessary. Rarely, in tricuspid atresia or mitral atresia or hypoplastic left heart syndrome, the interatrial communication is too small for adequate egress of blood from the right atrium (for tricuspid atresia),

or from the left atrium (for mitral atresia and hypoplastic left heart syndrome), a balloon atrial septostomy may be necessary to enlarge this communication.

49.6 Treatment

The initial management of babies with tricuspid atresia as well as functional single ventricle involves treatment of the congestive heart failure, if present, with digitalis and diuretics. In addition, it is essential to establish a reliable source of pulmonary blood flow if the pulmonary blood flow is insufficient and significant hypoxemia and acidosis are present. This can be accomplished with an infusion of prostaglandin E_l, while one prepares to surgically create a systemic-to-pulmonary artery shunt. In patients with excessive pulmonary blood flow, pulmonary artery banding may be needed to lessen congestive heart failure and protect the pulmonary vascular bed from damage.

Definitive treatment of all forms of single ventricle is accomplished using the modified Fontan procedure (Fig. 49.1). Since its original description, the Fontan operation has undergone numerous modifications. The goal of the Fontan operation is to direct all of the systemic venous return (blue blood) to the pulmonary artery without passing through a ventricle. Currently the most popular modification of the Fontan operation is the bicaval connection. In this procedure, the superior vena cava is disconnected from the heart and anastomosed, in an end-to-side fashion, to the right pulmonary artery. The inferior vena cave is connected to the underside of the right pulmonary artery using an interposition graft that is positioned either outside the heart or within the right atrium. This separates systemic and pulmonary venous returns and decreases ventricular volume overload.

In some patients a small communication ("fenestration") is created between the inferior vena cava to pulmonary artery connection and the pulmonary venous atrium. Patients who have had a "fenestrated Fontan" operation continue to have a right-to-left shunt and are mildly cyanotic. The fenestration reduces some of the postoperative complications and may reduce the risk of late development of protein-losing enteropathy.

49.7 Outcome

The outcome after the Fontan operation depends upon the number of disadvantageous factors for the Fontan operation that existed prior to the Fontan operation. Since patients with tricuspid atresia or single ventricle have only one functional ventricle, this ventricle is used to pump blood into the aorta. There is no ventricle to pump blood into the pulmonary artery. For blood to flow into the pulmonary artery, the pulmonary arteries have to be normal size and there must be normal pulmonary arterial resistance. In addition, left ventricle filling pressure and ejection fraction must be normal, and there must be no significant mitral valve stenosis or insufficiency. It is preferable if there are no significant abnormalities of systemic and venous return. It is advantageous for these patients to have sinus rhythm.

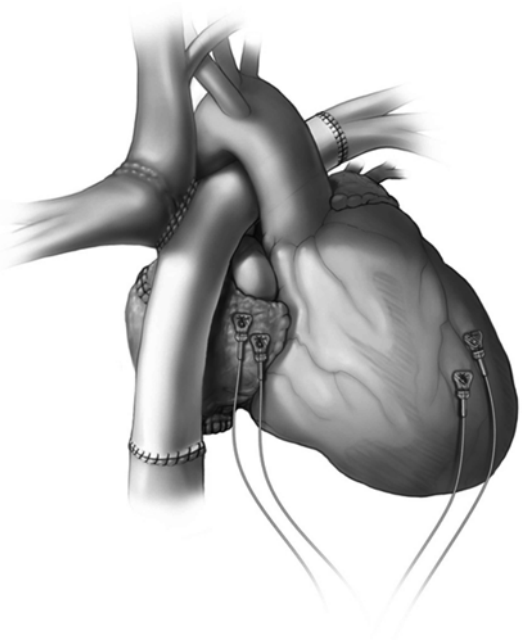

Fig. 49.1 Illustration of the modified extracardiac Fontan operation. The superior vena cava has been disconnected from the right atrium and anastomosed to the right pulmonary artery. A graft has been interposed between the inferior vena cava and the left pulmonary artery. This graft also has been anastomosed in a side-to-side fashion with the right pulmonary artery. Permanent pacemaker leads have been attached to the right atrial appendage and the ventricle

The long-term survival for patients who are ideal candidates for the Fontan operation is excellent and may exceed 85 % 10 years after operation. As the number of disadvantageous factors for the Fontan operation increase, however, the long-term outcome worsens. Long-term complications after the Fontan operation include atrial arrhythmias, protein-losing enteropathy, cirrhosis, hepatocellular carcinoma, and left ventricular dysfunction.

Human Genetics of Tricuspid Atresia and Univentricular Heart

50

Abdul-Karim Sleiman, Liane Sadder, and George Nemer

Contents

Abstract

Tricuspid atresia (TA) is a rare congenital heart condition that presents with a complete absence of the right atrioventricular valve. Because familial and/or isolated cases of TA are rare, little is known regarding the potential genetic abnormalities contributing to TA. Exploratory studies have determined potential

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etiologic chromosomal abnormalities including deletions in 22q11, 4q31, 8p23 and 3p as well as trisomies 13 and 18. In parallel, potential culprits include the *ZFPM2*, *HEY2*, *NFATC1*, *NKX2-5*, and *MYH6* genes. The aim of this chapter is to expose the genetic components that are potentially involved in the pathogenesis of TA in humans. A large variability in phenotypes and genotypes of cases of TA suggests a genetic network that involves many components yet to be unraveled.

50.1 Introduction

The evaluation of human genetics that characterize the pathogenesis of tricuspid atresia (TA) is ongoing, yet no single genetic or chromosomal defect has been singled out. In this chapter, the embryological pathways of human heart valve development will be described and the different genetic and chromosomal abnormalities that may interrupt this intricate process will be highlighted.

50.2 Clinical Genetics

Tricuspid atresia (TA) constitutes 1–3% of all congenital heart defects [1]. A compilation of 11 studies revealed a mean incidence of TA of 0.079/1000 live births with an interquartile range of 0.024–0.118. TA has no sex predilection, and the recurrence risk for TA in siblings is relatively low (1 %) and may be considered insignificant [2–4].

TA is classified into two types based on the presence or absence of transposed great arteries. Type I displays normally related great arteries and constitutes 66% of TA cases, whereas type II displays transposition of great arteries (dextro or levo) and comprises the remaining TA cases [5, 6]. Although TA can arise in an isolated form, it is also commonly associated with other cardiac anomalies, including atrial and ventricular septal defects (ASD/VSD) and patent ductus arteriosus (PDA). Less commonly associated conditions include Alagille syndrome and Ellis-van Creveld (EVC) syndrome. Transposition of the great arteries (TGA), on the other hand, is a condition where irregular twisting of the aorticopulmonary septum leads to an abnormal morphology, with the aorta originating from the right ventricle and the pulmonary artery from the left ventricle.

The reason behind concurrence of TGA with TA is still abstruse. Alagille syndrome is an autosomal dominant disorder in which the renal, cardiac, hepatic, skeletal, and visual systems are affected [7]. It has been associated with TA due to a common involvement with *HEY2* mutations [8, 9]. Finally, EVC syndrome is an uncommon autosomal recessive condition that reflects a form of chondroectodermal dysplasia. The reasoning behind coexistence of EVC and TA has not yet been delineated. TA is also notably associated with other cardiac defects in siblings and relatives including Ebstein's anomaly, tetralogy of Fallot, truncus arteriosus communis, ASD, mitral valve prolapse, and unspecified cardiovascular malformations without TA.

50.3 Embryology

A review of the embryology of the heart is essential to the understanding of TA and the underlying molecular and genetic pathways. During the course of embryological heart development, bilateral cardiac progenitor cells originate from the mesoderm, the fusion of which forms the primitive heart tube. This further differentiates into the endocardium and myocardium and is followed by formation of the epicardium from surrounding mesodermal tissue [10, 11].

Development of the atrioventricular valves is initiated by the formation of endocardial cushions. Mesenchymal proliferation from the anterior and posterior walls of the atrioventricular opening creates two elevations, referred to as endocardial cushions. Subsequently, these cushions will grow and fuse to form the definitive tricuspid and mitral valves, on the right and left side of the heart, respectively. Although the endocardial cushions play a large role in AV valve formation, myocardial remodeling also is implicated in the process [12, 13]. Specifically in TA, an endocardial-lined "dimple" marks the site of the absent tricuspid valve [14].

Notably, appropriate AV valve development is regulated by a large spectrum of genetic mechanisms and pathways, reflecting the susceptibility of this process to gene mutation and anomalous outcomes. The causes and predispositions of TA are numerous and complex. There is no indication of a single genetic, chromosomal, or environmental determinant for this condition. But specific genetic and chromosomal entities orchestrate the abnormal development of a fetal heart with TA.

50.4 Associated Chromosomal Defects

50.4.1 22q11 Deletions

Deletions on chromosome 22q11 can have adverse effects on any of the 30–40 genes present in that region, many of which are not studied yet. The diverse possibilities and combinations of resulting phenotypes led to the categorization of these syndromes into broader terms including DiGeorge syndrome, conotruncal anomaly face syndrome and velocardiofacial syndrome. All anomalies fall under the more general umbrella term of 22q11 deletion syndrome – a condition of cardiac abnormality, craniofacial irregularity, thymic aplasia and hypocalcemia/hypoparathyroidism [15, 16].

The first case reported with a microdeletion on chromosome 22q11 and TA with a common arterial trunk was in 2003 [16]. Recently, association was confirmed between deletions at chromosome 22q11 and TA phenotypes, the likelihood of 22q11 del. in TA being around 7%. Cases of sporadic hemizygous 22q11 microdeletion present with TA associated with other cardiac abnormalities such as VSD, TGA, PDA, and pulmonic stenosis have been reported [17, 18]. This indicates an overlap between 22q11 microdeletion genotypes and subsequent cardiac phenotypes. Del22q11.2 exhibits an increased risk of sibling recurrence, whereas congenital cardiac anomalies in general are not associated with an increased risk of

del22q11.2 in subsequent siblings. Although the pathogenesis of the TA phenotype due to 22q11 microdeletion has not been elucidated, there is significant indication of co-occurrence between the two.

50.4.2 Trisomies and Other Chromosomal Defects

TA has been reported with trisomies 13 and 18 [19]. Other chromosomal defects associated with TA include mutations on chromosomes 3, 4, and 8. Terminal deletions on the long arm of chromosome 4 at breakpoint 4q31 have been reported with congenital heart defects including TA, left-sided vena cava, and anomalous aortic arch [20]. Similarly, partial deletion of the short arm of chromosome 3 (3p) has been reported with double orifice mitral valve, complete atrioventricular canal, and TA [21]. Such relationships between chromosomal deletions and TA have been reported with interstitial deletions at chromosome 8p23.1, which harbors the GATA binding protein 4 (*GATA4*) gene that plays a pivotal role in heart development [22].

Although often demonstrated through single cases and small samples, the chromosomal defects implicated in TA are diverse, reflecting the intricacy of genotype-phenotype relationship in fetal heart development.

50.5 Genes

50.5.1 ZFPM2 (FOG2)

ZFPM2, also known as *FOG* (friend of GATA) family member 2, encodes a zinc finger protein; it maps to chromosome 8q22 and is mainly expressed in the human heart, brain, and testes. ZFPM2 presents significant modulation of cardiac GATA protein activity throughout development, interacting directly with the N-terminal zinc finger of these proteins, both *in vivo* and *in vitro* [23, 24]. A chief function of transcription factor GATA4 is the regulation of genes that are essential for complete myocardial differentiation, as indicated by its significant expression in the yolk sac endoderm and the mesodermal cells involved in heart development. Other functions of ZFPM2 include the maintenance of epithelial cell differentiation in the mammary gland, as well as interactions with GATA1, a transcriptional activator ensuring the normal development of hematopoietic cell lineages, and with C-terminal binding protein 2 (*CTBP2*), a corepressor of diverse transcription factors [25].

ZFPM2 is co-expressed with GATA4 in the developing heart. Although *Zfpm2*−/− genotypes in mice are implicated in the occurrence of TA (see Chap. 44), evaluation of the possible genetic mechanisms involved in human TA etiology is still underway. Some sequence variations in human *ZFPM2* (exon 8) are controversially associated with TA since they are also found in healthy individuals [26, 27].

50.5.2 HEY2

The *HEY* genes encode nuclear proteins of the hairy and enhancer-of-split related family with YRPW motif, which are known direct transcriptional targets of the Notch signaling pathways in *Drosophila* and vertebrates [28, 29]. The *HEY2* gene is a homologue of the zebrafish "gridlock gene," which is expressed during cardiac development prior to vessel formation and contributes to the arterial-venous cell fate decision. HEY2 is a suspected repressor of transcription downstream of Notch signaling in cardiac development. It is expressed in the embryonic heart, mainly in developing ventricles and arteries. Studies on *Hey2* mutant mice showed a weak association with TA (see Chap. 44). Still, there is potential correlation between TA and the specific single-nucleotide polymorphisms (SNPs) in the coding regions of *HEY2* [30].

50.5.3 NFATC1

NFATC1, also known as nuclear factor of activated T cells, is a DNA-binding protein which functions primarily in the regulation of T-cell activation and IL-2 expression alongside other heterologous transcription factors [31, 32]. It belongs to the Rel/NFkB family of transcription factors and is an essential player in endocardial cushion formation and remodeling as demonstrated by *Nfatc1−/−* knockout mice [33].

Two missense heterozygous SNPs in exons 2 (p.Pro66Leu) and 8 (p.Ile701Leu) of *NFATC1* have occurred in one patient with TA, concomitantly resulting in an 80% failure of NFATC1 translocation to the nucleus, while single mutations have no effect. Additionally, DNA-binding affinity and promoter activation is decreased in single and double mutant forms [34].

An unusual allele with 44 additional nucleotides bearing 6 SNPs contrasts the normal genotype that bears 4 SNPs repeated twice at 56 nucleotides downstream of the 3′ boundary of exon 7 with the intronic region [35]. However, the lack of homozygosity of this unique allele in TA subjects hinders the understanding of its contribution to the pathogenesis of TA.

50.5.4 NKX2-5

NKX2-5 is a human, homeobox-containing gene that is involved in regulating anatomical development and is exclusively expressed in cardiac cells. Homologues in Drosophila and mice have demonstrated major roles in cardiac differentiation in their respective species, suggesting a role of *NKX2-5* in human heart formation. It also was uncovered that *NKX2-5* has strong interactions with TBX5, a T-box containing transcription factor associated with Holt-Oram syndrome – an autosomal dominant disorder that affects the extremities and the heart. *NKX2-5* and *TBX5*

synergistically activate the expression of a gene-encoding cardiac-specific natriuretic peptide precursor type A (*NPPA*) [36].

In mice, *Nkx2-5* serves an important role as a transcription factor, directing the expression of certain ion channel genes involved in cardiac conduction and contraction. It contributes to the AV node development and atrial septal formation. Numerous mutations in humans were identified as being the underlying cause for familial atrial septal defects. Two siblings with *NKX2-5* p.Arg190Leu recently were found to have TA in addition to ASD-II and VSD. Like other *NKX2-5* mutations at the same position, this mutation is suspected to reduce the DNA-binding activity of NKX2-5 but offers no clues as to its role in the malformation of the tricuspid valve [37].

A second mutation in *NKX2-5* in a patient with complex CHD including TA results in a premature stop codon and thus a truncated protein (255 amino acid residues rather than 292) but with an intact homeodomain indicates that the phenotype is likely due to a change in the affinity of the NKX2-5 protein to other transcriptional partners [37].

Evidently, the role of *NKX2-5* mutations in the abnormal development of the tricuspid valve elucidates the intricacy of this process, as we continue to explore the involvement of specific transcriptional factors it may interact with.

50.5.5 MYH6

MYH6 is a gene that codes for a potent ATPase represented by myosin alpha heavy chain subunits in cardiomyocytes. It is an important factor in heart development that is predominantly expressed in atrial muscle [38–40].

MYH6 p.Ala230Pro variant significantly contributes to the cardiac phenotype of several aberrant heart morphologies. The variant was identified in the concurrence of TA, valvular and supravalvular pulmonary stenosis, hypoplastic right ventricle, and ASD. The same allele was observed in family members with different cardiac malformations or none at all. The alanine at position 230 is located in the motor domain of MYH6 and consequently affects the kinetic coupling of nucleotide binding in muscle myosin molecules. This, in turn, results in an altered affinity toward actin during the ATPase cycle. However, the effect this mutation has on the formation of the tricuspid vale is uncertain since MYH6 is restricted to the myocardium.

A MYH6 p.Glu501Stop variant has been identified in the presence of TA, restrictive ventricular septal defect, and hypoplastic right ventricle. It is a nonsense mutation thought to generate a truncated myosin peptide with missing lower domains (50 and 20 kDa) and neck and rod regions. The resulting protein retains most of the actin and nucleotide-binding capacities yet still exhibits other motor dysfunctions [39].

Conclusion

Most of the occurrences of tricuspid atresia that were identified with a genetic cause occurred as part of a multitude of congenital heart diseases. Genetic culprits ranged from extra chromosomes and chromosomal deletions to SNPs. The

involvement of some genes such as *ZFPM2* and *NKX2-5* is certain. Other genes such as *HEY2* however are less likely to be involved. The heterogeneity of the observed phenotypes and reported genotypes, and hence of their relationship, diminishes the contribution of each report to the greater picture, allowing only very little to be known about the mechanism of development of TA and the molecular pathways involved therein. Since only a few mutations were found to be direct culprits in patients with tricuspid atresia so far, analysis of copy number variation (CNV), yet to be undertaken to assess the genotypes of those patients, will provide an opportunity to discover new genes underlying tricuspid atresia.

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Molecular Pathways and Animal Models of Tricuspid Atresia and Univentricular Heart

51

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Contents

Abstract

The process of valve formation is a tremendously complex process and involves a very intricate interplay between appropriate pathways at appropriate times. Although we have not completely elucidated the molecular pathways that lead to normal valve formation, we have identified a few major players in this process.

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We are now able to implicate TGF-ß, BMP, and NOTCH as suspects in tricuspid atresia (TA), as well as their downstream targets: NKX2-5, TBX5, NFATC1, GATA4, and SOX9. We know that the TGF-ß and the BMP pathways converge on the SMAD4 molecule, and we believe that this molecule plays a very important role to tie both pathways to TA. Similarly, we look at the NOTCH pathway and identify the HEY2 as a potential link between this pathway and TA. Another transcription factor that has been implicated in TA is NFATC1. While several mouse models exist that include part of the TA abnormality as their phenotype, no true mouse model can be said to represent TA. Bridging this gap will surely shed a lot of light on the molecular pathway on TA and allow us to fill in many gaps.

51.1 Introduction

The process of valve formation is a tremendously complex process and involves a very intricate interplay between appropriate pathways at appropriate times. Although we have not completely elucidated the molecular pathways that lead to normal valve formation, we have identified a few major players in this process.

The transforming growth factor beta (TGF-ß) and bone morphogenetic protein (BMP) 4 pathways are critical in valve formation. They both require SMAD family member (SMAD) 4 molecules to propagate their intracellular message. SMAD4 is associated with GATA binding protein 4 (GATA4) and zinc finger protein, FOG family member 2 (ZFPM2), and mutations in either of these two transcription factors cause tricuspid atresia (TA).

Similarly, NOTCH is essential for normal valve formation, and its association with hes-related family bHLH transcription factor with YRPW motif 2 (HEY2) provides us with a possible link to TA.

Other transcription factors like nuclear factor of activated T-cell, cytoplasmic, calcineurin-dependent 1 (NFATC1) should not be overlooked.

Mouse models for TA are far from being perfect, but the trisomy 16 mouse, the Hey2 mutant mouse, and the ZFPM2 mutant mouse all provide us an insight about the molecule pathways of TA.

We are far from understanding the complete picture of TA. When studying the pathophysiology of any diseased organ, however, a good place to start is in trying to understand the physiology of the healthy organ in question.

The tricuspid valve, like the mitral valve, becomes fully functional at mid-gestation following a process that requires an intimate interaction between endocardial and myocardial cells. Using genetic data in humans and animal models will allow us to understand the molecular pathways involved in normal valve formation and will afford us a better idea about the molecular pathways involved in TA.

51.2 Normal Valve Development

Normal tricuspid valve development can be separated into three main events: inductive stimuli of endocardial cells, epithelial-to-mesenchymal transition (EMT), and maturation of the valves.

51.2.1 Inductive Stimuli

Studies performed in the mid-1980s using the collagen gel assay found that only a subset of the endocardial cells of the heart – those that line the atrioventricular (AV) canal and outflow tract – are capable of undergoing EMT. Indeed, when endocardial/myocardial cells were explanted from the (AV) canal region and placed on the gel assay, it was found that the endocardial cells underwent EMT, while the resulting mesenchymal cells migrated into the gel. If, however, explants of endocardial/myocardial cells from the ventricle were used, EMT would not occur. Furthermore, scientists mixed and matched endocardial and myocardial cells from different locations within the heart tube to find that only when both the endocardial and the myocardial explants originated from the AV canal region did EMT occur [1–3]. Such studies formed the bases of the belief that there must be a myocardial signal that primes the endocardium at the AV canal and allows it to undergo EMT. Certain extracellular matrix (ECM) components have been implicated in the inductive stimuli [2], as well as signals that include the TGF-ß superfamily of proteins [4].

51.2.2 Epithelial-to-Mesenchymal Transition

Following this initial inductive stimulus, the process of EMT begins. At this point, the developing heart has just finished looping [5]. Initially, the innermost endocardial layer is made up of cells that have an apical-basal polarity. Junctions on their lateral membranes connect them to each other, and they rest on a basement membrane. These characteristics define epithelial cells. As a result of the process of EMT, some of these cells will lose their polarity, change their morphology, dissociate from the endocardial layer, and migrate into the cardiac jelly. Once there, these, now mesenchymal cells, will play an integral part in tricuspid valve formation. Though much of EMT is still unclear, scientists have identified key regulators of this process. Two main growth factor pathways that act as upstream regulators of EMT are the TGF-ß/BMP pathway and the Notch pathway. Downstream targets of these pathways include transcription factors such as NK2 homeobox 5 (NKX2-5), T-box 5 (TBX5), NFATC1, GATA4, and SRY (sex-determining region Y)-box 9 (SOX9) [5, 6].

51.2.3 Maturation

The end result of EMT is the formation of the cardiac cushions, which will undergo maturation and form the cardiac valves. This process of maturation is not very well understood, but the mesenchymal cells formed by EMT will differentiate into fibroblasts that secrete specific ECM components necessary for valve formation [7].

The process of normal valve formation is the result of a concert of pathways and transcription factors that need to be regulated tightly. Much work has been done to understand the molecular bases of valve formation and abnormality, and while we have uncovered a lot, many questions still cloud our understanding of normal and abnormal valve formation. TA is among the pathological valve conditions that the molecular pathway of which is still somewhat murky.

51.3 Abnormal Valve Development: Underlying Molecular Pathways of TA/Univentricular Heart

Animal models for TA as well as genetic studies of patients with this condition have suggested that the two major families of secretory proteins that are involved in valve formation – TGF-ß/BMP and NOTCH – and their regulators and/or downstream target effectors are directly involved in the malformation of the tricuspid valve.

What follows is a discussion of the TGF-ß/BMP and the Notch pathways, their relation to tricuspid valve formation and TA, their overlap, and important downstream targets of both pathways that have been implicated in TA, namely, NFATC1 and Hey2.

Figure 51.1 illustrates how these different factors play a role in the heart in relation to the endocardium and the myocardium within the regions destined to become valves.

51.3.1 TGF-ß/BMP Family of Growth Factors

Aside from their vital role in heart development, the TGF-ß and BMP pathways play an essential role in embryonic development. In fact, the TGF-ß and the BMP subfamilies of secreted proteins are part of larger superfamily of growth factors known as the TGF-ß superfamily. This superfamily includes three major branches: the TGF-ß sensu stricto (TGF-ß in vertebrates), DVR (decapentaplegic-Vg-related, which includes BMP), and activin. These three different subfamilies share many characteristics such as the structure of the ligands, the receptors, the mechanism of action of the ligand-receptor complex, as well as the intracellular mediators of the ligands – the SMADs [8]. This similarity in the mechanism of action, especially in the SMAD mediators, will play an important role in the overlap between the BMP and the TGF-ß pathways as they relate to TA, as we shall discuss below.

Ligands of the TGF-ß superfamily require two receptors to function, TGF-ß receptors type I and II, both of which are receptor tyrosine kinases. The ligand binds

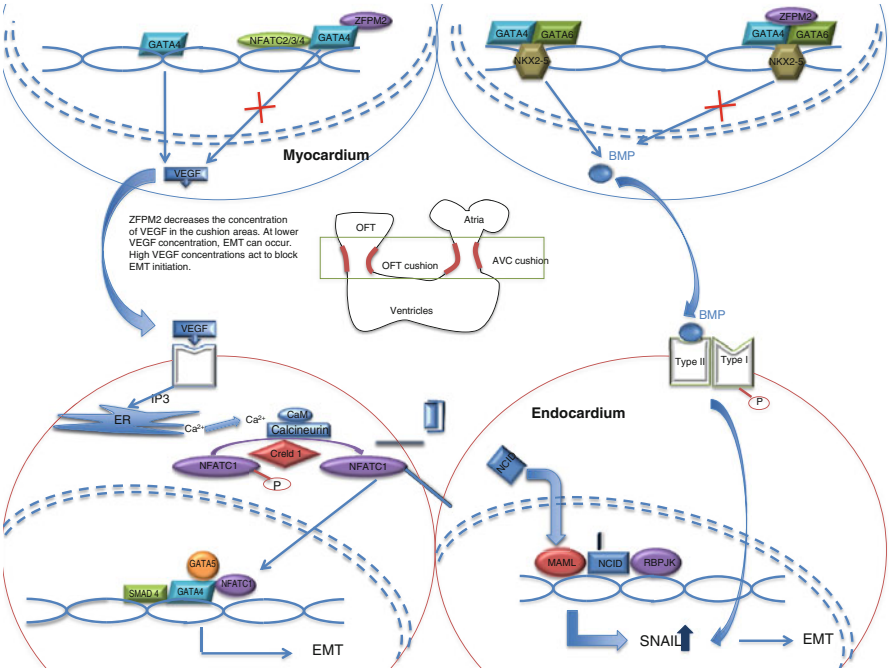

Fig. 51.1 The Notch receptor is present in endocardial cells prior to EMT and will bind to its ligand and release its NCID domain. This domain forms a nuclear complex with MAML and RBPJK and will upregulate SNAIL, which leads to EMT formation. Simultaneously, myocardial BMP2 signals will reach the endocardium and will stabilize the nuclear SNAIL signal. At the correct level, VEGF produced by the myocardium will bind to its receptor on the endocardium and result in an increase of Ca^{2+} from the ER via IP3. This Ca^{2+}, in concert with calmodulin, will activate calcineurin. Calcineurin will dephosphorylate NFATC1 and uncover its nuclear localization signal. It is believed that the step of NFATC1 dephosphorylation is also controlled by Creld1. Once in the nucleus, NFATC1 will bind to GATA4 and/or GATA5 among other proteins and will induce EMT

to receptor type II and will induce the type II receptor to bind to a type I receptor and phosphorylate it. The phosphorylated type I receptor is now activated and will in turn phosphorylate and activate a "receptor-activated SMAD" molecule (R-SMAD) [9]. The R-SMAD represents the intracellular mediator of the ligand and is responsible for transmitting the TGF-ß or BMP signal into the nucleus.

In humans, there are more than 30 different ligands in the TGF-ß superfamily [10]. There are also seven type I receptors and five type II receptors [9]. A ligand-receptor complex can be made by numerous combinations of ligand, type I and type II receptors.

While the type II receptor determines the ligand specificity, the type I receptor determines the SMAD specificity [8]. Ultimately, BMP ligands result in the activation of SMAD1, SMAD5, and SMAD8, while the TGF-ß ligands result in the activation of SMAD2 and SMAD3 [9]. All R-SMAD molecules must then necessarily bind to cytoplasmic SMAD4 molecule to form a functional unit that will in

turn enter the nucleus. Once in the nucleus, this SMAD4/R-SMAD functional unit will bind to specific transcription factors and regulate gene expression [9, 10]. Later on we will show that the SMAD-4 molecule, which links both the BMP and the TGF-ß pathways, interacts directly with GATA4, one of the most important regulators of valve formation whose cofactors have been implicated in TA. It is this link between GATA4 and SMAD4 that will tie the BMP and TGF-ß pathways to TA.

Figure 51.2 illustrates the similarities of the TGF-ß and the BMP pathways and their mechanism of action in the heart.

51.3.1.1 Role of TGF-ß Protein in Tricuspid Valve Formation

Although TGF-ß and BMP belong to the same superfamily and share many characteristics, they play distinct roles in tricuspid valve formation. These differences can be seen in the various functions that each pathway plays in EMT.

During embryogenesis, cells that are destined to form valves undergo three separate rounds of EMT, aptly named primary, secondary, and tertiary EMT. Each round of EMT is followed by a round of MET to reset the cells into the epithelial state in preparation for the next round of EMT. Primary EMT results in the formation of cardiac progenitor cells. Secondary EMT plays an important role in the formation of the cardiac tube. Finally, tertiary EMT results in valve formation [11]. TGF-ß and BMP play essential roles in tertiary EMT.

TGF-ß is one of the earliest molecules to be involved in EMT. Studies carried out in mice and chick embryos have shown that TGF-ß acts as an inductive stimulus for EMT, as well as a regulator of later steps in the EMT program. Scientists studied the various isoforms of TGF-ß in the chick embryo and found that TGF-ß2 is essential for EMT initiation, while TGF-ß3 is essential for mesenchymal cell invasion into the ECM [12]. In mice, the function of the TGF-ß genes was slightly different. Studies performed in mice using functional antibodies against different isoforms of TGF-ß concluded that TGF-ß1 and TGF-ß3 were not needed for EMT to occur. TGF-ß2, however, did play a role. *In vitro* studies showed that the number of mesenchymal cells that invaded the collagen assay upon TGF-ß2 antibody exposure was significantly reduced. Along the same lines, *in vivo* studies with *TGF-ß2*$^{-/-}$ mice showed an abnormality in EMT that resulted in hypercellular cushions [13].

Besides the TGF-ß ligand itself, the TGF-ß receptor has an important role in EMT (indicating perhaps that other pathways besides those activated by the TGF-ß ligand are at play). The importance of the TGF-ß receptor has been studied in mice and chick embryos. Studies in chick embryos showed that if TGF-ß receptor type II is blocked, EMT does not take place [6]. In mice, it appears that the TGF-ß ligand-receptor interaction is more complicated. *In vivo* studies showed that blocking TGF-ß receptor type II does not affect EMT. *In vitro* studies, on the other hand, showed that blocking the same receptor inhibited explanted tissue EMT. This implies that other compensatory mechanisms, possibly unrelated to the TGF-ß ligand, are able to make up for the loss of the receptor [5].

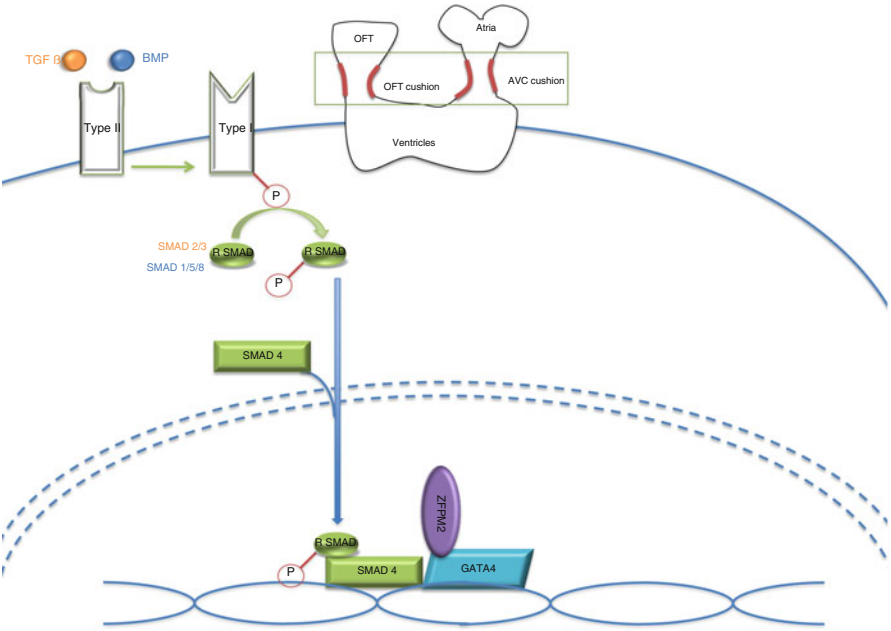

Fig. 51.2 TGF-ß or BMP ligand will bind to the type II receptor that will activate the type I receptor. The type I receptor will phosphorylate a particular R-SMAD molecule (SMAD2/3 for TGF-ß and SMAD 1/5/8 for BMP). This R-SMAD molecule will complex with a SMAD4 molecule to enter into the nucleus and interact with transcription factors such as GATA4

51.3.1.2 Role of BMP in Tricuspid Valve Formation

Although similar in importance to the TGF-ß pathway discussed above, BMP seems to have a more upstream role than that of TGF-ß in valve formation. Previous reports have shown that BMP2 is important in the initiation of the EMT process. Experiments done on mouse AV canal explants showed that in the absence of the myocardium, endocardial cells exposed to BMP2 are able to undergo EMT. Therefore, BMP2 released from the myocardial cells must be part of the inductive stimuli that kick off EMT in the endocardial cells. Furthermore, BMP2 resulted in an upregulation of TGF-ß in these cells, indicating that perhaps BMP2 is upstream of the TGF-ß pathway in EMT [5, 6]. BMP4 is more important in the process of valve maturation as opposed to EMT initiation [6]. BMP5, BMP6, and BMP7 have been investigated together in the mouse heart. Double knockout mice for BMP5 and BMP7 did not form a cardiac cushion, while double knockout mice for BMP6 and BMP7 showed a delay in the cardiac cushion formation that affected the outflow tract cushion more than it did in the AV cushion [6].

The effect of BMP on EMT can also be appreciated by blocking the BMP receptors. Indeed, blocking the BMP2 type I receptor in mice resulted in mice embryos that failed to undergo EMT and had a decrease TGF-ß expression – similar to BMP2 knockout mice [5].

51.3.1.3 The TGF-ß/BMP Pathway in TA: A Role for ZFPM2 and SMAD

While one can draw distinctions between the roles of TGF-ß and BMP in early valve formation, the fact that they belong to the same superfamily and therefore share a common mechanism of action means that these two pathways will overlap. They do so at the level of the SMAD molecules and more specifically SMAD4. Understanding the role of SMAD4 in both pathways and the role it plays alongside one of the main regulators of valve formation (GATA4) is the key to understanding the roles of TGF-ß and BMP in TA.

The SMAD proteins play a vital role in the downstream pathway of both TGF-ß and BMP. After binding of either BMP or TGF-ß to a surface receptor, a series of events occurs that ultimately results in the activation of a ligand-specific R-SMAD molecule. SMAD4 will then bind to the activated R-SMAD molecule and translocate it into the nucleus [9, 10, 14]. Once in the nucleus, the SMAD4/R-SMAD complex interacts with other transcription factors, including the master regulator of cardiac development, the GATA4 protein. Studies have shown that disruption of this interaction could lead to CHD mainly related to atrioventricular septation and valve formation. In fact, mice double heterozygous for both genes die in utero because of atrioventricular septal defects. Furthermore, *in vitro* experiments show a strong physical and functional interaction between SMAD4 and GATA4 [15].

In short, the BMP and TGF-ß pathways must signal through SMAD4 to relay their message into the nucleus. SMAD4, in turn, interacts with several transcription factors, including GATA4. The way that all of this ties in with TA is through GATA4's transcriptional partner ZFPM2.

ZFPM2 (also known as FOG2) is a zinc finger protein with 8 zinc finger domains. It is highly expressed in the heart where it interacts with GATA4 and mediates its activity [16, 17]. *ZFPM2*$^{-/-}$ mice die in utero between days E13–E14 from a cardiac phenotype that mirrors TA [18].

The molecular pathways that lead to TA are not yet well defined. We have at our disposal only pieces of the puzzle that seem to implicate an intricate relationship between upstream factors like TGF-ß and BMP and downstream targets like GATA4 ZFPM2 and SMAD4. The identification of transcriptional targets for the GATA4/SMAD4 and GATA4/ZFPM2 interactions would help establish a better genotype/phenotype correlation and a comprehensive mechanism that links the transcriptional outcome activities with the underlying defect.

51.4 The Notch-Hey2 Pathway

Besides the BMP and the TGF-ß pathways, the Notch pathway is one of the upstream regulators of EMT and valve formation [5, 6]. Not unlike the previous two pathways, the Notch pathway is vital for several cellular processes including differentiation and patterning. It distinguishes itself from the TGF-ß and the BMP pathways, however, by using ligands that are not secreted. The Notch pathway involves a membrane-bound receptor binding to a neighboring cell's membrane-bound ligand [19]. This makes the Notch signaling pathway contact dependent, unlike either the BMP or the TGF-ß signaling pathways.

There are four Notch receptors in mammals and they all share the same basic structure. An extracellular domain will bind to one of the five Notch ligands (Dll 1, Dll 3, and Dll 4 and Jag 1, Jag 2), an intracellular domain (NICD) that detaches from the receptor and moves into the nucleus, and a transmembrane domain [19, 20].

After the Notch binds to its ligand, a series of reactions ensue that ultimately result in the detachment of the NICD and its translocation into the nucleus. Once in the nucleus, the NICD forms a complex with the RBPJK (Recombinant signal Binding Protein for immunoglobulin kappa J region) transcription factor. This NICD/RBPJK complex recruits the co-activator protein MAML (MAsterMind-Like), and this entire complex upregulates the expression of several downstream targets, including Hey2 and SNAIL. Figure 51.3 illustrates this contact-dependent Notch signaling pathway.

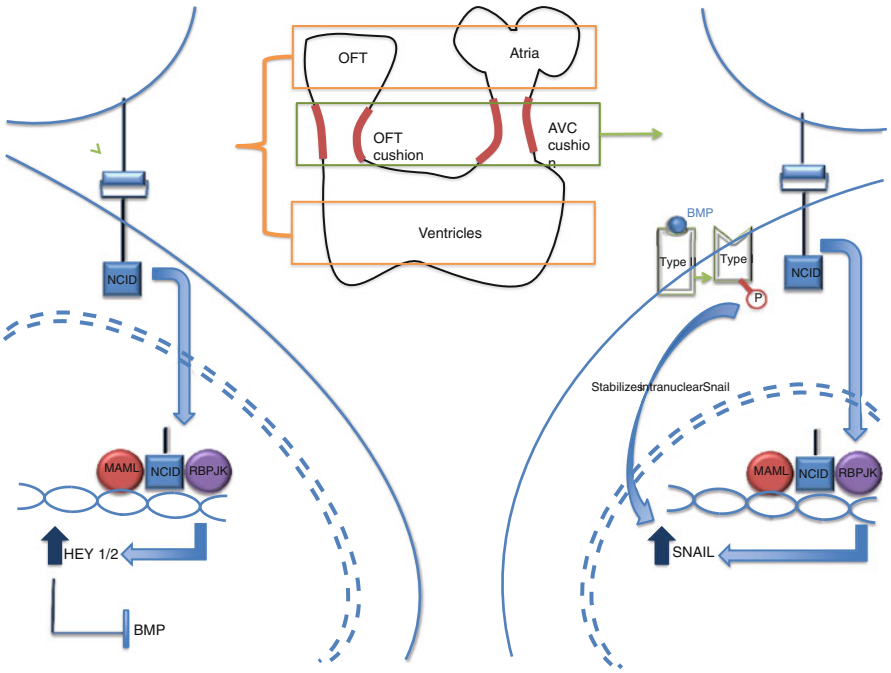

Fig. 51.3 After Notch binds to its ligand in a cell contact-dependent manner, it will release its NCID domain. This domain will travel into the nucleus and form a complex with MAML and RBPJK and upregulate different factors depending on what area of the heart this is taking place in. In the atria and ventricle (*right side* of the figure), the Notch pathway will result in the upregulation of HEY1/2 that will in turn block BMP signaling. This will work to inhibit EMT. In the cushion regions (*left side* of the figure), the Notch pathway results in the activation of SNAIL and will induce EMT. BMP will stabilize the SNAIL in the nucleus

51.4.1 Role of Hey2 Notch in Tricuspid Formation and TA

Like TGF-ß and BMP, Notch also plays an important role in normal valve formation. The importance of the Notch pathway in normal tricuspid valve formation was realized in studies that showed severely dysmorphic cardiac cushions upon inhibition of Notch in mice. Upregulation of Notch, on the other hand, results in hypercellular cushions [6]. Detailed spatial and temporal analysis of the expression of Notch during cardiac cushion development reveals an important role for Notch in the regulation of the EMT process in the tricuspid valves. Analysis of the spatial distribution of the Notch ligand, Dll4, just prior to EMT reveals its presence in high amounts in areas destined to become valves [21]. Predictably, Notch itself also was present in these atrioventricular canal (AVC) areas prior to EMT initiation. Furthermore, inhibition of Notch, either directly or by inhibiting its RBPJK transcription factor, resulted in endocardial cells that are unable to migrate into the cardiac jelly [19, 21], which indicates that Notch must play a role in EMT and tricuspid valve formation.

Besides allowing EMT to occur, the Notch signaling pathway is also important for patterning the premature cardiac cushion areas. It seems that some of the transcription factors activated downstream of Notch, specifically Hey1 and Hey2, determine which areas will undergo EMT by inhibiting essential EMT signals in the tissue in which they are present. Just prior to valve development in the mouse, Hey1 is present in the atrial myocardium and endocardium, and Hey2 is present in the ventricular endocardium. Thus, these downstream Notch cofactors inhibit EMT in the ventricle and atria and allow EMT to proceed only in the AVC and the OFT cushion regions where they are not present [21].

Unlike the relation between TGF-ß/BMP and TA, the relation between Notch and TA is more direct. The evidence that tied the Notch pathway to TA involved studies done on *Hey2*$^{-/-}$ mice. These mice mostly died in utero or survived to die shortly after birth. More important, however, are the shared cardiac defects among all mice with this genotype whereby 40% of them presented with tricuspid atresia [22].

51.4.2 Cross Talk Between the Notch and TGF-ß/BMP Pathways

Serving as a reminder of the intricacies involved in cardiac valve formation is the fact that the TGF-ß, the BMP, and the Notch pathways exhibit cross talk between them. Studies have shown that the Hey2 and Hey1 cofactors demarcate the boundaries of AVC and OFT cushion by downregulating BMP2 in all but these two areas in the heart [21]. Furthermore, it appears that high levels of BMP2 in the AVC areas maintain a high level of EMT specific genes, namely, the transcription factor Snail1. Coincidentally, Snail1 is also a downstream target of the Notch pathway, and Snail1 acts in a positive feedback loop with Notch [19].

There is also some evidence that links the Notch pathway to the TGF-ß pathway via R-SMAD molecules. Inhibition of the Notch pathway leads to a decrease in the SMAD3 levels within endothelial cells. Notch activation, on the other hand, leads to endothelial cell downregulation of SMAD1 and SMAD2 [20].

To highlight the complexities of the three pathways mentioned above, one should keep in mind that the spatial expression of the different ligands and receptors mentioned thus far is crucial. It appears that the Notch receptor is preferentially expressed in the endocardium during tricuspid valve formation, while its ligands are present both in the endocardium and myocardium. Similarly, BMP signals, especially the BMP2 signal, are myocardial in origin [5, 20].

51.4.3 Other Downstream Targets: NFATc1 and the Tricuspid Valve

Just like the TGF-ß/BMP and the Notch pathways that can be tied to TA via their respective downstream targets, the GATA4/ZFPM2 and the Hey2, respectively, other downstream transcription factors have been implicated in TA. Chief among those is the NFATC1.

NFATC1 is a transcription factor that was initially implicated in T-cell activation. It belongs to the Rel/NF-kB family and has, since its discovery, been described to interact with several regulators of differentiation during embryogenesis [23]. NFATC1 is a cytoplasmic protein that moves into the nucleus to bind DNA and regulate gene expression upon its dephosphorylation by calcineurin, a calcium-calmodulin-dependent phosphatase [23, 24].

NFATC1 plays a role in valve development as shown by the *Nfatc1* knockout mice that fail to develop mature valve leaflets [25]. The role of NFATC1 in valve maturation has been corroborated by the fact that it interacts with several other transcription factors, including, but not limited to, the GATA zinc finger protein family [26]. During early valve formation, NFATC1 expression is restricted to endocardial cells in areas of the heart that are destined to become cardiac cushions [27]. After the cardiac cushions have formed, expression of NFATC1/calcineurin at day E11 in the endocardial cells is required for cardiac valve elongation and maturation [28]. In parallel, human genetic studies identified two compound heterozygous mutations in the *NFATC1* gene in a patient suffering from TA [23]. Cellular analysis revealed that the NFATC1 mutated proteins affected with this compound heterozygous mutation resulted in abnormal NFATC1 activity including decreased nuclear localization, decreased NFATC1 DNA-binding affinity, a subsequent decrease in calcineurin-induced transcriptional activity, and finally, decreased ability of NFATC1 to bind to other transcription factors such as GATA5 and HAND2 – both of which play essential roles in cardiac valve development [23].

Several upstream signaling pathways besides calcineurin have been implicated directly in the regulation of NFATC1 including vascular endothelial growth factor (VEGF) and connexin 45, while others, including TGF-ß and BMP, have an indirect effect on NFATC1 [24, 29, 30]

Mice that are deficient for connexin 45 died in utero and had cardiac problems that were similar to *Nfatc1*$^{-/-}$ mice [31]. Furthermore, analysis of cardiac cells revealed that NFATC1 in these mice was mainly cytoplasmic and was unable to translocate into the nucleus [30].

Similarly, it has been shown that VEGF plays an important role in cardiac valve development by affecting NFATC1 activity. Indeed, cultures of chick endocardial cushion cells treated with VEGF had a significantly higher amount of nuclear NFATC1 than cells without VEGF treatment. Ultimately, VEGF was shown to cause an increase in proliferation of these endocardial cushion cells by activating the calcineurin/NFATC1 pathway [29]. Mouse studies have recently identified cysteine rich with EGF-like domains 1 (Creld1), a risk gene for non-syndromic atrial septal defects (ASD), as a regulator of NFATC1. Creld1-deficient mice had NFATC1 that maintained a phosphorylated state, which inhibited the nuclear translocation of NFATC1. Furthermore, Creld1-deficient mice did not respond to VEGF treatment, while control mice showed a normal NFATC1 response to VEGF, indicating that VEGF regulation of NFATC1 occurs through Creld1 [24].

As a testament to the complexities of the different pathways in valve formation and to our imprecise yet growing knowledge of these pathways, recent studies have identified a novel role of NFACT in the outflow tract (OFT) valve formation. There, it seems that NFATC1 is critical during two time points: the initiation of EMT and the maturation of the valves. At the first time point, NFATC1 plays a role similar to that discussed above. However, during the second time point, which occurs when the valves are beginning to mature, it seems that NFATC1 plays a different role. Then, it seems that NFATC1 is a critical factor in determining the cell fate by inhibiting endocardial cells from undergoing EMT and becoming mesenchymal cells. During the maturation phase, it seems that by inhibiting EMT, NFATC1 maintains cells in their endocardial nature. Here again one can note the overlap between the different pathways discussed thus far. For NFATC1 inhibits EMT during the maturation phase of the valves by downregulating SNAIL1 and SNAIL2, two factors have been described above as being downstream targets of the Notch pathway. By downregulating these two factors, NFATC1 essentially blocks endocardial cells from becoming mesenchymal cells, thereby inhibiting EMT [32].

51.5 Animal Models

Almost all the animal models that present with TA are mice models. These mice were for the most part not created to study TA specifically but rather a different cardiac abnormality or normal cardiac or valve development.

51.5.1 Trisomy 16 Mouse

As early as 1985, the trisomy 16 mouse model was being recognized as a potential model to study the "endocardial cushion defect" observed in patients with Down's syndrome [33]. Though this model succeeded in recreating some of the cardiac phenotypes observed in humans who have trisomy 21, it had additional phenotypes that were seen in patients who suffer from microdeletions on chromosome 22, the

reason being that the mouse chromosome 16 carries genes that are expressed on human chromosome 22 [33, 34].

Coincidentally, mice with trisomy 16 had features resembling human TA. Indeed, around half of the animals with trisomy 21 showed a muscular right atrial floor with an atrioventricular junction that was connected exclusively to the left atrium with no right ventricular connection [34]. Other non-TA cardiac phenotypes observed in mice with trisomy 16 include a common atrioventricular junction, a lack of atrioventricular septation, and a lack of subpulmonary infundibulum formation [34].

51.5.2 Hey2 Mutant Mice

Another mouse model that has been used to study TA is the Hey2 mutant mouse. Hey2 is one of the downstream targets of the Notch signaling pathway, and it plays a role in the patterning of the cardiac endocardium prior to EMT. Transgenic mice were created with a mutated Hey2 protein that lacked three domains: the helix-loop-helix domain, the YXXW domain, and the orange domain.

There were no reported cardiac phenotypes in Hey2 heterozygous mice. Homozygous mice, however, consistently had numerous cardiac defects. The most common defect observed in homozygous mice was a ventricular septal defect (VSD). Other defects included an ASD and a TA-like phenotype where there was an absence of tricuspid valve formation or complete valve stenosis. These defects were present in about 40 % of embryos with homozygous null mutations for Hey2, and there was thus partial penetrance depending on the genetic background [22].

51.5.3 ZFPM2 Mutant Mice

Deleting all eight exons of the *Zfpm2* gene resulted in mice that have been used as models to TA. *Zfpm2* heterozygous mice showed normal cardiac development. *Zfpm2*$^{-/-}$ homozygous mice however did not survive beyond day E13.5. Examination of the embryos showed several cardiac defects including pulmonary stenosis, VSD, ASD, TA, and a single AV valve that connected both atria to the left ventricle. The right ventricle was smaller in these animals, but the left ventricular compact zone was larger than in wild-type mice [18].

Conclusion

The variety of the phenotypes in each model and the overlap between the different phenotypes in the different models suggest that there is a great deal of overlap between different pathways in cardiac development, which makes understanding the molecular pathway of TA more challenging.

Among the various molecular pathways in TA, however, the TGF-ß/BMP pathway with its GATA4/ZFPM2 downstream target and the Notch pathway with its Hey2 downstream target seem to be of significant importance. In addition, it seems that NFATC1 is also a part of any story involving TA. And while

the TGF-ß/BMP and the Notch pathways are essential for many other developmental origins, the secret to the molecular pathway of TA could lie with NFATC1. The role that NFATC1 plays in valve formation sets it aside from other factors. At the time that corresponds to valve formation, the presence of NFATC1 in the entire embryo is restricted to areas destined to become valves. This unique feature might just indicate that the holy grail of TA is NFATC1. And while it will not account for all the unknowns, it is entirely plausible that this transcription factor could serve as the thread that unravels the details of the molecular pathway of TA.

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Part XV

Ebstein Anomaly

Clinical Presentation and Therapy of Ebstein Anomaly

52

David J. Driscoll

Contents

52.1 Introduction

Ebstein anomaly is relatively rare constituting <1 % of cardiac malformations and occurring in 1 of 210,000 live births. Although primarily a malformation of the tricuspid valve, the right ventricle is myopathic as well.

52.2 Pathologic Physiology

The embryologic basis of Ebstein anomaly is failure of the tricuspid valve leaflets to delaminate from the ventricular muscle. The septal leaflet is the least well delaminated. The anterior leaflet, albeit the most completely delaminated, is usually quite large but also may have tethered attachments to the right ventricular free wall. Thus, the apparent orifice of the tricuspid valve is displaced into the right

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ventricle. However, the true orifice is not displaced and the portion of the heart between the true and apparent orifice is the so-called atrialized portion of the right ventricle.

The abnormalities in Ebstein anomaly are not limited to the tricuspid valve. The right ventricle (Fig. 52.1) is not normal and always, to a greater or lesser degree, is myopathic. In some cases the left ventricle also may show evidence of fibrosis.

An atrial septal defect or stretched patent foramen ovale is present in 50–70 % of cases, and 30 % of cases are associated with a right-sided accessory bypass tract or Wolff-Parkinson-White (WPW) syndrome. Atrial arrhythmias are common in patients with Ebstein anomaly. These can be associated with ventricular preexcitation (WPW) or the dilated right atrium. Accessory conduction pathways should be identified and ablated during a preoperative electrophysiologic study.

The pathophysiology depends on the degree of dysplasia of the tricuspid valve, the degree of tricuspid valve insufficiency, or, in rare cases, tricuspid valve stenosis. In addition, determinants of clinical severity include the severity of right ventricular myopathy; the presence or absence of an interatrial communication; the presence, absence, and degree of right ventricular outflow tract obstruction; and the presence or absence of ventricular preexcitation.

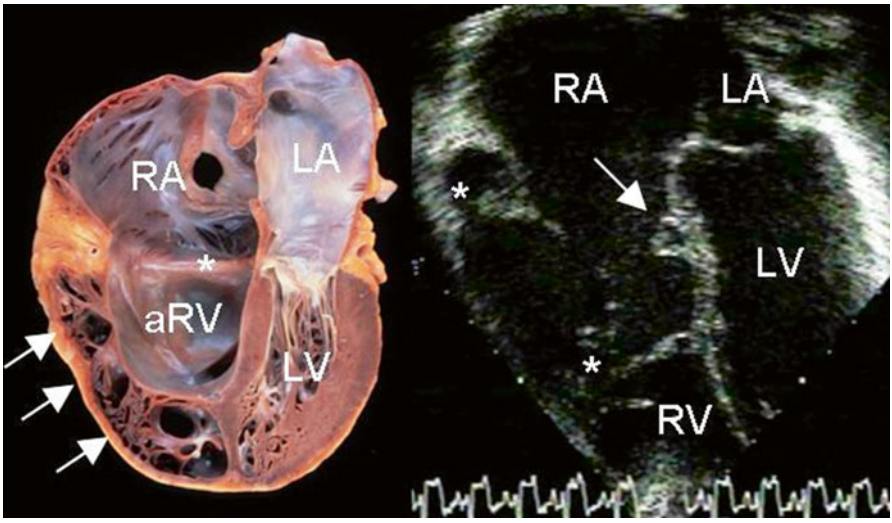

Fig. 52.1 Pathologic specimen of Ebstein anomaly (*left*) and echocardiographic image (*right*). The septal leaflet is plastered against the septum (failure of delamination), and the anterior leaflet is markedly enlarged and displaced into the right ventricle and tethered to the free wall of the right ventricle. There is a large atrial septal defect. *Arrows* on the left panel show thinned wall of right ventricle. *Arrow* on the right panel shows the hinge point where the septal leaflet is attached to the septum. Abbreviations: *RA* right atrium, *LA* left atrium, *aRV* atrialized portion of the right ventricle, *LV* left ventricle, *RV* right ventricle. * defines the enlarged anterior leaflet of the tricuspid valve

52.3 Clinical Presentation

The presentation and clinical course of patients with Ebstein are quite variable and dependent upon the severity of the malformation. On one end of the spectrum, fetal death can occur. On the other end of the spectrum, patients with mild forms of Ebstein anomaly can have a normal life span without medical or surgical intervention.

The classic presentation at birth is cyanosis and/or marked cardiomegaly. Newborns presenting with cyanosis do so because of right-to-left shunting through a patent foramen ovale (PFO) or atrial septal defect (ASD). Beyond the newborn period, patients may present because of the presence of a murmur, fatigue, dyspnea, cyanosis, atrial arrhythmias, or cardiomegaly discovered serendipitously.

52.4 Physical Examination

For patients with an ASD, cyanosis usually is apparent. Despite the presence of significant tricuspid insufficiency, because of the capacitance of the dilated right atrium, the jugular venous impulses usually are normal. The cardiac impulse may be quite diffuse as a result of cardiomegaly and a volume overloaded right ventricle. Classically, patients with Ebstein anomaly have been described as having a "quadruple rhythm." Rarely, if the valve is stenotic, a tricuspid diastolic murmur is present.

52.5 Echocardiography and Cardiac Catheterization Issues

The anatomy of the tricuspid valve can be delineated using echocardiography, and associated anomalies such as the presence of an ASD and right ventricular outflow tract obstruction can be identified. Right atrial size and right ventricular size and functions can be estimated. Left ventricular function also can be estimated. Other important information that echocardiography provides includes the size of the right ventricle and the position of the ventricular septum.

It rarely is necessary to perform cardiac catheterization in a patient with Ebstein anomaly. Indications for cardiac catheterization include hemodynamic assessment in selected circumstances if a concomitant bidirectional cavopulmonary shunt is being considered, particularly if there is depression of left ventricular function. It also may be necessary to exclude distortion of the pulmonary artery or the presence of pulmonary hypertension in cases in which a systemic to pulmonary shunt previously had been placed. In addition, if there is anatomic pulmonary valve stenosis, a balloon valvuloplasty may be necessary.

52.6 Treatment

Neonates with severe hypoxemia and extreme cardiomegaly are particularly challenging. Because of severe tricuspid valve insufficiency and neonatal elevation of pulmonary resistance, some of the neonates will have "functional pulmonary

atresia" without anatomic pulmonary atresia. These babies eventually respond to (prostaglandin) PGE1. However, if there is anatomic pulmonary atresia or stenosis, a systemic to pulmonary shunt or a balloon pulmonary valvuloplasty will need to be performed. Most will require intubation and mechanical ventilation. Infants who do not improve with these maneuvers may require neonatal operation.

Those infants without associated anomalies and less than extreme cardiomegaly who can be weaned from PGE1 and/or nitric oxide and remain well can be considered for operative repair at a later date when early mortality is substantially lower than in the neonatal period.

The principle concept of operation is to provide the patient with a competent, non-stenotic tricuspid valve, eliminate sources of right-to-left shunts, and reduce the size of the right atrium and, at times, the size of the right ventricle. Some patients may require ablation or division of an accessory pathway, and others may require a maze procedure for atrial arrhythmias. Hence one can repair or, if not repairable, replace the tricuspid valve.

52.7 Outcome

In a study of 539 patients who had operation for Ebstein anomaly at the Mayo Clinic, the 30-day mortality was 5.9 %. Late survival was 84.7 % at 10 years and 71.2 % at 20 years. The 1-, 5-, 10-,15-, and 20-year freedoms from any reoperation was 97 %, 91 %, 82 %, and 70 %, respectively. These patients had had the Danielson-type repair of the valve or valve replacement.

More recently for 89 patients who had the "cone" reconstruction at the Mayo Clinic, the early mortality was 1 %. Early reoperation for recurrent TR occurred in 12 patients (13 %). Re-repair was performed in 6 patients (50 %), and 6 (50 %) required valve replacement. Mean follow-up was 19.7–24.7 months. There was no late mortality or reoperation.

Human Genetics of Ebstein Anomaly

53

Gregor U. Andelfinger

Contents

Abstract

Ebstein anomaly is a congenital malformation of the tricuspid valve characterized by abnormal attachment of the valve leaflets, resulting in varying degrees of valve dysfunction. The anatomic hallmarks of this entity are the downward displacement of the attachment of the septal and posterior leaflets of the tricuspid valve, and additional intracardiac malformations are common. Familial recurrence and an association with structural genomic variation, including syndromic forms, point to a strong genetic influence in pathogenesis. Gene content of more commonly observed chromosomal imbalances suggests that multiple cardiac regulators can contribute to Ebstein anomaly. A specific association has emerged with the definition of left ventricular noncompaction and Ebstein anomaly, caused by autosomal dominant mutations in the beta-myosin heavy chain MYH7. The current gene catalogue discussed here suggests that diverse developmental pathways are affected in Ebstein anomaly and that variable expressivity and incomplete penetrance are commonly observed phenomena.

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53.1 Introduction

Ebstein anomaly is a rare disease and accounts for 0.5 % of all cases of congenital heart disease (CHD), with a birth prevalence of approximately 1:20,000 [1]. Several clinical observations favor a strong genetic influence in Ebstein anomaly. In the Baltimore-Washington Infant Study, genetic risk factors included twin pregnancies, a family history of cardiovascular malformations, and previous miscarriages [1]. A disproportionately high incidence of congenital heart disease was found in a genetic study of 26 families of patients with Ebstein anomaly [2]. Several reports, compatible with autosomal, recessive, or X-linked inheritance, demonstrate familial occurrence of Ebstein anomaly [3–7]. In several families, Ebstein anomaly also has been identified in the context of familial congenital heart disease with variable expressivity [8–11]. Associated intracardiac (38 %) and extracardiac malformations (19 %) are common in individuals with Ebstein anomaly (1), but there is no consistent association of this malformation with distinct genetic conditions. In 32 nonsyndromic patients, 22 patients (69 %) had isolated Ebstein anomaly, with familial recurrence of CHD in one (left ventricular noncompaction), and 10 (31 %) had additional CHD, including atrial septal defect ($N=7$), ventricular septal defect ($N=2$), pulmonary stenosis ($N=2$), dextrocardia, aortic coarctation, and patent ductus arteriosus ($N=1$ each) [12]. Arrhythmia is already present in 17 % of children and more than half of adults with Ebstein anomaly, suggesting that developmental and hemodynamic factors interact from very early stages of cardiac morphogenesis [13].

Canine tricuspid valve malformation (CTVM) bears a striking resemblance to human Ebstein anomaly. It has been noted that this malformation is enriched in specific dog breeds, suggesting either founder effects or other strong influences of the genetic background. We studied three Labrador retriever pedigrees in which CTVM segregated as an autosomal dominant trait with reduced penetrance [14]. Genome-wide linkage analysis in one kindred identified a CTVM susceptibility locus on dog chromosome 9 (CFA9) with a maximum multipoint lod score of 3.33. The two additional kindreds showed a conserved disease haplotype, suggesting a founder effect in apparently unrelated kindreds [14]. A disease-causing gene or mutation has not yet been identified, but of particular relevance is the replication of this locus in mapping efforts of the keeshond conotruncal defect [15].

In the 1970s, several studies suggested a very strong association between maternal lithium treatment during early pregnancy and the risk for Ebstein anomaly in the fetus [16]. Since lithium is an inhibitor of GSK3 (glycogen synthase kinase 3) and thus increases canonical Wnt signaling in a nonspecific fashion, this observation is of potential interest from a developmental point of view. However, more thorough reevaluation of the risks of prenatal lithium exposure has clearly shown that the teratogenic risk of first-trimester lithium exposure is lower than previously suggested [17]. In an animal model, lithium can even pharmacologically rescue decreased Wnt signaling which would otherwise result in defective heart development [18].

53.2 Nonsyndromic Single-Gene Causes of Ebstein Anomaly

The first report clearly linking Ebstein anomaly to a single gene was published in 1999 using a candidate approach [11]. In this study, the coding region of NKX2-5 (NK2 homeobox 5) was screened in probands with diverse forms of CHD and/or atrioventricular block. Four members of two families with a NKX2-5 mutation also had Ebstein anomaly or other tricuspid valve anomalies. However, this phenotype was not always present, even with full segregation of the NKX2-5 mutation with the disease trait in each of these families. Interestingly, a mouse model of the human p.Arg52Gly mutation, which is localized at a highly conserved position within the homeodomain, is highly penetrant and results in pleiotropic cardiac effects [19]. This model, in which the effects of the heterozygous knockin mimic findings in humans with heterozygous missense mutation in the NKX2-5 homeodomain, also results in Ebstein anomaly. These and other studies provide evidence that NKX2-5 mutations can contribute to Ebstein anomaly in humans, albeit with reduced penetrance [20]. However, in a series of 28 nonsyndromic Ebstein anomaly patients, no mutations of NKX2-5 were found, suggesting that mutations in this gene are not a major contributor to the phenotype [12].

Left-sided lesions occur in a large proportion of patients with Ebstein anomaly [21, 22]. A clinically very relevant association emerged since 2004 with the systematic description of Ebstein anomaly and left ventricular noncompaction (LVNC) [23–26]. In 2007, mutations in MYH7 encoding beta-myosin heavy chain were identified as the cause underlying the association of Ebstein anomaly with LVNC [27]. This study relied on genome-wide linkage analysis of a single large family with 12 members affected with LVNC, out of which four also had Ebstein anomaly. Since this first report, numerous similar MYH7 mutation-positive cases have been published [28–31]. To date, there is no clear genotype-phenotype correlation emerging for mutations in the rod versus the head of the myosin molecule. Approximately 40 % of patients with LVNC and Ebstein anomaly exhibit a decrease in their left ventricular function [31]. In MYH7 mutation-positive families with LVNC and Ebstein anomaly, these phenotypes are not fully penetrant: for LVNC, penetrance is estimated at 50–100 %; for Ebstein anomaly, penetrance is estimated at 20–50 % [27, 30, 32].

A large family with Ebstein anomaly in six members and sex-dependent expressivity of the phenotype was described [7]. Of note, most Ebstein anomaly cases in this family also had skeletal anomalies, whereas two members had the skeletal features without Ebstein anomaly. Male members were more severely affected than female members; one male had aortic stenosis but not Ebstein anomaly. These findings are compatible with a distinct monogenic entity of Ebstein anomaly, either as an autosomal dominant trait or an X-linked recessive trait with mildly affected female mutation carriers.

Recently, a homozygous SCN5A (sodium channel, voltage gated, type V, alpha subunit) mutation was identified in a large consanguineous family in which four affected children had a history of early cardiac arrhythmia encompassing sinus node dysfunction, conduction disease, and severe ventricular arrhythmias [33]. One of the affected children also had Ebstein anomaly.

In summary, several monogenic causes of Ebstein anomaly are known. The most frequently reported association is that of Ebstein anomaly with LVNC. Genetic testing for mutations in MYH7 and NKX2-5 is readily available and can aid in counseling and management of selected patients with Ebstein anomaly.

53.3 Structural Genomic Variation in Ebstein Anomaly and Syndromic Forms

The occurrence of extracardiac anomalies in about 20 % of cases with Ebstein anomaly has prompted systematic investigations of structural genomic variation as a potential cause. From case reports and patient series, a correlation of Ebstein anomaly with several disorders emerges; details are summarized in Table 53.1. However, even in defined disorders such as the 1p36 deletion syndrome, the penetrance of congenital heart disease is only 50–69 %, with even lower occurrence of Ebstein anomaly [12, 34]. Whereas the deletions at chr5q35 (NKX2-5) and 8p23.1 (GATA4) each harbor biologically plausible candidate genes, multiple cardiovascular malformation critical regions have been defined at 1p36, and a single causative gene for Ebstein anomaly at this locus has not yet been identified [34, 35]. Current knowledge suggests PRDM16 (PR domain containing 16) as a gene contributing to the left ventricular noncompaction/dilated cardiomyopathy phenotypes encountered in the 1p36 deletion syndrome [36], but it is also conceivable that deletions of 1p36 constitute a contiguous gene deletion syndrome with other or multiple contributing genes [34, 35]. Whether mutations in PRDM16 or other genes from the 1p36 interval can contribute to Ebstein anomaly remain to be studied. Haploinsufficiency of these genes alone is unlikely to be the sole cause of heart malformations in 1p36 deletion syndrome, since penetrance of all CHD is estimated to be 69 % and that of Ebstein anomaly 6 % in the largest systematic series published to date [34]. Along the same lines, it is interesting to note that the three patients with Ebstein anomaly described with deletions of chr18q21.3- > qter are all predicted to be haploinsufficient for NFATC1 (nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1), a key cardiac transcription factor [12, 53]. Other features in patients with this or similar deletions who do not have Ebstein anomaly still frequently share other cardiac phenotypes such as atrial septal defects and pulmonary valve stenosis [53].

Syndromic forms of congenital heart disease also can be caused by single genes or be of unknown genetic origin. In a larger case series with 44 patients with Ebstein anomaly, 12 (27 %) had syndromic presentation, and 7 of those had distinct disorders including CHARGE syndrome ($N=2$), VACTERL association, Noonan syndrome, Kabuki syndrome, Holt-Oram syndrome, and Cornelia de Lange syndrome (one each) [12]. An additional two patients with CHARGE syndrome have been reported in the literature, both of which carry mutations in CHD7 (chromodomain helicase DNA binding protein 7), as well as one additional patient each with Kabuki syndrome, Holt-Oram syndrome, Noonan syndrome, Williams syndrome, and Ellis-van Creveld syndrome without mutation screening

Table 53.1 Summary of chromosomal imbalances reported in Ebstein anomaly

Chromosomal disorder	Number of patients reported	Proven or suspected causal gene(s)	References
Deletion 1p36	9	DVL1, SKI, RERE, PDPN, SPEN, CLCNKA, ECE1, HSPG2, LUZP1, WASF2, PRDM16, PRKCZ, UBE4B, MASP2	[12, 34–44]
Microdeletion 5q35	2	NKX2-5	[44, 45]
Deletion 8p23.1	3	GATA4	[12, 46, 47]
Duplication 9p	2	Unknown	[48, 49]
Deletion and duplication 11q	1	ETS1	[50]
Duplication 15q	2	Unknown	[51, 52]
Trisomy 18	1	Unknown	[1]
Deletion 18q21.3- > qter	3	NFATC1	[12, 53]
Trisomy 21	8	Unknown	[54–61]

[62–67]. A single patient with thiamine-responsive megaloblastic anemia and Ebstein anomaly has been reported; interestingly, several other cardiac defects have been described in this syndrome, including one case of atrial standstill, which can be part of Ebstein syndrome [68–70]. A relatively high number of patients with Ebstein anomaly and Down syndrome have been reported in the literature (Table 53.1); however, one has to keep in mind that the denominator of all children born with trisomy 21 is much higher than for all other chromosomal imbalances listed here.

Conclusion

Clinical, epidemiological, and molecular evidence supports the hypothesis that Ebstein anomaly has strong genetic determinants. While the genetic causes are heterogenous, overlapping phenotypes are observed even in genetically distinct entities. Prime examples for these phenomena are left ventricular noncompaction in both MYH7 mutation and 1p36 deletion carriers or the spectrum of recurring right-sided malformations in patients with Ebstein anomaly and 18q21 deletion. Genes contributing to Ebstein anomaly affect diverse developmental pathways, with variable expressivity and incomplete penetrance being common observations in multiplex families. It is very likely that the causal genes identified to date act in concert with modifiers, either environmental or (epi)genetic. The most frequent chromosomal imbalances contributing to Ebstein anomaly are the microdeletions 1p36 and 8p23.1. It can be anticipated that use of molecular karyotyping and next-generation sequencing technologies will increase the gene catalogue involved in the pathogenesis of Ebstein anomaly.

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Molecular Pathways and Animal Models of Ebstein Anomaly

54

Gregor U. Andelfinger

Contents

Abstract

Ebstein anomaly is a congenital malformation of the tricuspid valve characterised by abnormal attachment of the valve leaflets, resulting in varying degrees of valve dysfunction. The anatomic hallmarks of this entity are the downward displacement of the attachment of the septal and posterior leaflets of the tricuspid valve, and additional intracardiac malformations are common. From an embryological point of view, the cavity of the future right atrium does not have a direct orifice connected to the developing right ventricle. This chapter provides an overview of current insight into how this connection is formed and how malformations of the tricuspid valve arise from dysregulation of molecular and morphological events involved in this process. Furthermore, the naturally occurring model of canine tricuspid valve malformation and the recently generated Nkx2-5 (NK2 homeobox 5) p.Arg25Gly knockin allele, which are the most faithful animal models of Ebstein anomaly reported to date, are described and set in contrast to the human model. Although Ebstein anomaly remains one of the least under-

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stood cardiac malformations to date, the studies summarised here provide, in aggregate, evidence for strong monogenic and oligogenic factors driving pathogenesis.

54.1 Introduction

Few cardiac malformations pose riddles as intriguing as Ebstein anomaly for both the clinician and the basic researcher. The anatomic hallmarks of this entity are the downward displacement of the attachment of the septal and posterior leaflets of the tricuspid valve. These leaflets are dysplastic and adherent to the ventricular wall. Characteristically, the anterior leaflet is enlarged and "sail-like". The downward displacement of the right-sided atrioventricular junction leads tricuspid insufficiency and dilation of the right atrium. The portion between the functional and anatomic valve annulus is termed "atrialised right ventricle". The right ventricle is myopathic. Associated anomalies, particularly secundum atrial septal defect and accessory atrioventricular (AV) pathways, are common, and an association with ventricular inversion (left-sided Ebstein anomaly) is well known. Left-sided anomalies including aortic and mitral valve anomalies as well as abnormal contraction of the left ventricle have been well documented during life and autopsy, including aortic valve malformations and left ventricular noncompaction (LVNC) [1–8]. Malformation of the tricuspid valve with abnormal attachment of the septal leaflet is also common in hypoplastic left heart syndrome [9, 10].

The morphologic features of Ebstein anomaly and associated malformations make it likely that the genes involved in the pathogenesis of this disease exert an influence on several different valve- and myocardium-specific developmental pathways. Still, in comparison to other congenital heart diseases (CHD), there is a scarcity of both molecular data and animal models for the investigation of this interesting malformation.

54.2 Development of the Right Ventricular Inlet and Tricuspid Valve

Valve development is a highly conserved process. It is initiated early in heart morphogenesis, and primitive cardiac valves arise after endocardial cushions are induced at the atrioventricular canal and the outflow tract regions. Endocardial cushion formation is initiated by signals from the outer myocardial layer which induce the endocardium to undergo an epithelial-to-mesenchymal transition [11]. Mesenchyme then invades the underlying extracellular matrix. The endocardial cushions of the AV canal have unique developmental properties and will fuse to form the atrioventricular septum and to divide the single heart tube into a mitral and a tricuspid inlet. Mesenchyme from the endocardial cushions also will contribute to the formation of the inlet portion of the right ventricle [12].

It is important to note that before septation, the cavity of the future right atrium does not have a direct orifice connected to the developing right ventricle (Fig. 54.1) [12–15]. The formation of the tricuspid valve is thought to occur in two phases: first, the development of a connection between the right atrium and the right ventricle, and second, the formation of the valves and their suspension apparatus.

In the first phase, the parietal wall of the right atrium will come into continuity with the parietal wall of the right ventricle through remodelling and posteroinferior expansion of the myocardium at the right junction. Concomitant with the development of the right atrioventricular connection, a myocardial ridge forms at the boundary between the AV canal and the embryonic right ventricle. This myocardial funnel develops to become the "tricuspid gully" [12], which at this stage does not have any leaflets of the tricuspid valve yet. It grows to become a myocardial gully that funnels atrial blood beneath the lesser curvature of the initial heart tube towards the middle of the right ventricle. Fenestrations in the floor of the gully create an additional inferior opening in the funnel, with the primary orifice of the developing tricuspid valve running parallel to the developing ventricular septum [13].

In the second phase, epicardially derived cells, myocardium and endocardial cushions all make contributions to the leaflets of the tricuspid valve [12, 13, 16]. With the appearance of the endocardial cushion, the lumen between the trabecular and compact layers of myocardium expand, leading to the formation of the anterosuperior and inferior leaflets during the seventh week of gestation. Delamination of the septal leaflet from the developing ventricular septum occurs much later during the twelfth week. The myocardial contributions to the septal leaflet come from both the myocardial gully and the ventricular septum.

In parallel to this process, the insulation between the muscle masses of atrium and ventricle is established by the fusion of the tissues of the atrioventricular sulcus

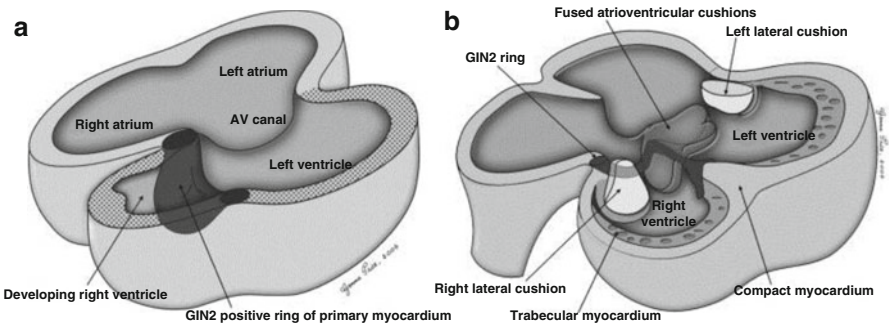


Fig. 54.1 (**a**) Location of the ring of musculature of the primary heart tube delineated by reaction to an antibody to the nodose ganglion of the chick. This is the myocardium of the so-called primary ring. (*AV* atrioventricular, *GIN2* antigen to chick nodose ganglion). (**b**) The first step in remoulding of the primary ring occurs concomitant with appearance of the primary ventricular septum. As the apical parts of the ventricle balloon form the primary tube, the inferior part of the primary foramen deepens to form the right ventricular inlet component between the right atrium and the dorsal part of the developing right ventricle. At the same time, the cushions within the atrioventricular canal become draped across the inferior part of the forming ventricular septum (Adapted from [13])

(located at the epicardial side of the junctional myocardium) with those of the atrioventricular cushions (located at the endocardial side of the junctional myocardium). This fusion leads to the formation of fibrous insulating tissues at approximately 7 weeks of development in the anteromedial portion of the right atrioventricular junction and is largely completed around the 12th week of development. In the normal adult heart, the only remaining myocardial continuity between atrial and ventricular myocardium is the atrioventricular axis of conduction [15].

Molecular pathways involved in the pathogenesis of Ebstein anomaly are likely to impact the cellular and morphological events of tricuspid valve formation at very early stages. Several signalling and transcription cascades are therefore prime candidates based on their spatiotemporal expression patterns or known functions in heart morphogenesis.

Gain- and loss-of-function studies, tissue explant studies and expression patterns point towards an important role of BMP (bone morphogenetic protein) signalling in endothelial-mesenchymal transformation of endocardial cushions. BMP2 is strongly expressed in AV cushions, whereas the major BMP receptors, including BmprII, Alk2 (Acvr1, activin A receptor, type 1) and Alk3 (Acvr3) are widely expressed in the entire heart, with no clear spatial restriction [17, 18]. Using a GATA (GATA binding protein) 6-Cre driver mouse, Gaussin et al. conditionally deleted Alk3 in the developing AV canal [19]. Interestingly, lineage analysis showed a clear contribution of AV canal cardiac myocytes to the future tricuspid mural and posterior leaflets, but not to the tricuspid septal leaflet. The mitral septal leaflet and the atrial border of the annulus fibrosus were also stained. When Alk3 was deleted in these cells, defects were seen in the same leaflets, i.e., the tricuspid mural leaflet and mitral septal leaflet were longer, the tricuspid posterior leaflet was displaced and adherent to the ventricular wall and the annulus fibrosus was disrupted resulting in ventricular preexcitation. These defects provide in fact some overlap with Ebstein anomaly and demonstrate that signalling by Alk3, the type 1a receptor for bone morphogenetic proteins, in the myocardium of the AV canal is required for the development of both the AV valves and annulus fibrosus, but they do not replicate the hallmark of the condition, namely, the apical displacement and non-delamination of the septal leaflet of the tricuspid valve. Conceivably, expression of Cre in the GATA6-Cre driver mouse may become progressively restricted in primary myocardium and thus insufficient to label all AV canal myocardial cells.

However, in extrapolation of these results, it can be presumed that key processes possibly involved in Ebstein anomaly are at least in part mediated through BMP signalling in the developing atrioventricular myocardium, including the processes leading to proper electric insulation of ventricles and atria [19]. Within the same morphological processes and molecular pathway, it was observed that positioning of the endocardial cushions in the ventricular lumen and formation of early trabeculae are part of one remodelling process which is under control of TGF-β2 (transforming growth factor, beta 2) [20]. In the Tgf-beta2-KO mouse, remodelling of the early myocardium is disturbed as indicated by defective trabeculae formation, persistence of valvular myocardium, disturbed myocardial phenotypes and differential defects at left and right side of the AV canal [20]. Bmp2 and Tgf-beta2 themselves

are under genetic control of Tbx (T-box) 20, disruption of which leads to defective AV canal myocardial and valve development [21].

Epicardially derived cells (EPDC) are known to contribute to the developing fibroblast population in the heart [22, 23]. EPDCs contribute to the development of the AV canal and the annulus fibrosus and populate the ventricular myocardial wall and interventricular septum [24, 25]. While early development of the myocardial compact layer is independent of EPDCs, the subsequent increase in wall thickness is impaired when epicardial development is disrupted [26, 27]. Using the mWt1/IRES/GFP-Cre mouse to trace the fate of EPDCs from embryonic day (ED) 10 until birth, Wessels et al. found that there is a dramatic disparity of the contribution of epicardial- versus endocardial-derived cells to the different leaflets of the right-sided AV valve: the parietal AV leaflets have a very strong epicardial contribution, whereas the septal leaflet of the tricuspid valve (and all leaflets derived from the major cushions) hardly have any epicardial contribution [16]. The differential contribution of EPDCs and other cell sources to the various leaflets of the atrioventricular valves, as well as their roles in myocardial growth and maturation, thus helps to understand how individual components of this region of the heart may be differentially affected in Ebstein anomaly. A corresponding cell population that heavily populates the septal leaflet of the tricuspid valve and also participates in the remodelling of the "tricuspid gully" [12], however, remains elusive to date. Equally unknown are the precise molecular factors that drive the apoptosis of the myocardial layer in the developing valves and their subsequent excavation and delamination [28, 29].

54.3 Mouse Models of Ebstein Anomaly

Although several mouse models exist that replicate some aspects of Ebstein anomaly, no single-gene model exhibiting all its features has been reported to date. A model of tricuspid atresia was reported in Fog2 (friend of GATA 2 or ZFPM2) knockout mice, most likely secondary to impaired attenuation of endothelial-to-mesenchymal transition within the developing cushions [30, 31]. The recently reported Nkx2-5 (NK2 homeobox 5) p.Arg25Gly knockin allele, which mimics a mutation found in humans, is the most faithful murine Ebstein model to date [32]. The human NKX2-5 p.Arg52Gly mutation destabilises the homeodomain of this transcription factor, reduces DNA binding as well as homo- and heterodimerisation with GATA4 and TBX5 [33]. In the 129Sv background, this knockin leads to a 100 % penetrance of CHD, in particular LVNC (100 %), ventricular septal defects (82 %), atrioventricular septal defects (18 %) and tricuspid valve anomalies (47 %). Incomplete delamination was seen in all mice with tricuspid valve anomalies, with all hallmarks of Ebstein anomaly in 8.5 % of mice. Detailed molecular assessment and timelines of maldevelopment of the tricuspid valve have not yet been reported in this mouse. In keeping with other studies [34, 35], penetrance of different phenotypic aspects of those mice was highly dependent on the background. After one cross into the C57B6 background, attenuation of most defects was noted, with the

exception of LVNC which still had a very penetrance [32]. As seen in other mouse mutants, these important observations suggest that the morphogenetic processes governing normal and abnormal tricuspid formation are likely to be under the control of a cooperative gene network [36].

54.4 Canine Tricuspid Valve Malformation As a Model for Human Ebstein Anomaly

Dogs have long been popular models for cardiac physiology studies because canine heart size approximates the human heart. The advantages of the canine as a potential model of genetic heart disease include short generation time and multiple offspring in one litter. Selective breeding strategies increase the likelihood of transmission of desirable traits, but also lead to an excess of genetic disorders [37]. Individual dogs may have more than 100 offspring and exert a strong influence on the genetic outfit of a particular breed ("popular sire effect") [38]. Therefore, founder effects play an important role in modern dog breeds, even in the absence of known common ancestors [39, 40]. As an example, there is statistical genetic and molecular evidence for cardiovascular traits as diverse as subaortic stenosis and cardiomyopathy that strong genetic factors shape the phenotype, albeit this can occur in complex fashion [41–43].

Canine tricuspid valve malformation (CTVM) is characterised by an apical displacement of the attachment of the tricuspid leaflets and adherence of the septal

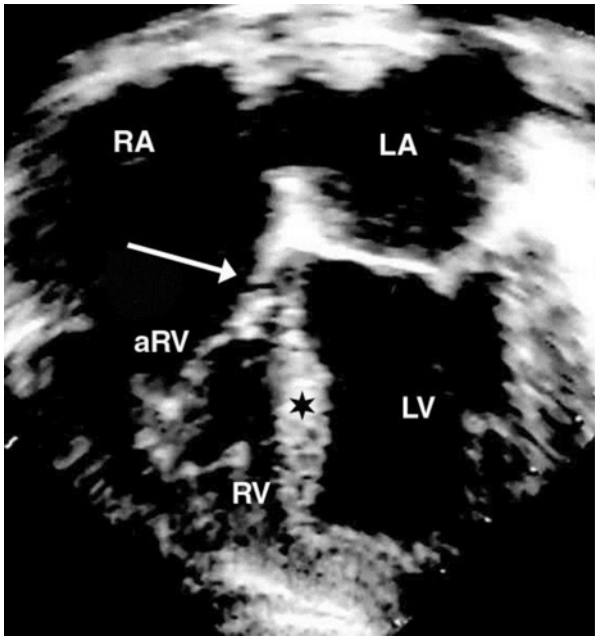


Fig. 54.2 Apical four-chamber echocardiogram in canine tricuspid valve malformation. *Arrowhead* downward displacement and abnormal adherence of the septal leaflet of the tricuspid valve [44]. *RA* right atrium, *LA* left atrium, *aRV* atrialised right ventricle, *RV* right ventricle, *LV* left ventricle, ★ interventricular septum

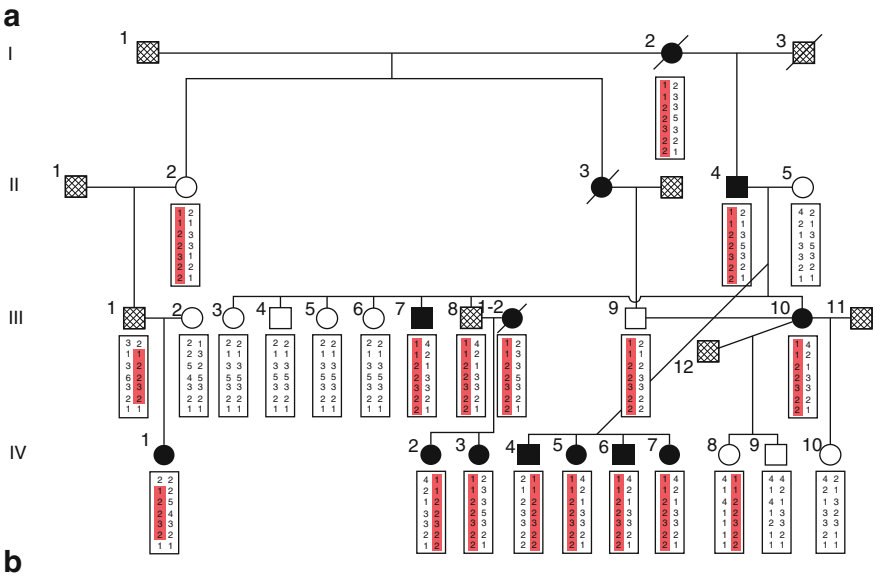
leaflet to the interventricular septum (Fig. 54.2). Other features include thickening of the valve leaflets, redundancy of the parietal leaflet and presence of a large, fused papillary muscle rather than normal small, discrete muscles. Additional congenital heart defects in affected dogs have been described, including mitral valve abnormality [45–49]. CTVM has been most frequently identified in Labrador Retrievers, but it has been observed in other breeds as well [47, 50–53]. As in humans, the clinical picture varies, with severity in affected dogs ranging from asymptomatic to premature death secondary to right heart failure [48, 50, 51]. In conclusion, CTVM mimics the morphological and clinical picture of Ebstein anomaly.

The availability of larger pedigrees and ascending genealogies from breeding documents has facilitated the genetic analysis of CTVM. All three available studies show clear evidence of heritability of CTVM, two of them in favour of a recessive mode of transmission [52, 53] and one of them in favour of an autosomal dominant transmission with reduced penetrance [44]. In this latter study, a total of 140 tested polymorphic markers excluded ~60 % of the genome before suggestive linkage to canine chromosome 9 (CFA9) was detected at REN75M10 with a two-point LOD score of 1.56. Subsequently, fine mapping with adjacent and linkage analysis yielded a maximum multipoint LOD score for the three most informative markers within the critical interval of 3.33, indicating odds of greater than 2000:1 that CTVM is linked to a locus on CFA9 (Fig. 54.3) [44]. Subsequent comparative mapping showed that this interval is homologous to a gene-rich region on human chromosome 17q, but a disease-contributing variant could not yet be isolated (Fig. 54.4) [54, 55].

Several observations are noteworthy: a single locus seems to control the predisposition in the Dogue de Bordeaux population in Israel towards both CTVM and subaortic stenosis [52]. It is unknown whether this locus is identical to the Labrador CTVM locus on CFA 9. In Labradors, two independent studies estimated heritability at 0.71 and penetrance at 68 % [44, 53]. In Keeshonds, conotruncal defects (CTD) are oligogenic, with the strongest support for a locus on CFA9 (LOD 3.7), fully overlapping with the CTVM locus mapped in Labradors [56]. Two other loci were mapped in this study to CFA2 and CFA15 (LOD scores of 2.71 and 3.03), and a two-locus analysis suggested that CTD-predisposing alleles of these three loci are necessary, at least in pairs, to produce CTD [56]. It is conceivable that breed-specific background effects in the Labrador, Keeshond and Dogue de Bordeaux dogs influence both penetrance and expressivity of CTVM, reminiscent of observations both in humans and mouse.

Conclusion

In Ebstein's malformation, the attached margin of the septal and inferior leaflets of the tricuspid valve are displaced apically, but never beyond the junction of the ventricular inlet with the apical trabecular component [12]. This junction corresponds to the anterior limit of the myocardial gully, the expansion of which corresponds to the atrialisation of the right ventricle. The septal leaflet is not delaminated properly from the ventricular septum, with a large offset between the hinge points of the tricuspid valve and the anterior leaflet of the mitral valve. In more severe cases of Ebstein anomaly, dysplasia of the septal leaflet can be so



d

Not available for genotyping or clinical assessment

Markers:	Disease haplotype in CTVM1:	Map distance in cR:
C03304	1	0.0
REN54L20	1	129.6
REN75M10	**2**	51.2
RARA	**2**	55.9
REN206J15	3	22.4
REN124K12	2	4.3
REN126A15	2	42.2

Fig. 54.3 (**a**) kindred CTVM1, (**b**) kindred CTVM2, (**c**) kindred CTVM3, (**d**) legend, (**e**) genotyped markers and map distances (*cR*). All affected dogs in kindreds CTVM2 and CTVM3 also fulfilled the diagnostic criteria for canine tricuspid valve malformation (CTVM). None of the dogs in the series reported here showed any other intra- or extracardiac malformation [44]

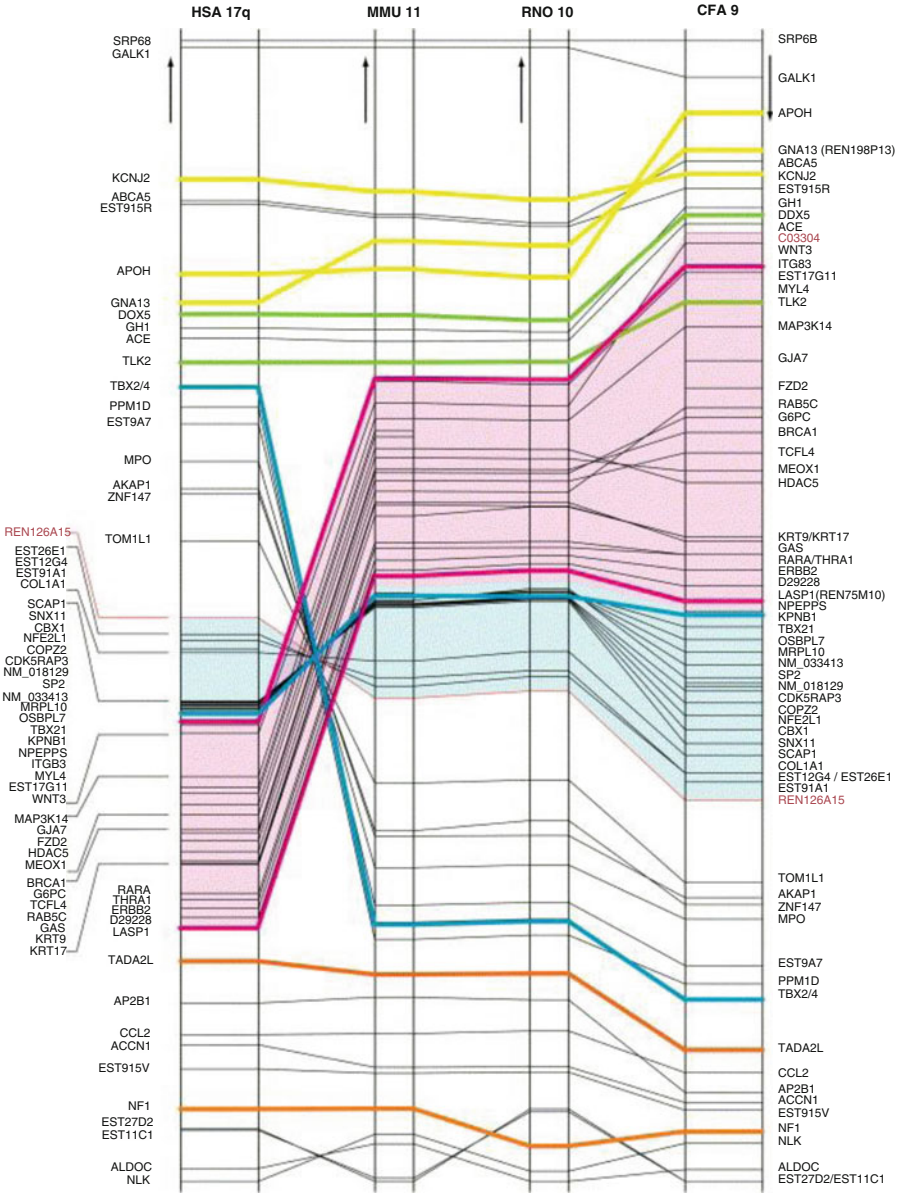


Fig. 54.4 *Horizontal axis*: human chromosome 17q (HSA17q) and its syntenic regions on mouse chromosome 11 (MMU11), rat chromosome 10 (RNO10) and dog chromosome 9 (CFA9). *Vertical axis*: Chromosomal positions of genes, with largest distances in individual species equalised to each other. Positions are drawn to scale. *Arrows* denote directionality from centromere to telomere. The recombinant markers for the ctvm interval, C03304 and REN126A15, are labelled by red lettering in the right-hand column. *Yellow*, *green*, *blue*, *purple* and *orange lines* facilitate the identification of genes involved in important rearrangement events. *Pink* and *blue overlays* identify human chromosomal segments that undergo macroreversals in a multiple genome rearrangement scenario. Brackets group genes in densely mapped areas in respective order [54]

severe that only a small orifice from the right atrium to the right ventricle persists [57–59]. It has been postulated that these features are in fact typical for the state of the normal tricuspid valve at 8 weeks of development [12].

Although the molecular substrates of Ebstein anomaly are still largely unknown, the elements outlined above leave ample scope to consider several specific processes in the development of this condition. Cell populations affecting tricuspid valve development arise from epicardial, endocardial and myocardial lineages; specific AV canal precursors for the tricuspid septal leaflet have not yet been identified. Molecular cascades governing the behaviour of these cell populations include the TGF-β signalling pathway, matrix proteins and transcription factors. The naturally occurring model of canine tricuspid valve malformation mimics human Ebstein anomaly; most likely, strong oligogenetic determinants interact in disease pathogenesis.

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Part XVI

Hypoplastic Left Heart Syndrome

Clinical Presentation and Therapy of Hypoplastic Left Heart Syndrome

55

David J. Driscoll

Contents

55.1 Introduction

Hypoplastic left heart syndrome (HLHS) can result from a number of abnormalities that result in a very small, unusable left ventricle and severe hypoplasia of the ascending aorta. Theses abnormalities include aortic valve atresia or severe stenosis and/mitral valve atresia and/or stenosis without a ventricular septal defect (Fig. 55.1).

55.2 Pathologic Physiology

At birth, systemic blood flow is supplied by the right ventricule pumping blood into the pulmonary artery and then both to the lungs and, via a patent ductus arteriosus, to the aorta. When the ductus closes, significant organ ischemia and death occurs. If the ductus does not close significantly, pulmonary edema occurs leading to death.

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There are very rare reports of patients surviving to childhood because of the development of early pulmonary vascular obstructive disease that limits pulmonary blood flow and pulmonary edema.

55.3 Clinical Presentation

Most infants with HLHS will present with cyanosis. Occasionally, the cyanosis will be mild and missed. These infants then present at 3–7 days of age in shock when the ductus arteriosus closes.

55.4 Physical Examination

At birth, many infants with HLHS will have cyanosis, an increased right ventricular impulse, and a systolic murmur. If the ductus is restrictive, all the peripheral pulses may be diminished or absent. Most will be tachypneic and have other signs of congestive heart failure. As alluded to above, if a 3–7-day-old infant presents with sudden onset of shock, one must consider a diagnosis of HLHS.

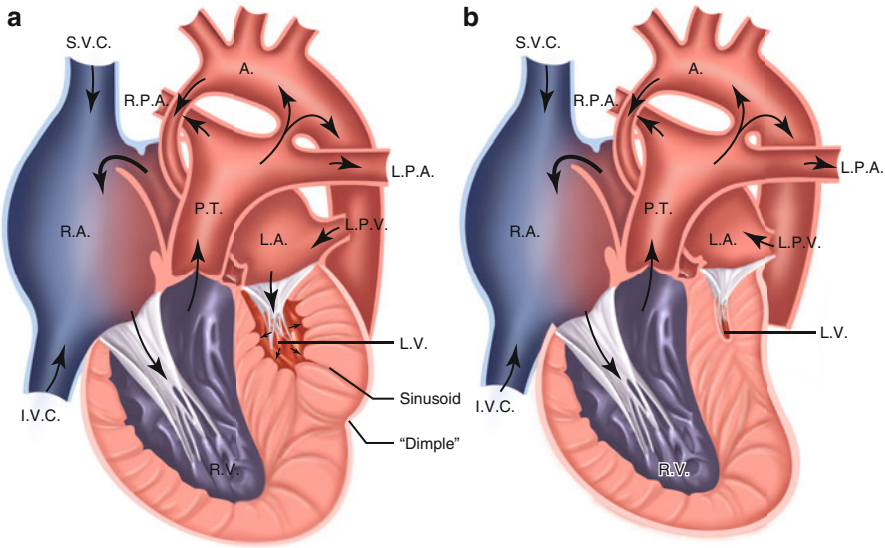

Fig. 55.1 Diagrammatic representation of HLHS. On the left is aortic valve atresia and on the right is mitral valve atresia. Note the hypoplastic left ventricle in both cases. Abbreviations: *SVC* superior vena cava, *IVC* inferior vena cava, *RA* right atrium, *RV* right ventricle, *PT* pulmonary trunk (artery), *RPA* right pulmonary artery, *LPA* left pulmonary artery, *LA* left atrium, *LPV* left pulmonary vein, *LV* hypoplastic left ventricle, *A* aorta (Diagram the courtesy of Patrick O'Leary, MD, Mayo Clinic College of Medicine)

55.5 Echocardiographic and Cardiac Catheterization Issues

The diagnosis of HLHS is made using echocardiography. Cardiac catheterization no longer is needed for diagnosis. In some centers, a "hybrid" approach to treatment requires interventional cardiac catheterization procedures.

55.6 Treatment

The initial treatment for patients with HLHS is to maintain patency of the ductus arteriosus using prostaglandin. The next step is to perform the Norwood procedure, usually done at 2–5 days of age. There are many modifications of this operation, but essentially the aorta and pulmonary artery are reconstructed such that the right ventricle empties into the neoaorta. The pulmonary artery confluence is removed from the main pulmonary artery and oversewn. In the original description of the Norwood procedure, pulmonary blood flow then was supplied via a modified Blalock-Taussig shunt. A more recent modification involves using a small Gortex tube to connect the right ventricle to the pulmonary artery (the Sano modification).

At about 4–6 months of age, the so-called second stage procedure is performed. This involves ligating the modified Blalock-Taussig shunt or the Sano shunt, repairing any distortions of the pulmonary tree and performing a bidirectional Glenn anastomosis.

The third and final stage is performed at 2–6 years of age and is a modified Fontan operation. Usually, this is performed using an extracardiac conduit from the inferior vena cava to the underside of the right pulmonary artery.

An alternative treatment strategy to a Norwood operation is cardiac transplantation in infancy. The 5 year survival of this strategy is similar to that of the Norwood strategy and, in some centers, greater. However, there is a wait time for a donor heart to become available, and during that wait period, the patient may succumb. In addition, cardiac transplantation is associated with risks of rejection, infection, post-transplant lymphoproliferative disease, renal failure, and graft coronary artery disease.

55.7 Outcome

HLHS is perhaps the most severe form of congenital heart disease. The mortality from the first stage of surgical repair, the Norwood operation, ranges from 5 % to 30 % depending upon the experience of the surgeon and the associated anomalies. There continues to be a hazard for death between the first and second (bidirectional Glenn anastomosis) stages. There is an additional mortality risk at the time of the second stage. At the time of the third stage (modified Fontan operation), there is an additional 3–8 % risk of death. After the Fontan operation, there is an ongoing hazard for death, arrhythmia, cirrhosis, and protein-losing enteropathy.

Human Genetics of Hypoplastic Left Heart Syndrome

56

Woodrow D. Benson

Contents

Abstract

Hypoplastic left heart syndrome (HLHS) is a severe congenital cardiovascular malformation characterized by hypoplasia of the left ventricle, aorta, and other structures on the left side of the heart. The pathologic definition includes atresia or stenosis of both the aortic and mitral valves. Despite considerable progress in clinical and surgical management of HLHS, mortality and morbidity remain concerns. One barrier to progress in HLHS management is poor understanding of its cause. Several lines of evidence point to genetic origins of HLHS. First, some HLHS cases have been associated with cytogenetic abnormalities (e.g., Turner syndrome). Second, studies of family clustering of HLHS and related cardiovascular malformations have determined HLHS is heritable. Third, genomic regions that encode genes influencing the inheritance of HLHS have been identified. Taken together, these diverse studies provide strong evidence for genetic origins

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of HLHS and related cardiac phenotypes. However, using simple Mendelian inheritance models, identification of single genetic variants that "cause" HLHS has remained elusive, and in most cases, the genetic cause remains unknown. These results suggest that HLHS inheritance is complex rather than simple. The implication of this conclusion is that researchers must move beyond the expectation that a single disease-causing variant can be found. Utilization of complex models to analyze high-throughput genetic data requires careful consideration of study design.

56.1 Introduction: What Is HLHS?

Hypoplastic left heart syndrome (HLHS) is a severe form of congenital heart disease (CHD). As the name suggests, hypoplasia of the left ventricle (LV) resulting in a functional single ventricle is a cardinal feature, but the pathologic definition includes atresia or stenosis of both the aortic and mitral valves, along with hypoplasia of the ascending aorta. Intact ventricular septum and normally related great arteries are also factored in the definition [1–3]. A subclassification scheme, based on the pathologic anatomy of the aortic and mitral valves, is widely employed. Although emphasis has been on left-sided heart structures, dysplasia of both tricuspid and pulmonary valves often is reported [4–7]. Despite a similar approach to surgical management for hearts with LV hypoplasia, the HLHS definition used for studies of genetic discovery typically excludes anatomic variations of LV hypoplasia such as unbalanced atrioventricular septal defect and double-outlet right ventricle with mitral valve atresia. This exclusion is based on the belief that the developmental origins of these varied LV hypoplasia phenotypes differ from the origins of HLHS.

56.1.1 HLHS Prevalence

HLHS accounts for approximately 4–8 % of all CHD, and the prevalence has been estimated from 0.06 to 1.20 with a median of 0.22 per 1000 live births [8, 9]. HLHS is viable in utero and rarely results in fetal loss. HLHS is slightly more common in males; however, there is no ethnic or geographical association. Despite live birth of ~2000 cases of HLHS annually, the burden to society is substantial. The direct costs, medical morbidity, and utilization of a disproportionate share of pediatric cardiac resources make HLHS a central problem in pediatric cardiology.

56.1.2 HLHS-Related Phenotypes

An increased prevalence of CHD in families identified by an HLHS proband was noted in the Baltimore-Washington Infant Study [9, 10]. Additional observations that first-degree relatives of HLHS probands had increased occurrence of bicuspid

aortic valve (BAV) [9, 11] lead to the hypothesis that cardiac malformations characterized by obstruction to blood flow in the LV outflow tract are causally related. In addition to HLHS and BAV, these malformations include aortic valve stenosis and aortic coarctation (collectively referred to as left-sided malformations). Additional supportive evidence for a causal relationship stems from several other observations including (1) the association of underlying BAV with aortic stenosis in patients of all ages [12, 13], (2) longitudinal observations in the fetus that identified HLHS as part of the in utero natural history of aortic stenosis [14, 15], (3) occurrence of these defects in patients with Turner syndrome [16], and (4) findings of identical twins with discordant phenotypes of BAV and HLHS [17]. Further, BAV, HLHS, and other left-sided cardiac malformations have been shown to be heritable [17–19]. However, little is known of the size or extent of these effects. This may be an important consideration in the design of genetic discovery studies, i.e., can these phenotypes be pooled or should they be separately analyzed in order to minimize noise from phenotypic variation?

56.2 Genetic Studies of HLHS

Several lines of evidence support a genetic cause for HLHS. First, there are numerous reports linking occurrence of HLHS to chromosomal abnormalities, e.g., Turner syndrome (monosomy X) and Jacobsen syndrome (chromosome 11q deletion) [20, 21]. Less frequently, trisomy 18, Smith-Lemli-Opitz syndrome, VACTERL association, CHARGE syndrome, Wolf-Hirschhorn syndrome, Rubinstein-Taybi syndrome, Noonan syndrome, and Holt-Oram syndrome have been reported [22–25]. However, these reports need to be analyzed cautiously as lack of phenotype detail and variation in CHD phenotype criteria blurs the genotype-phenotype correlations. For example, LV hypoplasia observed in Noonan syndrome or Holt-Oram syndrome may be associated with unbalanced atrioventricular septal defect rather than HLHS.

A second line of evidence supporting genetic origins of HLHS is that heritability (h^2, a statistical measure of genetic effect size) indicates that HLHS is determined largely by genetic factors. In a family-based study where all participants were screened for CHD by echocardiography, Hinton et al. [17] determined HLHS heritability was very high. In addition, the recurrence risks for HLHS and any CHD were 8 % and 22 %, respectively. Further, a tenfold increase in bicuspid aortic valve was noted. McBride et al. [19] also found high heritability using a family-based analysis of phenotypically related left-sided heart malformations.

Further evidence comes from use of linkage analysis to identify genomic regions that encode genes influencing the inheritance of HLHS. Hinton et al. [26] used nonparametric linkage analysis and identified two significant loci on chromosomes 10q22 and 6q23. These findings confirmed that nonsyndromic HLHS is genetically heterogeneous. Interestingly, ~21 % of kindreds contributed to linkage at each locus, suggesting these loci account for a substantial number of HLHS cases. Further, a suggestive HLHS locus on 11q22, previously identified in a case of HLHS with a balanced translocation t [10;11] (q24;q23), validated these analyses [27].

When the linkage approach was extended to a family-based cohort ascertained by either an HLHS or BAV proband, subset linkage analysis showed a significant improvement in the logarithm of odds (LOD) score at chromosome 14q23, providing evidence that some HLHS and BAV cases are genetically related [26]. In a family-based cohort ascertained by probands with left-sided malformations that included aortic valve stenosis, aortic coarctation, and HLHS, McBride et al. [28] found evidence for suggestive linkage to chromosomes 2p23, 10q21, and 16p12. They concluded that overlapping linkage peaks provide evidence for a common genetic etiology. Mitchell et al. [29] performed a genome-wide association study (GWAS) using a trio design where probands were ascertained by varied left-sided malformations including HLHS. Major findings included association at a chromosome 16 locus and suggestive association at loci on chromosome 3 and 10.

Several investigators have reported findings of genetic variants in single genes using mutation analysis of candidate genes in series of cases or small families. Genetic variants in connexin 43 (*GJA1*/*Cx43*) [30], NK2 homeobox 5 (*NKX2-5*) [31–33], Notch 1 (*NOTCH1*) [34, 35], and V-erb-B2 avian erythroblastic leukemia viral oncogene homolog 4 (*ERBB4*) [36] have been reported in HLHS. Ware et al. [37] identified patients with HLHS who had mutations in Zic family member 3 (ZIC3), a transcription factor associated with heterotaxy syndrome. More recently [38], patients with left-sided heart defects were found to be enriched for *de novo* variants in histone-modifying genes (H3K4me-H3K27me pathway); interestingly, patients with conotruncal defects and heterotaxy demonstrated similar enrichment. In addition to these reports of germinal mutations, there is a single report that identifies somatic mutations in heart and neural crest derivatives expressed 1 (*HAND1*) [39].

Genetic studies of HLHS patients have also evaluated copy number variations (CNVs), which are genomic regions of DNA gains or losses >1000 base pairs [40–45]. CNVs are detectable as a result of recent advances in molecular cytogenetics, particularly using microarray-based methods. Utilizing such methods to scan the genome, it has become evident that a significant proportion of the normal healthy human genome harbors CNV of unknown medical significance. However, other CNVs, usually large and often *de novo*, are considered pathogenic. Several investigators have examined the role of CNV in CHD, and 3 recent family-based studies examine the role of CNV in HLHS. Hitz et al. [40] sought to determine the impact of structural genomic variation in multiplex families ascertained by a proband with a left-sided heart malformation including some cases with HLHS. They searched for unique or rare copy number variations present only in affected members. Their findings indicate that unique CNVs contribute to at least 10 % of left-sided heart malformations cases. Carey et al. [41] evaluated patients with single ventricle, many of whom had HLHS. Putatively pathogenic CNVs had a prevalence of 13.9 %, which was significantly greater than the 4.4 % rate of such CNVs among controls. The authors concluded that pathogenic CNVs seem to contribute to the cause of single ventricle forms of CHD in $\geq$10 % of cases and are clinically subtle but adversely affect outcomes in children harboring them. Warburton et al. [42] compared CNV rates in two types of CHD, HLHS versus conotruncal defects, and found

no significant difference. However, they found a substantially higher rate of *de novo* CNVs in probands with CHD than in control families (9 vs. 2 %). Among the *de ovo* or rare inherited CNVs, there were 12 CNVs that the authors considered likely to be causally related to CHD.

56.3 HLHS Exhibits Complex Inheritance

Taken together, these diverse studies provide strong evidence for genetic origins of HLHS and related cardiac phenotypes. Initially, analyses of pedigrees ascertained by an HLHS proband were interpreted as indicating simple Mendelian inheritance of HLHS [46]. However, review of literature indicates that despite considerable effort, identification of single genetic variants that "cause" HLHS has remained elusive. These results may indicate that HLHS inheritance is complex rather than simple, a finding concluded by several studies [26, 40]. The implication of this conclusion is that researchers must move beyond the expectation that a single disease-causing variant can be found. Although analytical methods to evaluate the effects of 2 or more genetic variants are well established, the ability to apply these methods in a high-throughput manner must be developed. Furthermore, researchers must realize that utilization of these more complex models requires careful consideration of study design and statistical power. Optimal approaches to discovering pathogenetic variants in complex diseases remain unclear [47].

56.4 Pathogenetic Mechanisms of HLHS

What types of genes might be involved in the genetic underpinnings of HLHS? Concepts of cardiac development have greatly influenced our understanding of the formation of the mesoderm derived, 4-chambered vertebrate heart. Human genetic studies have identified mutations in genes important for early heart formation that cause CHD, supporting the idea that these birth defects are caused by alterations during cardiogenesis [48–52]. There has been considerable interest in CHD such as HLHS, in which individual chambers or valves of the developing heart are selectively impaired [48]. A widely accepted hypothesis is that HLHS develops as a result of embryonic alterations in blood flow, such as premature narrowing of the foramen ovale [1] or aortic valve obstruction [53]. In this light, it is noteworthy that valve malformation is a prominent part of the HLHS phenotype as evidenced by the frequent occurrence of left- and right-sided valve dysplasia in HLHS probands and the presence of BAV in family members [17]. The "no-flow, no-grow" hypothesis of heart maldevelopment also is supported by studies in the embryonic chick heart, which alter blood flow during cardiac development [54]. An alternative hypothesis for HLHS etiology focuses on the summation of separate genetic modules. Recent studies have investigated chamber-specific regulatory mechanisms, e.g., TBX5 (T-box 5) and IRX1 (iroquois homeobox 1), leading to formation of morphologically, functionally, and molecularly distinct cardiac chambers [48, 49]. In this

context, it has been suggested that LV hypoplasia may result from a primary defect in myocardial growth during development. Unfortunately, there are presently no experimental models of HLHS to elucidate the relative contribution of these two hypotheses, but defining the genetic underpinnings of HLHS should enlighten the debate.

Conclusion

While once uniformly lethal, for the past two decades, a three-staged surgical approach for HLHS has dramatically reduced mortality [55]. Despite this progress in clinical care, mortality and morbidity of HLHS remain significant. Further, progress in patient management has provided little insight into the cause of HLHS. This lack of knowledge has been a barrier to progress in risk appraisal and assessment of clinical outcomes. In addition, there has been limited information for reproductive decision making for both parents of children with HLHS and survivors of HLHS surgical intervention who are now reaching reproductive age. Further, without understanding the origins of HLHS, care of patients is based solely on clinical phenotype rather than the underlying mechanism of cardiac maldevelopment. Studies to date strongly support significant genetic underpinnings of HLHS. However, although numerous genetic abnormalities have been associated with HLHS, at this time it is not possible to reconcile all genetic findings into a comprehensive pathogenetic model. Together these genetic findings indicate genetic heterogeneity and complex inheritance of HLHS. Ultimately, we expect that identification of genetic underpinnings may result in better understanding of outcomes. New biological insights into HLHS will facilitate development of alternative therapeutic strategies.

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Molecular Pathways and Animal Models of Hypoplastic Left Heart Syndrome

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Abstracts

Hypoplastic left heart syndrome (HLHS) is a rare and severe defect in which the structures of the left side of the heart are severely underdeveloped. Only a very small minority of HLHS cases can currently be explained. This review summarizes how growth of the left-sided structures of the heart is initiated very early during development and which mechanisms could be defective in HLHS. Numerous cascades driving development of ventricular cardiomyocytes have been described and are put into perspective. Current genetic, epigenetic, and hemodynamic concepts in HLHS pathogenesis are discussed in the context of both animal and human models of impaired growth of left-sided structures of the heart. Understanding the contribution of these factors may be crucial for stratification of therapeutic interventions in HLHS.

57.1 Introduction

Hypoplastic left heart syndrome (HLHS) is a rare congenital heart defect in which the structures of the left side of the heart are severely underdeveloped. Often used as an umbrella term, HLHS comprises several anatomic subtypes, with different degrees of hypoplasia/atresia of left-sided heart structures, including the mitral valve, the left ventricle, the aortic valve, and the aorta. A multitude of factors are implicated in the pathogenesis of HLHS, making a clear definition of causative factors more difficult than for other forms of congenital heart disease. This is highlighted by the fact that contrary to other congenital heart malformations, no major genetic driver or molecular pathway has been identified and no defined animal model for studying the molecular origin of HLHS exists to date. The incomplete understanding of HLHS is in stark contrast to its high heritability, estimated by some but not by all investigators at 99 % when associated with additional cardiovascular malformations and at 74 % by itself [1]. Studies that utilized linkage analysis identified multiple regions significantly linked to HLHS, bicuspid aortic valve (BAV), and left ventricular outflow tract malformations but have so far failed to identify a major genetic driver for HLHS [2, 3].

For the purpose of this chapter, we will arbitrarily divide HLHS pathogenesis into two major processes that play a role in the normal development of the heart: (1) cellular processes/molecular cascades of ventricular development and (2) flow-related growth. An inherent factor in ventricular development is primary growth defects of cardiomyocytes that might impede the heart's capacity to establish an intact left ventricle [4, 5]. We will therefore start by considering the different origins of myocardial progenitors from the first and second heart field and the recent rethinking of early heart development by introduction of the ballooning model of chamber development (Fig. 57.1). We then continue by drawing comparisons between endocardial and myocardial development to provide a deeper understanding of their contribution to chamber formation. A different viewpoint on ventricular development is provided by flow-related growth models in which the decrease or absence of blood flow to the left side of the heart, often secondarily caused by

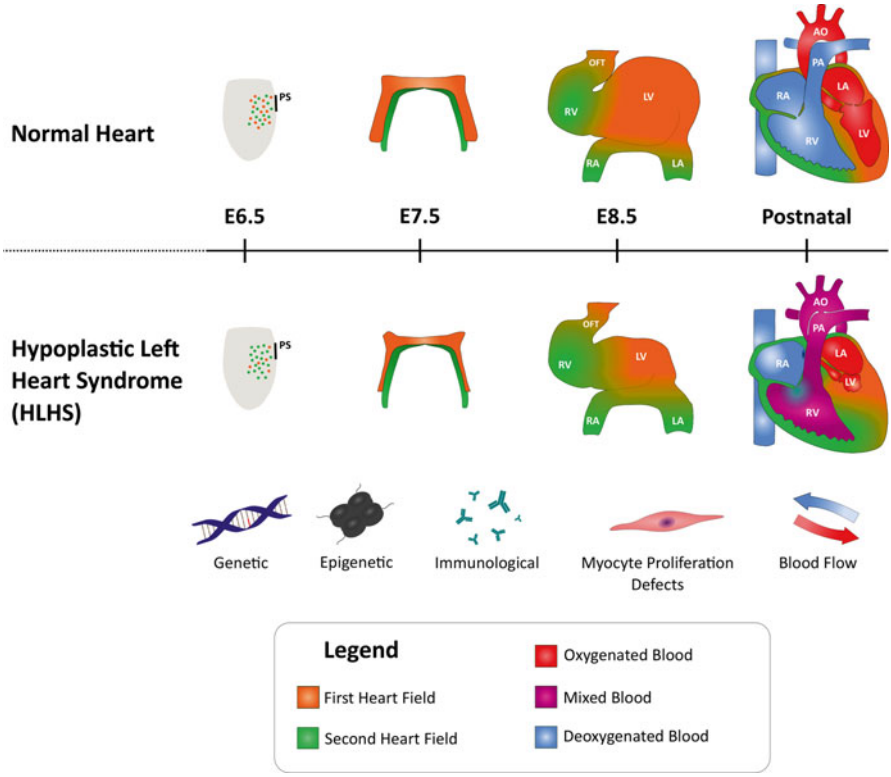

Fig. 57.1 Schematic representation of heart development in mice (*upper row*) and hypothetical depiction of factors leading to HLHS, as summarized from the literature (for references, see text). Abbreviations: *OFT* outflow tract, *RV* right ventricle, *LV* left ventricle, *RA* right atrium, *LA* left atrium, *AO* aorta, *PA* pulmonary artery, *PS* primitive streak, *E* embryonic stage in days postconception

outflow tract obstructions, is considered the main cause for an incorrectly developing left heart [6]. This mechanistic explanation of hypoplasia known as the "no flow-no grow" hypothesis states that the amount of blood flow through a vascular system determines its growth and development. Flow-related growth models therefore present a more environmentally driven development of the left heart structures in contrast to molecular models based on inherent genetic components. Multiple lines of investigation in zebrafish, chick, and mice using altered blood flow patterns support the idea of "no flow-no grow," whereas genetic analyses in human HLHS patient cohorts point toward shared genetic denominators between affected individuals. We will discuss how both flow-dependent and flow-independent mechanisms act in coordinated fashion in the development of the left heart structures.

Following the introductory part about left heart development, we will discuss the molecular pathways that are involved in HLHS. We will start from the early transcriptional programs that are established in the developing heart, proceed with the

Notch signaling pathway, and discuss cell cycling and growth factor contribution to left heart hypoplasia. We will finish this part by considering epigenetic and immunological factors in HLHS. Subsequently, we will take a look at previous and current animal models used in researching HLHS and evaluate the difficulties in utilizing model systems for this rare syndrome. The chapter closes with a view ahead into the future challenges and opportunities for researchers and clinicians in the quest for improving our understanding of HLHS.

57.2 Heart Fields and Cell Lineages in HLHS

57.2.1 Origins of Ventricular Myocardial Cells: Cardiac Mesoderm and the First and Second Heart Field

Heart cells emerge at gastrulation stages and are clonally related mainly to endodermal and mesodermal lineages [7]. One of the earliest markers of cardiogenic cells is Mesp1, a basic helix-loop-helix (bHLH) transcription factor required for mesodermal cells to generate a linear heart tube after leaving the primitive streak [8]. However, Mesp1 expression is not restricted to cardiovascular lineages and acts mainly through inducing a combinatorial set of specific cardiogenic target genes in early precursor cells [9]. Other early regulators of cardiac specification of mesoderm include the T-box transcription factor Eomes [10], which is an activator of Mesp1, as well as the cell fate factors Numb and Numbl, which are regulators of an undifferentiated and expansive population of cardiogenic precursor cells [11].

Long viewed as the sole contributor of cells for the mature heart, the early linear heart tube does not contain the whole population of cells that later make up the adult heart. Different genetic labeling experiments now provide ample evidence that the cardiac crescent is made up of distinct cell populations called the first and second heart field [12, 13] (Fig. 57.1). Clonal analysis in mice revealed that cells from the first heart field contribute primarily to left ventricle, both atria, and the right ventricle but not to the outflow tract, whereas cells from the second heart field mainly contribute to the outflow tract, the right ventricle, and both atria, but not the left ventricle [13, 14] (Fig. 57.1). The second heart field itself can be divided into an anterior and posterior part. Elegant explant experiments in mice showed that cells from the anterior second heart field were attributed to the outflow tract and right ventricular myocardium, whereas cells from the posterior second heart field make up the atrial myocardium [15]. While the phenotype of *Numb/Numbl* double knockout mice seems to arise from a role of these two genes in the second heart field and therefore is not reminiscent of HLHS, it is intriguing to speculate that similar mechanisms exist for precursor populations destined for a left ventricular fate. Recent clonal analysis of the earliest cardiovascular progenitor cells revealed two temporarily distinct Mesp1 progenitor cell populations in the first and second heart field [9]. The identification of distinct origins of progenitor cells might suggest that primary growth defects of the left ventricle itself may arise from perturbations in the genetic program of a cellular subpopulation within the first heart field.

57.2.2 The Ballooning Model of Cardiac Chamber Formation

After its formation, the linear heart tube starts to loop and the previously ventrally located side of the heart, primarily fated to constitute the left ventricle, starts to enlarge, differentiate, and form the primitive left and right ventricle [16]. This local expansion of myocytes is known as the "ballooning model of chamber formation" [17]. Prior to the concept of "ballooning," it was hypothesized that all segments of the linear heart tube were pre-patterned to become anatomic regions of the adult heart. In contrast, the current concept of heart development allows for contribution of several distinct cell lineages and localized expansion of these cell populations (for a comprehensive review of recent concepts in cardiac development, see [18]). Newly formed myocardial cells constituting the linear heart tube initially display slow proliferative properties [6, 19]. Cell expansion of chamber myocardium at the looping heart tube stage is achieved by a local increase in proliferation and cell size at the outer curvature leading to the formation of the ventricular chambers [20]. During this stage, the contractile machinery of the ventricles develops by cell differentiation at the outer curvature [21]. At the same time, the myocardium of the inner curvature, the outflow tract, and the atrioventricular canal remain in a less differentiated state. This local repression of differentiation is mediated by a set of T-box transcription factors specifically expressed by precursors of the non-chamber myocardium [22].

The cell cycle itself is by far less well understood in (cardiac) myocytes as opposed to most other cell types. Genes implicated in myocardial cell cycle regulation serve multiple processes essential for ventricular growth, such as cellular hypertrophy (e.g., cyclins and cyclin-dependant kinases (CDK) [23]), sarcomere integrity (e.g., myocardin (Myocd) [24]), and postnatal quiescence (e.g., pocket proteins Rb and p130 [25]). The same cell cycle mechanisms are required postnatal for hypertrophic growth and DNA synthesis [26]. In agreement with these observations, knockout models for several cell cycle regulators are embryonic lethal due to cardiac defects, either as constitutive or lineage-specific loss-of-function models [27].

57.2.3 The Endocardium, a Hemodynamic Sensor and Constitutive Component of the Developing Heart

The primitive heart tube consists of the inner, endocardial layer, and the outer myocardial layer, which come to lie in a sack formed by the thin pericardial layer. All three cardiac tissues arise from the same precursors in the epiblast [28]. Endocardium and myocardium are separated by cardiac jelly, produced by the myocardium. At looping of the linear heart tube, both the endocardium and myocardium simultaneously turn rightward on the ventral side of the heart. Hemodynamic factors like shear stress and retrograde flow actively modulate chamber formation at myocardial and endocardial levels in the ballooning outer curvature [29, 30]. The endocardium represents the flow-sensitive entity in the developing heart and regulates chamber

morphogenesis through the modulation of cell size and growth via the hemodynamically sensitive transcription factor *Klf2a* [31]. Mechanical forces thus induce transcriptional programs and contribute to the regulation of myocardial chamber volume, endocardial cell morphology, and endocardial cell proliferation.

A blood flow-independent stimulant for cell proliferation at the growing outer curvature is bone morphogenetic protein (Bmp) signaling. In particular, *Bmp10* is necessary for correct ventricular development. Bmp10 expression can be found in the developing murine heart as soon as E9.0 first at the trabeculated part of the ventricular chamber and later in the atrial wall [32]. In line with early expression patterns, *Bmp10* knockout mice show a strong reduction of cardiomyocyte proliferative activity and elevated expression of p57Kip2, a cyclin-dependent kinase inhibitor that is implicated in inhibiting reentry of cardiomyocytes into cell cycling [33, 34]. Bmp signaling from the myocardium thus influences the proliferation of endocardial cells in collaboration with but unaffected by hemodynamic forces. In addition to hemodynamic and Bmp signaling, there is a tightly regulated growth balance between myocardial and endocardial cell lineages, as evidenced by the overgrowth of myocardium in the absence of *Etv2*, an ETS domain transcription factor required for endocardial development [35, 36]. The presence of the endocardial cell layer and its localized interaction with the myocardium present one of the key moments in the development of the ventricular chambers and therefore deserve increased attention in the search for the molecular origins of HLHS. In the following section, we will further discuss the molecular factors that participate in ventricular development and discuss their role in HLHS etiology.

57.3 Molecular Pathways in Left Heart Development

Development of the four-chambered heart is facilitated by a spatially and temporally tightly regulated network of transcription activators, repressors, and signaling cascades. Left heart structures are controlled by factors that act early during heart development as shown by the presence of left ventricular myocardial progenitors in the linear heart tube. The developmental differences between the right and left heart regions are reflected by chamber-specific factors that are exclusively expressed in cells of either the left or right side of the heart.

57.3.1 Ventricular Development Is Regulated by a Set of Specific Transcription Factors

Mesp1, a basic helix-loop-helix transcription factor (bHLH), is the earliest known cardiac marker and initiates the cardiac transcription factor cascade which will lead to the generation of cardiac mesoderm [8, 37, 38]. Mesoderm-derived progenitor cells of the first and second heart field become cardiac specific by expression of NK2 homeobox 5 (Nkx2-5), one of the earliest markers of cardiomyocyte differentiation [39–41]. Two factors that display exclusive right/left ventricular expression

in the looping heart are Hand2 (dHand) and Hand1 (eHand), both bHLH transcription factors [42]. Although initial chick experiments using antisense oligonucleotide knockdown showed coexpression of both dHand and eHand in the linear heart tube, suggesting genetic redundancy, subsequent studies in mice revealed a differential expression pattern in the developing heart [43]. After the linear heart tube stage, dHand expression in mice is mainly found in the outflow tract and right ventricular cells, while eHand is found in the outflow tract and the early left ventricular structures [44]. The left ventricular contribution of eHand and the right ventricular contribution of dHand are also consistent with their spatial expression in the looping heart where eHand is solely found in the outer curvature [45]. Expression of eHand in turn is regulated by Nkx2-5 in mice, as the Nkx2-5 knockout mouse shows absence of eHand transcripts. Another important factor in ventricular development that is independent of eHand but directly regulated by dHand and Nkx2-5 is the Iroquois homeodomain transcription factor 4 (Irx4) [46]. Irx4 is expressed in mice from E7.5, where it is found in the cardiac primordia, to E11.5 when it is exclusively found in the myocardium of the ventricles. Irx4 contributes to ventricle chamber formation by activating ventricle-specific genes such as ventricular myosin heavy chain 1 (Myh15) and suppressing atrial-specific genes such as atrial myosin heavy chain 1 (Myh7) [47]. The early formation of the left ventricle is thus governed by the specific interaction of cardiac transcription factors that are spatially and temporally orchestrated and conserved throughout vertebrate evolution. Defects in this intricate system might contribute to the development of a hypoplastic left heart but more data will be necessary to evaluate the contribution of transcription factor defects to HLHS pathology in humans.

57.3.2 Notch Signaling at the Frontier of the Endocardium and Myocardium

The notch signaling pathway in cardiac development is best known for its role in outflow tract development, and multiple studies have associated mutations in the NOTCH1 receptor with aortic valve malformations [48–50]. The identification of NOTCH1 mutations in HLHS patients is therefore not very surprising, as HLHS is often thought to be secondary to outflow tract obstructions like BAV [51]. Notch signaling however might not only secondarily contribute to HLHS but also primarily by endocardial activation of myocardial trabeculation at the outer curvature of the ballooning heart [52]. Activation of Notch by endocardial-myocardial interaction has two distinct functions in the compaction of ventricular myocardium: (1) differentiation of compact and trabecular myocardium through EphB4/EphrinB2 endocardial-mediated Neuregulin 1 (Nrg1) activation, which in turn causes EphB4/EphrinB2 signaling activation in the myocardial cell layer [52]; and (2) inducing Bmp10 signaling at the growing poles of trabecular myocardium to provide a proliferative pool of cardiomyocytes. The control of these two processes by activated Notch is negatively regulated by Fkbp1a (Fkbp12), a peptidyl-prolyl cis-trans isomerase that is expressed ubiquitously and required for cardiac morphogenesis/

homeostasis [53–55]. The effects that disrupted Notch signaling can impose on the developing ventricular system are manifold, and more research will be necessary to completely understand its developmental contribution to HLHS.

57.3.3 Cell Cycle Regulation and Ventricular Growth

As highlighted earlier in this chapter, myocardial progenitor cells are initially small, highly proliferative cells that in mammals lose the ability to divide and are arrested in their cell cycle progression at postnatal stages. Defects in cell cycle regulation have been associated with HLHS, and several studies have investigated differential gene expression in hypoplastic left hearts. Interestingly, gene expression analysis using microarray and qPCR experiments on human atrial septum samples from HLHS patients and cardiac surgery patients not affected by HLHS revealed differential expression of numerous cell cycle regulators and chromatin remodeling factors [56]. Proper cardiomyocyte differentiation and proliferation seems to be impaired by the upregulation of cell cycle repressors WEE1 G2 checkpoint kinase (WEE1), RNA binding motif, single-stranded interacting protein 1 (RBMS1), and numerous CDK inhibitors, which interfere with the transition between cell cycle states [56, 57]. Another layer of the transcriptional program in HLHS is mediated by factors regulating chromatin remodeling and chromatin structure. Increased expression of the histone deacetylase 2 (HDAC2) and MYND domain-containing transcription factor (SMYD1) that can cooperatively regulate activation and repression of cardiac specific factors like HAND1 and IRX4 indicate transcriptional repression in the HLHS atrial septum [58]. These studies were limited however by the fact that gene expression was only measured in atrial septum of HLHS infants but not in other cardiac structures. Differences found in the atrial septum might therefore not reflect expression patterns that contribute to left ventricular development in HLHS. Nevertheless, experimental evidence points to an important role of myocyte cell cycling at an early stage of myocyte differentiation in the pathogenesis of HLHS [5]. The molecular origins of these proliferation defects in the developing human heart however remain enigmatic for now.

57.3.4 Epigenetic and Immunologic Contributions to HLHS

In addition to the genetic contributors enumerated above, other factors may play synergistic roles in the etiology of HLHS. Maternal immune response has recently been proposed to be a possible cause of HLHS [59]. According to this model, antibodies produced by the pregnant mother in response to infection are transmitted through the placenta to the fetus, where they damage the developing heart, either directly or indirectly by affecting the blood flow in the forming chambers. This hypothesis was recently tested using a fetal rat immunization model [60]. Offspring of females that were immunized with cardiac myosin (CM) prior to pregnancy showed a high burden of left-sided structural congenital malformations, of which

hypoplasia of the left ventricular cavity was the most common one. The malformed development of left heart structures in this model could be interpreted in two different ways: (1) possibly, autoimmune-mediated mechanisms could impede left ventricular growth through direct cellular damage. Given the scarcity of recurrence of HLHS, however, this seems less likely. (2) Inhibition of sarcomere function during left ventricular development, such as through impaired systolic or diastolic properties, may lead to secondary cellular growth defects. This mechanism could also hold true in the absence of direct medical relevance of an autoimmune-mediated process in human HLHS.

One of the major limitations in studying extremely rare cardiac malformations like HLHS has always been the lack of specific cell culture models due to the difficulty in obtaining primary tissue. Recent advances in stem cell biology have empowered researchers and clinicians with the ability to reprogram differentiated cells into induced pluripotent stem cells (iPSCs) [61]. Two recent studies utilizing this technology found that iPSCs derived from HLHS patients show decreased cardiomyocyte differentiation potential compared to controls accompanied by repression of the major cardiac transcription factor genes *NKX2-5, T-box (TBX) 2*, the Notch/Hey signaling pathway, and significantly lower levels of heart and neural crest derivative-expressed (HAND) transcripts [62, 63]. Inhibition of NKX2-5 transcriptional activation was mediated by reduced methylation of histone marks H3K4 and H3 acetylation but an increased methylation of H3K27 marks on the *NKX2-5* promoter region. These two independent findings suggest that repression and activation of key cardiac regulators orchestrates ventricular chamber formation in a structural and temporal manner and thus contributes to the molecular identity of HLHS.

57.4 Animal Models of HLHS

Animal models have been used extensively to study congenital heart malformations and gain insights into the general development of the heart. While a variety of different species have been utilized to study cardiovascular malformations, there are mainly three widely used established models, the zebrafish, the mouse, and the chick. Each of these models has certain characteristics advantageous for different lines of investigation. Unknowns in the etiology of HLHS present major challenges for researchers trying to establish model systems to investigate the onset and progression of disease. Conceptually, it is evident that the drawback of the zebrafish model to study hypoplasia of the left heart is its two-chamber architecture, with the lack of a specific left ventricle. However, ease of experimental manipulation, the capacity to examine myocardial regeneration, and the tight molecular conservation of vertebrate cardiogenesis make the zebrafish an indispensable model to study underlying processes of ventricular growth and regeneration [64–66]. Mice and chicken on the other hand show orthologous architecture in comparison to the human heart, making them excellent candidates for the investigation of HLHS.

57.4.1 The Chicken Heart as a Model for Hypoplasia of the Left Heart Structures

Chickens are evolutionarily further from humans than mammals like mice and rats, but they essentially share identical molecular and morphological cascades of heart development [67]. The similarities in chamber formation therefore enable the investigation of molecular patterns of human congenital heart malformations like HLHS in the chick. The first and currently only viable model system to investigate the progression of HLHS was created almost four decades ago using left atrial ligation [68]. Utilizing a nylon device in the region of the left atrioventricular canal and restricting blood flow to the developing left ventricular chamber, it was possible to recreate morphogenetic changes commonly seen in human HLHS patients. Especially interesting is the close resemblance of malformations like mitral valve atresia and premature closure of the foramen ovale to the human conditions seen in HLHS. These findings reinforced the idea of "no flow-no grow" in the developing heart chambers and provided a first basis for further investigation of hypoplastic left heart development. A more recent study utilized this model of restricted blood flow to show the impact of ventricular blood load on myocyte proliferation [69]. In addition to ligating the left atrioventricular canal, the authors also partially clipped the right atrial appendage to increase the blood flow to the left ventricle. Following this adjustment in blood flow, control as well as HLHS clipping model hearts showed an increase in myocyte proliferation in left heart structures. Developing chamber myocardium thus exhibits a certain plasticity that can be triggered by changes in ventricular load during development even in already hypoplastic circumstances. Hypoplasia of the ventricles early in development thus seems to be a reversible process highlighting the possibility for intervention at an early stage in the human embryo. As a clinical vignette, this approach has been successfully exploited in selected cases of severe left ventricular outflow tract obstruction in human fetuses, which have benefited from catheter-based treatment to restore normal flow through the left ventricle, with secondary improvement of ventricular growth [70]. Hemodynamically induced hypoplasia in chicken hearts, however, only mimics the mechanical forces and does not reflect intrinsic molecular defects triggering the development of HLHS in humans. It does however provide a basis for studying cellular responses of hypoplastic structures and the development of new treatment and diagnostic strategies.

57.4.2 The Absence of Murine Models Highlights the Complexity of HLHS

The complexity and oligogenic origin of HLHS becomes most evident by the fact that despite the extensive large-scale efforts in describing knockout mouse models [71], there is as yet no single reproducible mouse model for isolated HLHS. Neither forward nor reverse genetic approaches have proven successful to date. Large-scale ENU mutagenesis screens in mouse did not recover monogenic HLHS models, although one polygenic model has been reported [72] (Mouse Mutant Resource

Web Site, The Jackson Laboratory, Bar Harbor, Maine. www.informatics.jax.org/reference/J:175213, [Feb 2015]). Knockout studies of *Hand1, Hand2*, and many others in mice offered important insights about the importance of these factors in ventricular development, but do not replicate HLHS per se [73]. Rescue of these embryos by contribution of wild-type trophoblasts leads to subsequent cardiac failure at E10.5 due to absent looping and cardiomyocyte differentiation defects. Absence of Hand1 during heart development leads to absence or severe hypoplasia of the left ventricle [73]. Unfortunately, the Hand1 mouse model as well as other models are embryonic or early postnatal lethal and do not replicate the progressive nature of HLHS. Several viable knockout mouse models however have been described to exhibit heart malformations that replicate certain features of HLHS, including several models with aortic stenosis (ADAM metallopeptidase domain 19 (*Adam19*), Notch1, GATA binding protein (Gata) 5, nitric oxide synthase 3 (*Nos3*), *Nkx2-5*, epidermal growth factor receptor (Egfr)) [48, 74–79].

57.4.3 Mechanical Flow Alterations in Fetal Lambs as a Proxy for Human HLHS

Studies performed in non-model organisms often utilized mechanical alteration of blood flow in an early developmental stage as a means to introduce development of HLHS like ventricular structures. In one of the earliest of such studies, ventricular blood flow in fetal lambs during midgestation was altered through partial obstruction of left ventricular inflow or outflow [80]. Lambs with LV inflow obstruction showed decreased weight ratio of LV/RV as well as a decrease of about 50 % in ventricular mean volume, mimicking the left ventricular HLHS phenotype in humans. Obstruction of the LV outflow tract showed a similar trend with a decrease in left ventricular volume and an increase in wall thickness. Interestingly, an artificial increase in LV afterload in this model caused hyperplasia of the left ventricular myocytes and thereby a large gain of LV mass. These findings hint at a more complex concept than “no flow-no grow” and highlight the ability of ventricular myocytes to induce localized proliferation due to external signals like shear stress and tension. While some interesting observations were made by simulating HLHS development using the fetal lamb model, there are major disadvantages to it. Experimental manipulation, generation time, and cost of the studies are just a few of the problems that are associated with large animal models. Furthermore, the induction of the hypoplastic left heart in this model is solely due to an alteration of blood flow by a single morphological cause, disregarding other factors such as genetic and environmental factors. The fetal lamb model of HLHS therefore remains limited in its power as a translational model of human HLHS.

Conclusion

In the preceding sections, we reviewed and discussed the current knowledge of molecular pathways and animal models in hypoplastic left heart syndrome (HLHS). We highlight the multifactorial nature of HLHS at the intersection of

different genetic, epigenetic, and environmental pathways implicated in the development of the left heart structures. This temporal and structural complexity of development represents a significant challenge in isolating the causes of HLHS. In addition, flow dependence of cardiac morphogenesis and its interaction with genetic programs are increasingly recognized as a driver in HLHS. Animal models replicate several isolated aspects of HLHS, although to date there is no faithful replicate that bears direct clinical reference to the full spectrum of human HLHS.

First described more than five decades ago [81, 82], HLHS has become a lesion that can be prenatally recognized and has several palliative treatment options. Surgical treatment of HLHS is performed in three stages with considerable success, but without a clear understanding of its molecular causes [83]. At this time, progress in basic research and the molecular understanding of HLHS lags behind the successes in the clinical arena. Also, a straightforward translation of the current findings from functional model systems into clinically actionable parameters has proven to be complex. The creation of highly reproducible and physiologically relevant animal models and a more precise delineation of the oligogenic etiology of HLHS are required to further improve clinical care. Novel high-throughput technologies will make it possible to examine additional layers of complexity in HLHS, such as the role of non-coding variants, epigenetic alterations, and environmental influences, which are all potential contributors to the complex phenotype of HLHS. A clear molecular understanding of reversible versus irreversible determinants of HLHS as well as their long-term consequences on right heart function will help improve stratification of therapeutic interventions, in particular for prenatal strategies aiming at restitution of ventricular growth.

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Part XVII

Cardiomyopathies

Clinical Presentation and Therapy of Cardiomyopathies

58

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Contents

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58.1 Introduction

There are three distinct cardiomyopathies: hypertrophic, dilated or congestive, and restrictive. Recently, the fourth type, "ventricular noncompaction," has been described, but it is yet unclear if this truly is newly recognized cardiomyopathy or, rather, is a manifestation of dilated or congestive cardiomyopathy.

58.2 Hypertrophic Cardiomyopathy

58.2.1 Pathologic Physiology

Hypertrophic cardiomyopathy (HCM) is a condition in which myocardial thickness is increased (Fig. 58.1). There may or may not be left ventricular (and in some cases, right ventricular) outflow tract obstruction. If there is left ventricular outflow tract obstruction, the condition is termed "hypertrophic obstructive cardiomyopathy" (HOCM). If there is no left ventricular outflow tract obstruction, it is termed "hypertrophic nonobstructive cardiomyopathy. HCM can be associated with chest pain, shortness of breath, exercise intolerance, syncope, and sudden death. It is the most common identifiable nontraumatic cause of death on the athletic field.

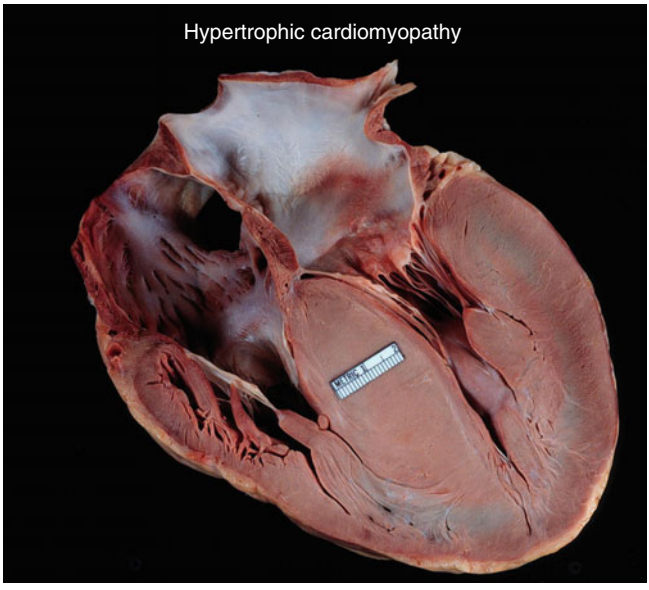

Fig. 58.1 Pathologic specimen of hypertrophic cardiomyopathy. Note the markedly thickened septum and left ventricular posterior wall (Reproduced or adapted from Driscoll, David, Fundamentals of Pediatric Cardiology, Lippincott Williams & Wilkins, 2006, with permission of the author and publisher)

The terms "obstructive and nonobstructive hypertrophic cardiomyopathy" usually implies an autosomal dominant condition that results from mutations in one of several.

58.2.2 Clinical Presentation

Patients with HC usually come to medical attention in one or more of the following ways: detection of a murmur, an abnormal electrocardiogram, from family screening or a positive family history, from evaluation of syncope, chest pain, palpitations, or out-of-hospital cardiac arrest.

58.2.3 Physical Examination

For patients with nonobstructive forms of HCM, there is no characteristic murmur. There may or may not be a prominent apical impulse and an S4. In some patients, obstruction can be unmasked and a murmur will appear with maneuvers to lower systemic vascular resistance such as inhalation of amyl nitrate or assuming a standing position.

Patients with obstructive HCM will have systolic ejection murmur. Unfortunately, in subtle cases one can mistake this murmur for an innocent flow murmur. There may be a bifid pulse.

58.2.4 Echocardiography and Cardiac Catheterization Issues

Classically there will be asymmetric septal hypertrophy and systolic anterior motion of the mitral valve. Echocardiography and Doppler will allow assessment of the absence, presence, and degree of left and right ventricular outflow tract obstruction. Echocardiography allows quantification of the thickness of the ventricular walls. Cardiac catheterization is unnecessary for diagnostic purposes but may be utilized in some adults for alcohol ablation of the obstructing septum.

58.2.5 Treatment

The most important concern is the increased risk of sudden death. Recent population-based studies indicate that the risk is 1 % per year. The ability to identify patients at high risk for sudden death is still crude, but the following features may be associated with higher risk for sudden death: diagnosis in childhood, septal thickness exceeding 30 mm, nonsustained ventricular tachycardia, failure of normal increase of systolic blood pressure during exercise, family history of premature death associated with HC, significant left ventricular outflow tract obstruction, and prior cardiac arrest.

The current paradigm for treating HC is to identify those patients at high risk for sudden death and implant an automatic internal cardiac defibrillator (AICD). For symptomatic

patients who are not at high risk for sudden death and do not qualify for implantation of an AICD, treatment with a beta blocker or amiodarone can be considered.

For patients with significant left ventricular outflow tract obstruction, surgical myotomy/myectomy or alcohol ablation can be done. There is increasing evidence that elimination of the obstruction prolongs life and relieves symptoms.

58.2.6 Outcome

Untreated, there is 1 % risk of death per year. For patients with significant left ventricular outflow tract, obstruction myotomy/myectomy reduces the risk of death. For patients at high risk for sudden death (see above), implantation of an automatic defibrillator reduces the risk of sudden death.

58.3 Dilated Cardiomyopathy

Dilated or congestive cardiomyopathy is a descriptive diagnosis implying that the left ventricle is dilated with reduced systolic function (Fig. 58.2). Normal left ventricular ejection fraction is 50–60 %. Anything less than 50 % is abnormal and could indicate the presence of dilated cardiomyopathy.

58.3.1 Pathologic Physiology

Dilated cardiomyopathy (DCM) can result from a variety of specific disorders. If no specific disorder is identified, it is termed "idiopathic DCM." Some of the

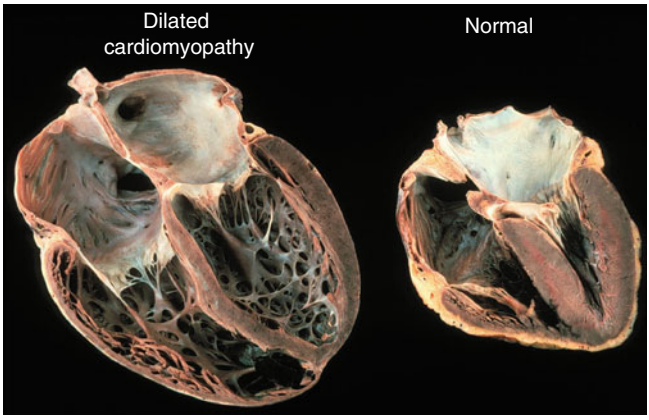

Fig. 58.2 Normal heart on the right compared to dilated cardiomyopathy on the left

specific disorders include: carnitine deficiency, beta-oxidation defects; alcohol-induced, drug-induced (i.e., Adriamycin, cocaine), and tachycardia-induced disorders, selenium deficiency; mitochondrial defects; myocarditis; coronary artery anomalies (anomalous origin of the left coronary artery from the pulmonary artery); thyrotoxicosis; storage diseases; amyloidosis; and postpartum cardiomyopathy, among many others. It generally is thought that in the absence of any explanation for the cardiomyopathy, a prior unrecognized viral myocarditis may be the culprit. Numerous mutations have been described as causing dilated cardiomyopathy. Additional genes will be described in the future.

The genetic aspects of DCM depend upon the underlying cause of the DCM. However, for idiopathic dilated cardiomyopathy, 20–30 % of index cases will have family members with abnormal ventricular function.

58.3.2 Clinical Presentation

Infants and young children may present with a murmur of mitral valve insufficiency or signs and symptoms of congestive heart failure. Older patients may present with fatigue, shortness of breath, exercise intolerance, syncope, and/or arrhythmia.

58.3.3 Physical Examination

It is possible for a patient to have DCM and to have a perfectly normal physical examination. However, most patients will have an abnormal physical examination. In infants, there may be tachypnea, intercostal and/or subcostal retractions, tachycardia, sweating, and evidence of poor weight gain. The first sound usually is normal. The second heart sound may be increased if there is associated pulmonary hypertension. There may be a holosystolic mid-frequency murmur of mitral regurgitation and a diastolic gallop rhythm at the apex of the heart. Older patients may have similar auscultatory findings to the infant. Older patients will be less likely to have retractions but may have pulmonary rales. It is impossible to make a firm diagnosis of dilated cardiomyopathy based on the physical examination alone.

58.3.4 Echocardiography and Cardiac Catheterization Issues

The echocardiogram is essential to establish a diagnosis of DCM. The sine qua non is reduced ejection fraction and increased diastolic and/or systolic left ventricular dimensions. It is critical, especially in infants, to establish that both coronary arteries originate from the aorta, that is, to absolutely exclude the presence of anomalous origin of the left coronary artery from the pulmonary artery.

58.3.5 Treatment

Infants with cardiomyopathy secondary to anomalous origin of the left coronary artery from the aorta generally will do very well if the condition is diagnosed early and operation is performed to attach the anomalous coronary artery to the aorta. Patients with cardiomyopathy secondary to carnitine deficiency will do very well if the diagnosis is made early in life and appropriately treated with carnitine replacement. Patients with acute myocarditis can improve as the myocarditis wanes. Patients with tachycardia-induced cardiomyopathy usually will improve significantly. Frequently, the cardiomyopathy will resolve completely once the tachycardia is controlled.

There have been tremendous improvements in the pharmacologic therapy of myocardial dysfunction in the past 15 years. Patients with depressed myocardial function should be treated with appropriate doses of an ACE inhibitor, beta blocker, spironolactone, and diuretics. The role of digitalis in this setting continues to be debated.

58.3.6 Outcome

An old rule of thumb is that 33 % of patients with idiopathic dilated cardiomyopathy will get better with normalization of ejection fraction, 33 % will get worse and die, and 33 % will survive but systolic function will not return to normal. Five- to 10-year survival for patients with idiopathic dilated cardiomyopathy ranges from 50 to 70 %. In most studies treatment with afterload-reducing agents, diuretics, beta blockers, and spironolactone seemed to improve symptoms and survival, but in some studies the benefit was minimal. With cardiac transplantation, the 10-year survival is approximately 60–70 %.

58.4 Restrictive Cardiomyopathy

Restrictive cardiomyopathy is a relatively rare form of cardiomyopathy in which diastolic function is abnormal. The ventricles are “stiff.” This results in elevated atrial and end diastolic pressures and dilated atria. In adults, amyloidosis is the most common cause of restrictive cardiomyopathy. In children, the cause of primary restrictive cardiomyopathy is unknown. The prognosis for restrictive cardiomyopathy in infants and children is very poor, and cardiac transplantation is recommended soon after the diagnosis is made.

58.5 Ventricular Noncompaction Cardiomyopathy

For many years clinicians have been aware of hearts in which the myocardium appeared “feathery” and “embryonic appearing.” More recently this has been thought to represent embryonic arrest in the normal development of the

myocardium. As the left ventricular myocardium forms, it "compacts" eliminating deep crevices. Thus, the appearance of left ventricle noncompaction is one of the deep crevices in the left ventricle particularly at the apex. There have been a number of studies suggesting that this is a unique form of cardiomyopathy that can be associated with arrhythmias and sudden death. The diagnosis is made by the echocardiographic appearance of the left ventricular myocardium. Unfortunately, the distinction between what is normal and what is abnormal is not perfectly clear. The diagnostic criteria and the best manner of treating this condition continue to evolve.

58.6 Arrhythmogenic Right Ventricular Cardiomyopathy

Arrhythmogenic right ventricular dysplasia/cardiomyopathy (ARVD/C) is a genetically mediated cardiomyopathy characterized, primarily, by potentially lethal ventricular arrhythmias and fibrofatty infiltration of the right ventricular myocardium. It may account for as many as 10 % or unexplained sudden deaths in young individuals. Causative mutations have been mainly described in genes encoding desmosomal proteins such as desmoplakin, plakophilin 2, desmoglein 2, desmocollin 2, and plakoglobin. Death can be associated with exercise and some clinicians advise against vigorous exercise for these patients. Family members of index patients should be screened for this condition. Patients with a history of cardiac arrest or potentially lethal arrhythmias are treated with an automatic internal cardiac defibrillator (AICD). There are no specific physical examination findings.

Human Genetics of Cardiomyopathies

59

Alexa M.C. Vermeer, Arthur A.M. Wilde,
and Imke Christiaans

Contents

Abstract

Over the past few decades, there has been notable progress in knowledge and implication of genetics in cardiomyopathies. Twenty-five years ago we started to recognize the genes; nowadays dozens -of genes associated with cardiomyopathies have been described. Genes and specific mutations can be unique for a certain cardiomyopathy or have specific phenotypic characteristics, but most genes,

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especially genes encoding for sarcomeric proteins, are associated with different cardiomyopathy subtypes. The large variability in disease penetrance, in disease symptoms and prognosis, and in some families even in cardiomyopathy subtype makes genetic counseling of great importance. Finding a causal mutation in a patient allows identification of relatives at risk of cardiomyopathy and enables presymptomatic assessment of the risk on sudden cardiac death (SCD). The advent of next-generation sequencing (NGS) techniques like cardiogenetic gene panels with sequencing of dozens of genes in one go does not only improve detection of causal mutations but also increases the chance of variants of unknown significance (VUS). These VUS make genetic counseling even more important and challenging. Reclassification of VUS into benign variants, disease modifiers, or causal mutations will be the main focus of research in coming years.

59.1 Introduction

Diagnosis of idiopathic cardiomyopathy is characterized by the absence of other (i.e., environmental or external) causal factors that can be detected in the patient's history (e.g., alcohol abuse in dilated cardiomyopathy (DCM)), physical examination (e.g., myotonia from myotonic dystrophy in DCM), cardiac investigation (major coronary artery disease in DCM), or other means [1]. This diagnosis by exclusion is often the most difficult part in diagnosing a hereditary cardiomyopathy. When we use the terms idiopathic hypertrophic cardiomyopathy (HCM), DCM, arrhythmogenic (right ventricular) cardiomyopathy (A(RV)C), and noncompaction cardiomyopathy (NCCM), we mean that no clinical causal factors have been identified. This does not exclude the possibility that the cardiomyopathy is hereditary. Restrictive cardiomyopathy is not addressed in this chapter since its genetic basis is not yet clear.

59.2 Inheritance Pattern

Cardiomyopathies in general are thought to be monogenetic diseases. This means that a mutation in a single gene is the main causal factor in the development of the disease. In most cardiomyopathies the inheritance pattern is autosomal dominant. Clues in a pedigree for autosomal dominant transmission are affected males and females in multiple generations and male-to-male transmission. X-linked and recessive transmission does occur in cardiomyopathies, but in those cases/families, extracardiac signs are often present. An example of recessive inheritance is Naxos disease, named after the island of Naxos where the first cases were identified, which is ARVC with woolly hair and palmoplantar hyperkeratosis. Fabry disease, a metabolic disease which causes HCM as well as acroparesthesia and renal failure, is inherited as an X-linked disorder. These so-called phenocopies are not extensively addressed in this chapter, but some details can be found in Table 59.1.

Table 59.1 Genes for cardiomyopathies and cardiomyopathy phenocopies

Disorder/gene	Frequency in phenotype (%)/phenotype
HCM	*Frequency in phenotype* (%)
MYBPC3	30–40 %
MYH7	30–50 %
TNNT2, TNNI3, MYL2, MYL3, ACTC1	<5 %
TTN[a], TPM1, TNNC1, MYOZ2, CSRP3, ACTN2, LDB3, TCAP, VCL, JPH2, CALR3, MYLK2, ANKRD1, CAV3, MYH6, NEXN, MYPN, PLN, CRYAB, FHL1, MTTL1	NA
HCM phenocopies	*Phenotype*
PRKAG2	LVH/preexcitation (Wolff-Parkinson-White syndrome)/conduction disease
LAMP2	Danon disease
FXN	Friedrich ataxia
GLA	Fabry disease
PTPN11	Noonan, Leopard, CFC syndrome
RAF1	Noonan, Leopard
KRAS2	Noonan, Leopard, CFC syndrome
SOS1	Noonan
TTR	Amyloidosis
BRAF1	CFC syndrome
MAP2K1	CFC syndrome
MAP2K2	CFC syndrome
HRAS	Costello syndrome
GAA	Pompe disease
GDE	Glycogen storage disorder III
Mitochondrial	LVH “plus” syndrome
DCM	*Frequency in phenotype* (%)
TTN[a]	18–27 %
MYH7	4–10 %
LMNA	5–6 %
MYBPC3	4 %
MYPN	3–4 %
MYH6	3 %
SCN5A	2–3 %
TNNT2	2–3 %
ANKRD1	2 %
TPM1	1–2 %
TNNC1	1 %
TNNI3, TCAP, CSRP3, DES, DSP, PLN, LAMA4, ACTC1, ACTN2, ABCC9, CRYAB, NEXN, SDHA, VCL, FHL2, PDLIM3, GATAD1, RBM20, TMPO	NA

(continued)

Table 59.1 (continued)

Disorder/gene	Frequency in phenotype (%)/phenotype
DCM phenocopies	*Phenotype*
LMNA	Emery-Dreifuss muscular dystrophy, limb-girdle muscular dystrophy type 1B
DMD	Duchenne/Becker muscular dystrophy
DES	Desminopathy
LDB3	Myofibrillar myopathy
TAZ	Barth syndrome
SGCD	Limb-girdle muscular dystrophy type 2 F
TCAP	Limb-girdle muscular dystrophy type 2G
FKRP	Limb-girdle muscular dystrophy type 2I
TTN	Limb-girdle muscular dystrophy type 2 J, early-onset myopathy with fatal cardiomyopathy
FKTN	Muscular dystrophy
BAG3	Progressive myofibrillar myopathy
HFE	Hereditary hemochromatosis
MYH7	Laing distal myopathy
DSP	Carvajal syndrome
EYA4	DFNA10 nonsyndromic hearing loss and deafness
PSEN1	Early-onset alzheimer disease
PSEN2	Early- and late-onset alzheimer disease
DOLK	Congenital disorder of glycosylation
Mitochondrial	Kearns-Sayre syndrome
ARVC	*Frequency in phenotype* (%)
PKP2	11–43 %
DSG2	12–40 %
DSP	6–16 %
JUP, DSC2, PLN, TMEM43, LMNA	NA
NCCM	*Frequency in phenotype* (%)
MYH7	21 %
ACTC1, TNNT2, TNNI3, TPM1, PLN, DSP, LMNA, SCN5A, DTNA, MIB1, PRDM16, MYBPC3	NA
NCCM phenocopies	*Phenotype*
TAZ	Barth syndrome
LDB3	Myofibrillar myopathy

Abbreviations: *HCM* hypertrophic cardiomyopathy, *DCM* dilated cardiomyopathy, *ARVC* arrhythmogenic right ventricular cardiomyopathy, *NCCM* noncompaction cardiomyopathy, *LVH* left ventricular hypertrophy, *CFC* cardiofaciocutaneous, *NA* not ascertained or <1 %

[a]Pathogenicity of mutations in this gene is still unclear

Autosomal dominant inheritance sometimes is difficult to recognize from the pedigree. This is due to reduced disease penetrance – especially in females – and to the large intra- and interfamilial variability of disease expression. Reduced disease penetrance means that not all people with the mutation develop a cardiomyopathy during life. In families with a cardiomyopathy, penetrance increases with age but may not be complete. The laymen can interpret this phenomenon as "skipping a generation." The large variability means that not all people with the same cardiomyopathy have the same disease course or develop the same symptoms. Even in a family in which all affected family members have the same mutation, some carriers of the mutation can have heart failure at young age, some can be asymptomatic throughout life, and some can die suddenly without previous symptoms.

In autosomal dominant inheritance, one gene mutation causes the disease. However, we also encounter patients with two or more mutations. These mutations can be located in the same gene on different alleles (compound heterozygous or homozygous) or in different genes (digenic). In HCM the presence of two mutations is relatively frequent (about 3–5 %). The presence of more than one mutation often is associated with a more severe phenotype: younger age of onset, more pronounced abnormalities on cardiac imaging (e.g., severe hypertrophy in HCM), and a higher risk of life-threatening arrhythmias [2–6].

59.3 Involved Genes

59.3.1 Hypertrophic Cardiomyopathy

Mutations in HCM are almost exclusively located in genes encoding sarcomeric proteins. The sarcomere represents the basal contractile unit of striated muscles, such as cardiac muscle, and is made up of thick and thin filaments. During contraction, the thin filaments slide past the thick filaments, shortening the sarcomere. In about 50–60 % of patients, a disease-causing (pathogenic) mutation can be detected [4, 7]. Main disease genes are myosin-binding protein C (*MYBPC3*) and beta-myosin heavy chain (*MYH7*) (Table 59.1) [4, 7–9]. Many countries or populations have specific so-called founder mutations. These mutations derive from a common ancestor and often comprise a large part of the detected mutations in that country or population.

No clear genotype-phenotype correlations are present in HCM, although older literature suggests this. Some correlations can be found in large cohorts; however, they cannot be used for prognosis or therapeutic options in the individual patient.

59.3.2 Dilated Cardiomyopathy

In contrast to HCM, mutations in DCM patients are found in a minority of cases and are often unique for the family. The recent discovery of the titin gene (*TTN*), the

largest gene in the human genome in which mutations can be found in about 25 % of patients, has changed the mutation detection rate enormously [10]. Titin is highly expressed in the heart, where it regulates sarcomere contraction and signaling. Despite the fact that many variants in this gene are found, it is uncertain whether or not variants in *TTN* are pathogenic. This is partly because *TTN* missense variants are very common with 23 on average per individual in the Exome Variant Server database and because truncation variants are frequently also found in healthy control populations [11]. Other genes responsible for *DCM* encode proteins of the sarcomere, the nuclear envelope, the cytoskeleton, ion channels, desmosomes, and proteins involved in calcium homeostasis.

Genotype-phenotype correlations are present in DCM. DCM patients with a mutation in the *LMNA* (lamin A/C) gene almost all have conduction disease and are at higher risk of developing arrhythmic and thromboembolic events [12, 13]. Mutations in phospholamban (*PLN*) are associated with a higher risk of ventricular arrhythmias and severe heart failure [14].

59.3.3 Arrhythmogenic Right Ventricular Cardiomyopathy

Currently eight genes have been identified responsible for 60–65 % of all ARVC cases; five genes encode for components of the cardiac desmosome (plakoglobin (JUP), desmoplakin (*DSP*), plakophilin 2 (*PKP2*), desmoglein 2 (*DSG2*), and desmocollin 2 (*DSC2*)) and three non-desmosomal proteins (transmembrane protein 43 (*TMEM43*), lamin A/C (*LMNA*), and *PLN*) (Table 59.1). The most frequently mutated gene is *PKP2*, with a detection rate up to of 40 % [15–18].

No clear genotype-phenotype correlations are present except that the presence of multiple mutations is associated with a more severe phenotype. However, this lack of correlation also can be caused by difficulty in diagnosing ARVC – reflected by the long list of diagnostic criteria – and the lack of large study populations.

59.3.4 Noncompaction Cardiomyopathy

NCCM is genetically heterogeneous. Most NCCM-associated mutations can be found in genes that encode for sarcomeric proteins, with *MYH7* as the most frequently mutated gene [19, 20]. Little is known about specific genotype-phenotype correlations.

The exact causal mechanism is also unknown. It has been suggested to be a block in embryonic trabecular development, but recent studies have identified mutation carriers with a normal heart who developed the noncompacted myocardium later in life [19, 21].

59.4 Overlapping Phenotypes

Although in clinical evaluation the type of cardiomyopathy seems essential for the cardiologist and patient, in reality clear-cut-specific cardiomyopathies are not always seen. In the last 20 years genetic testing has revealed that disease variability is large and, also, that the same genes and even the same mutations can give rise to different cardiomyopathies (Fig. 59.1). Almost all combinations of cardiomyopathies are seen in families, but these combinations also can be present in a single patient at the same time or in consecutive order. Carriers of a *PLN* mutation can have a clear ARVC phenotype and develop a clear DCM phenotype later on. In all types of cardiomyopathies, treatment and prognosis are mainly determined by the results of cardiac evaluations and not by the results of the genetic test. However, it is essential to be aware of the possibility of overlapping phenotypes in a patient or family, since it is unlikely that a patient with HCM with NCCM features has two different diseases.

59.5 Genetic Testing and Counseling

Since for most cardiomyopathies no clear genotype-phenotype correlations are present, a genetic diagnosis can confirm the disease in a patient but often does not add to therapy or prognosis. The main benefit of finding the causal mutation is that there is a better option to detect relatives at risk for the cardiomyopathy and associated life-threatening arrhythmias. The incomplete and age-dependent disease penetrance in cardiomyopathies makes it difficult to identify the relatives at risk, and in case of normal echocardiographic findings, the relative needs lifelong follow-up. Finding the causal mutation in a patient means that one can perform predictive DNA testing for healthy relatives. First-degree relatives are at 50 % risk of having the causal mutation. Carriers of the mutation are at risk of developing disease and are advised to undergo regular cardiac evaluations. Noncarriers are not at risk of developing the cardiomyopathy. They do not need cardiac evaluations and can be discharged from (further) cardiac follow-up. Besides, they cannot have transmitted the mutation to their offspring, so they also are no longer at risk.

The Working Group on Myocardial and Pericardial Diseases of the European Society of Cardiology has given advice for the screening of asymptomatic relatives of a cardiomyopathy patient based on the presence or absence of a disease-causing mutation in the family. This advice is summarized in Table 59.2. For most cardiomyopathies, they also advise discontinuing clinical screening for at-risk relatives between age 50 and 60 years if no symptoms are present. Recent studies have shown, however, that disease still can become manifest after this age. For example, in HCM increased mortality also is present after age 50, which suggests that continued clinical screening is needed in asymptomatic relatives [22, 23].

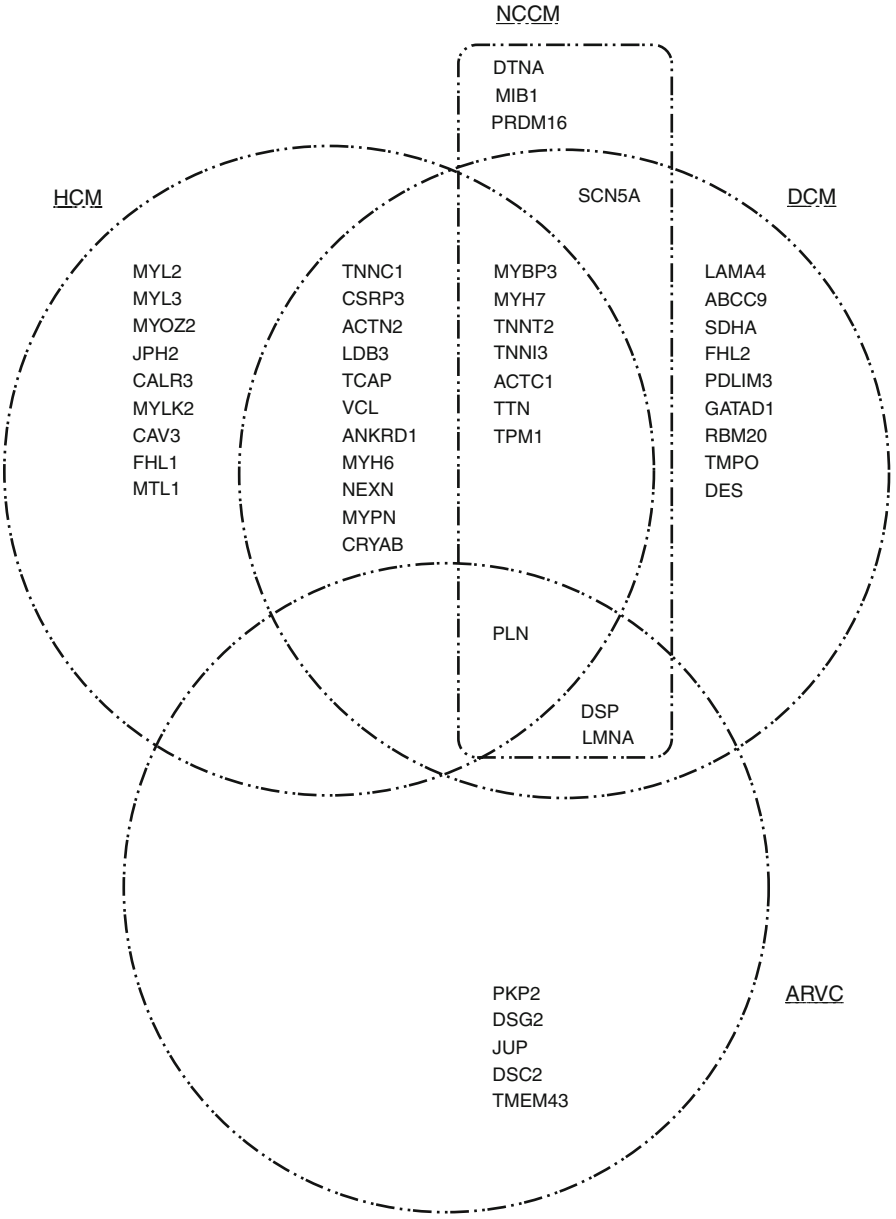

Fig. 59.1 Overlap in genes causing inherited cardiomyopathies (Adapted from van Spaendonck-Zwarts et al. [29])

Table 59.2 General recommendations for cardiac evaluations in asymptomatic relatives of cardiomyopathy patients without clinical signs of disease

	HCM[a]	DCM[a]	ARVC[a]	NCCM[a]
Asymptomatic relatives from families without a known causal mutation				
Cardiac evaluation	ECG, echocardiography	ECG, echocardiography (and Holter ECG if conduction disease in relative)	ECG, echocardiography, Holter ECG, signal-averaged ECG	ECG, echocardiography
Start of cardiac evaluation	10–12 years	10–12 years	10–12 years	Newborn
Frequency of cardiac evaluation [b]	10–20 years, every 1–2 years; >20 years, every 2–5 years	10–20 years, every 1–2 years; >20 years, every 2–5 years	10–20 years, every 1–2 years; >20 years, every 2–5 years	<20 years, every 1–3 years; >20 years, every 2–5 years
Mutation carriers				
Cardiac evaluation	Initially ECG, echocardiography, exercise test, Holter ECG; additional evaluations ECG and echocardiography	Initially ECG, echocardiography, exercise test, Holter ECG; additional evaluations ECG and echocardiography (and Holter ECG if conduction disease in relative)	Initially ECG, echocardiography, exercise test, Holter ECG, signal-averaged ECG; additional evaluations ECG and echocardiography, signal-averaged ECG	Initially ECG, echocardiography, exercise test, Holter ECG; additional evaluations ECG and echocardiography
Start of cardiac evaluation	10–12 years	10–12 years	10–12 years	Newborn
Frequency of cardiac evaluation	10–20 years, every year; >20 years, every 1–3 years	10–20 years, every year; >20 years, every 1–3 years	10–20 years, every year; >20 years, every 1–3 years	<20 years, every year; >20 years, every 1–3 years

Adapted from Charron et al. [1]

[a]Phenocopies are not covered here

[b]Only in familial cases of DCM and NCCM repeated cardiac evaluation is mandatory. In sporadic cases relatives can be evaluated once during puberty or adulthood

59.6 Genetics of Cardiomyopathies in the Future

For many cardiomyopathies the genetic mutation cannot be detected. This can mean that many disease genes are still unknown, but it can also mean that not all cardiomyopathies are monogenetic. In research we no longer identify new causal genes that are disease causing in more than a few percent of the cardiomyopathy population – with the exception, possibly, of *TTN*. Besides, NGS techniques allow us to sequence a large number of disease genes or even the entire genome rapidly. Several recently published studies have shown promising results of the application of NGS techniques. Identification of novel causal variants or variants that are potential disease modifiers in cardiomyopathies seems to be the outcome of NGS [24–26]. However, in addition to promising variants, more often one finds variants of unknown significance (VUS). These are genetic variants of which it is not known or not certain whether they can cause a cardiomyopathy or not or add to disease expression as a modifier. Frequently more than one VUS in a single patient is identified with expansion of NGS techniques to the entire exome or genome. VUS are also found in genes previously not known to be associated with cardiomyopathies. Besides, recent studies have shown that a number of variants previously annotated in the literature and databases as disease causing are now frequently found in healthy controls, which makes their causality extremely doubtful [27, 28].

For now, the frequent detection of VUS makes genetic counseling and testing more challenging and calls for the need of a specialized genetic counselor to interpret genetic test results.

Conclusion

Currently many cardiomyopathy-related genes have been identified. However, for many cardiomyopathy families a disease-causing mutation cannot be detected. NGS techniques will increase the yield of genetic diagnosis, but also add a new layer of complexity to genetic counseling due to the frequent detection of VUS. The interpretation of complex genetic results generated by NGS techniques remains challenging.

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Molecular Pathways and Animal Models of Cardiomyopathies

60

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Contents

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Abstract

Cardiomyopathies are a heterogeneous group of disorders of heart muscle that ultimately result in congestive heart failure (CHF), sudden cardiac death, and/or arrhythmias. Contraction of heart muscle is the prime force for efficient pumping of the blood during systole, and relaxation is main force that collects a sufficient volume of blood during diastole, preparing ventricles for next efficient contraction. Efficient contraction and relaxation is maintained by cadre of intracellular and intercellular molecular signaling though paracrine communication. However, persistent unfavorable imbalance of these molecular pathways results in systolic and/or diastolic dysfunction leading to failure of the heart to maintain hemodynamic balance. Rapid progress in genetics as well as in molecular and cellular biology over the past 25 years has greatly improved the understanding of pathogenic signaling pathways in inherited cardiomyopathies. This chapter will focus on intracellular and intercellular molecular signaling pathways that are activated by a genetic insult in cardiomyocytes to maintain tissue and organ level regulation and resultant cardiac remodeling in certain forms of cardiomyopathies. In addition, animal models of different clinical forms of human cardiomyopathies with summaries of triggered key molecules and signaling pathways will be described.

60.1 From Genetic Abnormality to Cellular Pathology and Cardiomyopathy: "Final Common Pathways"

It is widely accepted that inherited cardiomyopathies are a group of heterogeneous diseases of heart muscle resulting from genetic alterations in cardiac myocytes, the chief contractile cell type in the heart [1]. The "final common pathway" hypothesis elucidating the mechanisms of inherited cardiomyopathies, first described by Towbin et al. in the late 1990s, suggests that genes encoding proteins with similar functions and/or location or involved in the same pathway are responsible for a consistent cardiomyopathy phenotype with distinctive morpho/histological cardiac remodeling [2]. Further, disruption of particular protein pathways may intersect with other intracellular and intercellular pathways, resulting in an overlapping phenotype (Fig. 60.1). Apoptosis, necrosis, autophagy, gene expression, and metabolic and arrhythmogenic disturbances – which may present as the sole feature or as overlapping signs – result in definitive forms of cardiac remodeling including fibrosis, cardiomyocyte hypertrophy, and atrophy. Typically, molecular signaling activates associated compensatory responses and cooperates with other modifiers such as genetic modifiers and environmental, stress, or toxicity related that, in turn, may or may not influence the final cardiomyopathy phenotype. Alterations in cellular morphology and size, gene expression patterns, and metabolic shifts in cardiomyocytes initially compensate and maintain cardiac function in the subtle, preclinical stages of cardiomyopathy. However, when compensatory mechanisms fail, additional neuroendocrine signaling and other pathways are activated on an organ-specific or whole-organism level, leading to CHF. Thus, inherited forms of cardiomyopathy, irrespective of the specific morpho/clinical condition, may or may not present signs of CHF.

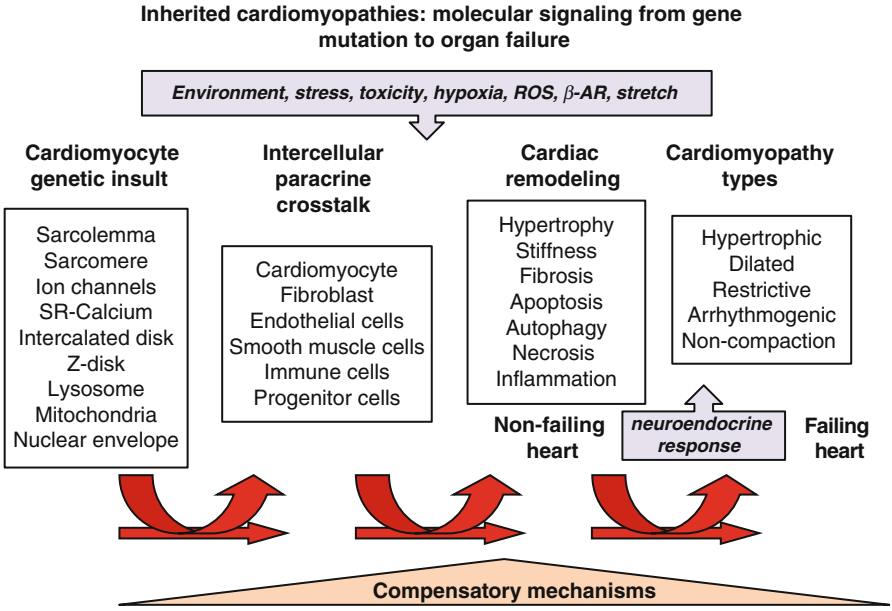

Fig. 60.1 "Final common pathway" hypothesis supplemented by intra- and intercellular signaling

60.2 Compensatory Stress Regulators in Inherited Cardiomyopathy

In inherited cardiomyopathies, cellular pathology originates from the initial genetic assault; however, the phenotypic expression of the specific cardiomyopathy type will be distinguished later when the effects of cardiac remodeling are perceptible. Thus, individuals carrying gene mutations may not present clinical signs of cardiomyopathy until adulthood, supporting a temporal mechanism by which chronically altered cellular responses and cardiac remodeling lead to the clinically relevant cardiomyopathy phenotype. Cellular responses to maintain normal cardiomyocyte function include changes in Ca^{2+} transients and mechanotransduction, the number and sensitivity of sarcomeres, shifts in metabolic processes, and gene expression.

60.2.1 Calcium Transients

Sarcoplasmic Ca^{2+} concentrations directly and critically control the contraction and relaxation of the sarcomere. In the initial compensated period of the genetically determined cardiomyopathy, contractility and heart rate are facilitated through altered Ca^{2+} transients, and this process is controlled by activation of ion channels, G protein-coupled receptors (GPCRs), stretch-activated channels, and many other biologic and chemical receptors. However, if Ca^{2+} overload stress is sustained, this activates calmodulin–calcineurin, a serine/threonine phosphatase

through dephosphorylation of nuclear factor of activated T cells (NFAT). Activated calmodulin–calcineurin then translocates into the nucleus, changing gene expression and triggering abnormal contractility and pathological hypertrophy [3].

60.2.2 Mechanotransduction Signaling

Mechanosensitive mechanisms are intracellular signaling events that alter and regulate gene expression in response to mechanical stretch. Altered stretch recruits the integrin-induced phosphorylation of focal adhesion kinase (FAK), activating downstream Rous sarcoma (SRC) signaling. The integrin–talin complex, components of the costamere, connects the sarcomere to the sarcolemma and extracellular matrix (ECM) through the Z-disk, which consists of numerous mechanosensitive proteins including α-actinin 2, nebulette, myopalladin, cardiac ankyrin repeat protein (CARP), and filamin C. Mutations in all these Z-disk proteins induce pathological signaling pathways in response to sustained stretch and initiate the development of cardiomyopathies [4].

60.2.3 Metabolic Substrate Utilization

In the healthy heart, phosphocreatine is the main reserve source of ATP during acute stress. In the initial compensatory stages of cardiomyopathy, the heart favors burning free fatty acids, which yield about three times more ATP than glucose, but require more oxygen to metabolize [5]. As contractility or relaxation is altered, the demand for ATP increases, yet the levels of phosphocreatine are progressively reduced. In the decompensated stage of cardiomyopathy, energy substrate utilization shifts from fatty acid oxidation to glucose, a less efficient resource for producing energy [6].

60.2.4 Fetal Gene Program

In adult cardiomyocytes, contractility is inversely proportional to levels of atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), β-myosin heavy chain (β-MyHC), and α-skeletal actin (α-SMA), genes that are normally expressed during embryogenesis [7]. Circulating ANP and BNP in plasma induce diuresis and vasodilation, while β-MyHC exhibits lower ATPase activity and enhanced ATP use. To compensate for increased myocardial wall stress, expression of these genes is induced through immediate early genes (IEGs) encoding transcription factors such as SRC, cellular FBJ osteosarcoma oncogene (c-FOS), transcriptional factor AP-1 (c-JUN), early growth response-1 (EGR-1), myelocytomatosis c (c-MYC), rat sarcoma (RAS) oncogenes, and mitogen-activated protein kinases (MAPK). Persistent expression of these fetal genes may contribute to contractile dysfunction and prolonged relaxation of cardiomyocytes independent of Ca^{2+} handling [8].

60.2.5 Intercellular Crosstalk in the Myocardium

Besides cardiomyocytes, composing approximately 56 % of the adult murine heart, fibroblasts (27 %), endothelial cells (7 %), and smooth muscle cells (10 %) reside in the ECM containing numerous transient immune cells [9]. The interaction between all these cell types via ongoing reciprocal secretion of autocrine and paracrine factors is essential in preserving normal cardiac function. This process is regulated by signaling molecules such as integrins, endothelin1 (ET1), bone morphogenetic proteins (BMPs), platelet endothelial cell adhesion molecule 1 (PECAM1), vascular endothelial (VE)-cadherin, vascular endothelial growth factor (VEGF), and transforming growth factor beta 3 (TGFβ3). Genetic mutations may trigger pathological signaling between these messengers.

60.3 Pathological Cardiac Remodeling and Signaling Pathways in Cardiomyopathy

60.3.1 Cardiomyocyte Hypertrophy

Cardiomyocyte hypertrophy (increase in size) and atrophy (decrease in size) are the most common alterations that occur when cardiac cells respond to genetic abnormalities. Two types of hypertrophy exist at the cellular level: concentric and eccentric. Concentric hypertrophy is an increase in myocyte width-to-length ratios that occur as a result of addition of sarcomeres in parallel as physiological adaptive responses to keep pace with hemodynamic demand. Persistent stress may transform physiological hypertrophy into a pathological state when reduced volumes of ventricular chambers affect cardiac output, ultimately resulting in the development of CHF [10]. In contrast, eccentric hypertrophy is an increase in myocyte length-to-width ratios associated with increased end-to-end addition of sarcomeres, primarily associated with decreased force production and often associated with DCM and CHF. In inherited cardiomyopathies, triggering of intracellular and extracellular hypertrophic signal-transduction pathways is dependent on the location of the genetic mutation. Major signal transduction cascades such as G protein-coupled receptors (GPCRs), protein kinase B (PKB), or AKT, MAPK, and tumor necrosis factor alpha (TNF-α) have been shown to play a significant role in the development and progression of cardiac hypertrophy.

60.3.2 GPCR Signaling

GPCRs are integral membrane proteins consisting of seven membrane-spanning domains that are competent to respond to paracrine and autocrine factors including adrenergic factors, angiotensin II (AngII), and ET1 [11]. The sarcomere, when composed of mutant proteins, exhibits blunted myofilament Ca^{2+} sensitivity, reduces ATP efficiency, and inhibits the sequestration of Ca^{2+} from the cytosol. Further,

mutation-induced contractile dysfunction causes activation of GPCR signaling by ET1 and AngII and increases release of Ca^{2+} from the sarcoplasmic reticulum (SR), activating calmodulin and myocyte enhancer factor 2 (MEF2). GPCR signaling is also associated with activation of the AKT signaling pathway.

60.3.3 AKT Signaling

Many adaptive processes in the heart such as protein synthesis, apoptosis, gene expression, and metabolism are regulated by phosphoinositide 3-kinase (PI3K). Activation of PI3K on the cardiomyocyte sarcolemma initiates activation of PKB/AKT [12]. When PKB/AKT-mediated phosphorylation of glycogen synthase kinase 3 beta (GSK3β) inactivates it, hypertrophic transcriptional effectors including erythroid transcription factor (GATA4), β-catenin, c-MYC, and NFAT are activated within the heart. Compensated hypertrophy and increased contractile efficiency are induced by AKT1 and AKT2 [13]; however, chronic activation of the PI3K/AKT pathway via mammalian target of rapamycin (mTOR) may lead to pathological cardiac hypertrophy [14].

60.3.4 MAPK Pathway

The MAPK family consists of four subfamilies of kinases: extracellular signal-related kinases (ERK1/2), c-Jun N-terminal kinases (JNK1-3), p38 kinases, and ERK5 that convert extracellular stimuli into a wide range of cellular responses [3]. In cardiomyocytes, MAPK signaling is initiated by GPCRs, insulin-like growth factor-1 (IGF-1) and fibroblast growth factor receptor (FGFR), TGFβ, and by cytokines, stretch, AngII, or ET1. When activated via the three-module cascade of phosphorylating kinases, MAPKs then translocate into the nucleus and activate transcription factors, resulting in reprogramming of cardiac gene expression.

Activated ERK1/2 stimulates the transcription of genes such as BNP and ETS domain-containing protein 1 (Elk1) [15]. JNKs and p38 are activated mainly by inflammatory cytokines, ischemia, oxidative stress, heat shock, endotoxins, as well as secondarily by growth factors and GPCR [16]. JNKs activation increase expression of ANP, alpha smooth muscle actin (α-SMA), TGFβ1, Elk1, p53, and collagen type I in cardiac hypertrophy and inhibit NFAT4, NFATc1, and signal transducer and activator of transcription-3 (STAT3). The p38 module promotes expression of pro-inflammatory cytokines such as interleukin-1β (IL-1β), IL-6, and TNF-α through the integrin-FAK-SRC-RAS pathway [17].

60.3.5 TNF-Alpha

TNF-α is a cytokine that initiates an inflammatory response via coupling with TNF receptor and activating nuclear factor kappa-light-chain-enhancer of activated B (NF-κB), protein kinase C (PKC), stress-activated protein kinases (SAPKs), and

JNKs in response to stress. Once activated, TNF-α triggers expression of matrix metalloproteinases (MMPs), a family of enzymes that degrade the ECM components. Levels of TNF-α and MMPs have been found to be concordantly increased in DCM and the failing human heart [13]. The NF-kB cascade is initiated upon activation of NF-κB-inducing kinase (NIK) and IKK complex, triggering subsequent inhibitor of κB alpha (IkB-α) degradation. Activated NF-κB translocates to the nucleus causing cardiac hypertrophy.

60.3.6 Cardiomyocyte Atrophy

Cardiac atrophy is a pathogenic event associated with mechanical unloading of the myocardium and is typically a result of maladaptive processes of apoptosis, necrosis, or excessive autophagy.

60.3.6.1 Apoptosis

Apoptosis is the process also known as programmed cell death as defined by certain morphologic changes, such as DNA fragmentation, progressive cardiomyocyte atrophy, and death [18]. Apoptosis is induced via an intrinsic pathway by cytochrome *c* released from mitochondria or via an extrinsic pathway by activation of cell membrane-bound death receptors (like FAS or TNF-α) by coupling with the corresponding cytokines. Both pathways ultimately activate downstream effector caspases. Induction of apoptosis plays a critical role in the transition from compensatory hypertrophy to decompensated CHF [19]. As adult cardiomyocytes are terminally differentiated, apoptosis is detrimental to cardiac function not only due to cardiac myocyte loss but also by generating fibrosis and decreased heart compliance. Many of the signaling pathways, including AKT and β-adrenergic stimulation with cytochrome *c* release from mitochondria [20], activation of Fas by upregulated Fas ligand, and TNF-α or degradation of cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein (cFlip), inhibitors of Fas, are common in the failing heart [21].

60.3.6.2 Necrosis

Necrosis, the premature death of cells, is typically a result of the unregulated digestion of cell components due to activation of various death receptors. Necrosis is almost always detrimental due to loss of sarcolemmal integrity, uncontrolled release of cellular products into the ECM, and consequent inflammatory responses. The wavefront phenomenon of myocardial atrophy with pathological remodeling such as inflammation, fibrosis, hypertrophy, and ventricular dilation with preceded necrosis is well documented in human ARVC [22]. The underlying mechanisms for this remain unknown, although disruption of sarcolemmal integrity and/or mitochondrial permeability with abnormal Ca^{2+} homeostasis may play an important role.

60.3.6.3 Autophagy

Autophagy is a natural process of conserving and recycling of cytoplasmic components, playing important cell survival roles by removing oxidated proteins and

damaged components during stress. Autophagy is controlled by sequential actions of autophagy-related genes (Atgs), such as Beclin1 (Atg6), microtubule-associated protein 1 light chain 3 or LC3 (Atg8), and class III PI3K that are responsible for vesicle elongation and conjugation. In contrast, the mTOR pathway or class I PI3K acts to inhibit autophagy. Although autophagy is a cytoprotective mechanism, pro-apoptotic factors released from the damaged mitochondria may lead to apoptotic cell death [23]. Several signaling pathways, reactive oxygen species (ROS), and increases in cytosolic Ca^{2+} levels not only trigger apoptosis but potently induce autophagy. In the heart, excessive autophagy causes cell death and cardiac atrophy. Thus, autophagy in the failing heart caused by DCM appears to be a sign of failed cardiomyocyte repair [24].

60.3.7 Cardiac Fibrosis

Evidence of interstitial and/or perivascular fibrosis is one of the major hallmarks of cardiomyopathies and is known to disrupt excitation–contraction coupling between cardiomyocytes, increases myocardial stiffness, and decreases contractility. Fibrosis is primarily produced by resident fibroblasts in the heart; however, there is evidence for collagen production by cardiomyocytes [25]. There are two forms of fibrosis that exist: reactive and replacement. Several pro-fibrotic factors such as AngII, platelet-derived growth factor (PDGF), connective tissue growth factor (CTGF), and the renin–angiotensin–aldosterone system (RAAS) along with prominent downstream mediators including TGFβ1 cause fibrotic responses in the heart.

60.3.7.1 Angiotensin II

AngII directly induces NADPH oxidase activity and increases SMAD2 levels augmenting the nuclear translocation of phosphorylated SMAD3. As result, stimulated TGFβ induces fibroblast proliferation and differentiation into collagen-secreting myofibroblasts leading to cardiac fibrosis.

60.3.7.2 CTGF

CTGF is a protein mainly expressed in fibroblasts in the healthy heart, but is also secreted by cardiomyocytes during cardiac remodeling [26]. Increased amounts of CTGF in human cardiac hypertrophy and CHF have been shown to contribute to fibrosis. However, a mouse heart with CTGF overexpression did not have a fibrotic phenotype, making CTGF's role in fibrosis unclear.

60.3.7.3 TGFβ

TGFβ is a cytokine involved in regulation of many signaling pathways involved in cellular differentiation, homeostasis, cardiac fibrosis, and hypertrophy. When TGFβ binds to its serine/threonine kinase receptors, the downstream SMAD signaling pathway activates to stimulate collagen production. TGFβ also signals through TGFβ-activated kinase 1 (TAK1) to activate activated transcription factor 2 (ATF2), which are directly correlated with cardiac hypertrophy and fibrosis [26].

60.3.7.4 SMADs

SMAD proteins are a family of transcription factors that consist of three groups: receptor-activated (SMADs 1, 2, 3, 5, and 8), co-mediator (SMADs 4 and 10), and inhibitory (SMADs 6 and 7). Receptor-activated or phosphorylated SMADs associate with co-SMADs and then translocate to the nucleus, where they interact with transcriptional factors and co-activators and alter gene expression.

60.3.7.5 Rho

Ras family G proteins regulate intracellular actin dynamics through Rho/ROCK signaling. TGFβ and mechanical force promote the nuclear translocation of myocardin-related transcription factor A (MRTF-A) in cardiac fibroblasts inducing a myofibroblast-like cell-type gene expression through Rho/ROCK signaling [26].

60.3.7.6 KLF

Kruppel-like transcription factors (KLF) are a large family of transcription factors with an important role in cell differentiation and tissue development. KLF15, a negative regulator of cardiac fibrosis, inhibits the SMAD3 activity on the CTGF promoter [26]. TGFβ downregulates KLF15 in cardiac fibroblasts and myocytes leading to fibrosis. The other KLF member upregulated through AngII, KLF5, and activates TGFβ expression thereby connecting AngII and TGFβ signaling in cardiac fibrosis.

60.3.8 Electrical Tissue Remodeling and Arrhythmogenesis

Electrophysiological remodeling in cardiomyopathy occurs at the cellular and tissue levels, and it is not fully understood how exactly these changes contribute to electrical instability and increased risk of arrhythmias. Ca^{2+} transients, ion channels located within the sarcolemma and intercalated disks, sarcomere sensitivity and cell–cell coupling through gap junctions and desmosomes are all crucial components in regulating cardiac electrical remodeling.

60.3.8.1 Calcium Cycling

Arrhythmogenesis is triggered through disturbance of the orchestrated interactions of key calcium-handling proteins including ryanodine receptor-2 (RyR2), troponins, calsequestrin, triadin, junctin, sarcoplasmic reticular Ca^{2+}–ATPase 2 (SERCA2), and phospholamban [27].

60.3.8.2 Action Potential Sarcolemmal

Action potential (AP) prolongation, a result of downregulation of repolarizing K^+ currents, an increase in late Na^+ current, and changes in intracellular Ca^{2+} transport contribute to the development of arrhythmias. The cardiac Na^+ channel (Nav1.5) interacts differentially with either syntrophin–dystrophin complex to the lateral membrane or to the intercalated disks by synapse-associated protein 97 (SAP97). For example, loss of Nav1.5 results in significant lateral conduction velocity

slowing and prolongation of AP with delayed repolarization in dystrophin-deficient *mdx* mouse hearts [28].

60.3.8.3 Gap Junction Coupling

Decreased gap junction coupling, due to altered post-Golgi transport and downregulation of connexin 43 (Cx43), leads to loss of electrical cell–cell coupling and slows conduction velocity [29]. Reorganization of desmosomal proteins such as desmoplakin (DSP) and plakoglobin (JUP) is suggested to play a role in the development of arrhythmogenic and fibro-fatty remodeling in the heart via interfering with Wnt/β-catenin signaling [30].

60.3.9 Failing Heart

A pathologic causative genetic insult followed by sustained maladaptive remodeling results in the development of decompensated cardiomyopathy when the failing heart is unable to keep up with hemodynamic demands at all levels, from the molecule to the whole organism. Molecular cell level alterations of end-stage cardiomyopathy and CHF respond to irreversible cardiac remodeling with significant changes in membrane ion currents and intracellular Ca^{2+} metabolism, fibrosis, hypertrophic or atrophic remodeling, and cell death. Cell–cell coupling abnormalities include reorganization of gap junctions and desmosomal proteins. Cardiac function is significantly depressed with depleted force development and slowed relaxation [31].

60.4 Animal Models of Human Cardiomyopathies

Numerous small and large animal models have been developed to discover novel mechanisms and clarify known molecular and cellular pathogenic mechanisms of cardiomyopathies discussed above (Table 60.1). Characterization of the mechanisms of cardiomyopathies using the study of animal models is challenging owing to the complexity of disease-causing mechanisms and modulators of pathology. The accessibility of transgenic, knockout, and knockin murine models has, however, been one of the most successful approaches for studying genetic cardiomyopathies. The zebrafish (*Danio rerio*) model with morpholino knockdown remains one of the most effective technologies for discovering and functionally studying novel cardiomyopathy candidate genes. Table 60.1 summarizes animal models of human cardiomyopathy with a focus on cellular and cardiac remodeling-associated key molecular signaling models.

60.4.1 Hypertrophic Cardiomyopathy

Animal models of HCM mostly carry human mutations in sarcomeric protein-encoding genes such as α-MHC, α-tropomyosin, and troponins, and these models have demonstrated that HCM mutations enhance contractile properties with

Table 60.1 Animal models of human-inherited cardiomyopathies and associated signaling pathways

Cell	Gene/protein	Human phenotype	Animal model	Reference	Remodeling	Signaling
Sarcolemma	Sarcoglycan (delta)	DCM	Murine KO	Cordier et al. (2000) *Mol Ther* 1:119–129 [32]	Focal necrosis, vascular spasm, fibrosis	Destabilization DGC, membrane permeability defect, Ca+ inbalance
			Murine KI S151A	Rutschow et al. (2014) *Eur J Hum Genet* 22:119–125 [33]	Mild cardiomyopathy	
	Sarcospan		Murine KO	Araishi et al. (1999) *Hum Mol Genet* 8:1589–1598 [34]	Progressive DMD with extensive degeneration and regeneration	Disruption of ECM–sarcolemma–cytoskeleton connection
	Laminin-α2	DCM	Murine KO	Miyagoe-Suzuki et al. (2000) *Microsc Res Tech* 48:181–191 [35]	Dilation of ventricles	
	Dystrophin	DMD BMD XL-DCM	Murine	Sicinski et al. (1989) *Science* 244:1578–1580 [36]	Dilated ventricles	Destabilization DGC, sarcolemma–actin connection, Ca+ alteration
			Zebrafish	Guyon et al. (2003) *Hum Mol Genet* 12:601–615 [37]	Mutant zebrafish are less active	
			Canine	Jones et al. (2004) *J Neurol Sci* 217:143–149 [38]	DMD and DCM phenotype	
	α-Dystrobrevin	DMD LVNC	Murine KO	Yoshida et al. (2000) *Hum Mol Genet* 9:1033–1040 [39]	Muscle dystrophy, mild cardiomyopathy	Alteration in cyclic GMP levels
	Caveolin3	DCM	Murine KO	Woodman et al. (2002) *J Biol Chem* 277:38988–38997 [40]	Hypertrophy, dilation, and reduced contractility	ERK1/2 activation, Src
			Murine TG P104L	Kuga et al. (2011) *Hum Mol Genet* 20:2975–2983 [41]	Hypertrophy, enhanced contractility, apoptosis	nNOS production, altered ER stress response
			Zebrafish KO	Nixon et al. (2005) *Hum Mol Genet* 14:1727–1743 [42]	Disruption of muscle differentiation, cardiac edema	Myoblast fusion defects

(continued)

Table 60.1 (continued)

Cell	Gene/protein	Human phenotype	Animal model	Reference	Remodeling	Signaling
Sarcomere	Myosin heavy chain	DCM HCM LVNC	Murine TG R403Q	Kamisago et al. (2006) *Novartis Found Symp* 274:176–189 [43]	Cardiac dysfunction, myocyte disarray, hypertrophy, fibrosis	
	Titin	DCM HCM	Zebrafish	Xu et al. (2002) *Nat Genet* 30:205–209 [44]	Cardiac edema, poor contraction, and normal sarcomeres are absent	Blockage of sarcomere assembly
	Tropomyosin	DCM	Murine KO	Rethinasamy et al. (1998) *Circ Res* 82:116–123 [45]	Homozygous null mice are embryonic lethal (E8-E11.5)	
		DCM	Murine TG E54K	Rajan et al. (2007) *Circ Res* 101:205–214 [46]	Dilated LV, impaired systolic, and diastolic functions	Decreased Ca2+ sensitivity and tension generation
		HCM	Murine TG E180G	Prabhakar et al. (2001) *J Mol Cell Cardiol* 33:1815–1828 [47]	Ventricular concentric hypertrophy, fibrosis, and atrial enlargement	Increased myofilament sensitivity to calcium
		HCM	Murine TG D175N	Muthuchamy et al. (1999) *Circ Res* 85:47–56 [48]	Myocyte disarray, hypertrophy, and impaired contractility and relaxation	Thin filament enhanced Ca2+ sensitivity
	Troponin T	Diverse CM	Murine TG MyHC	Tardiff et al. (1998) *J Clin Invest* 101:2800–2811 [49]	Mild hypertrophy, disarray, reduced number of myocytes	Multiple cellular mechanisms
			Murine TG R92Q	Tardiff et al. (1999) *J Clin Invest* 104:469–481 [50]	Fibrosis, mitochondrial pathology, diastolic dysfunction, shorter sarcomere lengths	Induction of ANP and β-MHC, increased basal sarcomeric activation
			Zebrafish KO	Sehnert et al. (2002) *Nat Genet* 31:106–110 [51]	Sarcomere loss and myocyte disarray	Misregulation of thin-filament protein expression

	Troponin I	HCM	Murine TG I145GLY	James et al. (2000) *Circ Res* 87:805–811 [52]	Cardiomyocyte disarray, fibrosis, diastolic dysfunction, death	Increased skinned fiber sensitivity to calcium and hypercontractility
			Rabbit TG R146G	Sanbe et al. (2005) *Circulation* 111:2330–2338 [53]	Cardiomyocyte disarray, fibrosis, and connexin43 disorganization	Altered fractal pattern of the repolarization phase
			Murine KO	Huang et al. (1999) *Circ Res* 84:1–8 [54]	Acute heart failure, shortened sarcomeres	Reduced myofilament Ca sensitivity, elevated resting tension
	Myosin-binding protein C	HCM	Murine TG	Yang et al. (1998) *J Clin Invest* 102:1292–1300 [55]	Sarcomere disorganization and dysgenesis, pCa2+-force curve shift	Inefficient incorporation of stable truncated protein into the sarcomere
			Murine KO	Palmer et al. (2004) *Mol Cell Biochem* 263:73–80 [56]	Severe cardiomyopathy, reduced myofilament stiffness	Abnormal sarcomere shortening velocity
			Cat TG	Meurs et al. (2005) *Hum Mol Genet* 14:3587–3593 [57]	Sarcomeric disorganization	

(continued)

Table 60.1 (continued)

Cell	Gene/protein	Human phenotype	Animal model	Reference	Remodeling	Signaling
Intermediate filament	Desmin	DCM	Murine TG R173del 179	Wang et al. (2001) *Circulation* 103:2402–2407 [58]	Disruption of the desmin network and myofibril alignment, intrasarcoplasmic granular aggregates	Blunted response to beta-agonist stimulation
			Zebrafish	Li et al. (2013) *J Gen Physiol* 141:335–345 [59]	Disorganized muscles, small larvae, diminished swimming activity	Normal active force generation, vulnerability during eccentric work
			Murine KO	Milner et al. (1996) *J Cell Biol* 134:1255–1270 [60]	Loss of lateral alignment of myofibrils, mitochondrial abnormalities, necrosis	Multisystem disruption of muscle architecture and degeneration
Z-disk	Myopalladin	DCM HCM RCM	Murine TG Y20C	Purevjav et al. (2012) *Hum Mol Genet* 21:2039–2053 [61]	Disrupted intercalated disks, hypertrophy, and heart failure	Desmin, desmoplakin, connexin 43, and vinculin disruption
			Murine KI Q529X	Huby et al. (2014) *J Am Coll Cardiol* 64:2765–2776 [62]	Fibrosis, diastolic dysfunction, T-tubule enlargement	Desmin, MLP, CARP, ERK1/2 dysregulation, altered mechanosensation
	MLP	DCM	Murine	Arber et al. (1997) *Cell* 88:393–403 [63]	DCM with hypertrophy and heart failure, disruption of cardiomyocyte cytoarchitecture	Altered mechanosensation

	Nebulette	DCM	Murine TG	Purevjav et al. (2010) *J Am Coll Cardiol* 56:1493–1502 [64]	DCM, mitochondrial abnormalities	Stretch-induced alteration of Z-disk assembly
	Nexilin	DCM	Zebrafish	Hassel et al. (2009) *Nat Med* 15:1281–1288 [65]	Z-disk damage, heart failure	Stretch-induced Z-disk destabilization
	Telethonin	DCM	Murine KO	Knoll et al. (2011) *Circ Res* 109:758–769 [66]	Heart failure following biomechanical stress	Modulation of nuclear p53 turnover after biomechanical stress
			Zebrafish	Zhang et al. (2009) *Hum Mol Genet* 18:4130–4140 [67]	Deformed muscle structure and impaired swimming ability	Disruption of sarcomere–T-tubular interaction through ILK
	Cypher/ ZASP	DCM	Murine KO	Zhou et al. (2001) *J Cell Biol* 155:605–612 [68]	Ventricular dilation, Z-disk disruption, muscle weakness	Z-line via interactions with a-actinin or other Z-line components
Iband	CARP	HCM DCM	Murine TG αMHC	Song et al. (2012) *PLoS One* 7:e50436 [69]	Attenuated cardiac hypertrophy in response to pressure overload and isoproterenol	Reduced TGF-β and ERK1/2, MEK and Smad3 to pressure overload
			Murine KO	Bang et al. (2014) *PLoS One* 9:e93638 [70]	No cardiac phenotype	
Gap junction	Connexin 43	ARVC	Murine KO	Thomas et al. (1998) *Circulation* 97:686–691 [71]	Conduction abnormalities, no gross pathology	Slowing of intercellular channels in sinoatrial node pacemaker and ventricular conduction cells
			Murine TG αMHC	Ewart et al. (1997) *Development* 124:1281–1292 [72]	Conotruncal abnormalities, partial rescue of Cx43 KO mice	

(continued)

Table 60.1 (continued)

Cell	Gene/protein	Human phenotype	Animal model	Reference	Remodeling	Signaling
Desmosome	Desmoplakin	ARVC	Murine KO	Garcia-Gras et al. (2006) *J Clin Invest* 116:2012–2021 [73]	RV dilation, apoptosis, necrosis, fibro-fatty infiltration	Cell–cell contact disruption
			Murine TG	Yang et al. (2006) *Circ Res* 99:646–655 [74]	RV dilation, apoptosis, fibro-fatty infiltration	
	Plakophilin 2		Murine KO	Grossman et al. (2004) *J Cell Biol* 167:149–160 [75]	Embryonic lethality, cardiac wall rupture, reduced trabeculation, cytoskeletal disarray	Disruption of cell–cell junction assembly
	Desmocollin 2		Zebrafish	Heuser et al. (2006) *Am J Hum Genet* 79:1081–1088 [76]	Contractile dysfunction, loss of desmosomal plaque and midlines	Cell–cell contact disruption
	Desmoglein 2		Murine TG N271S	Pilichou et al. (2009) *J Exp Med* 206:1787–1802 [77]	Necrosis, atrophy, fibrosis, death, arrhythmias, biventricular dilatation	Cell–cell contact disruption and cell atrophy due to necrosis
			Murine TG	Krusche et al. (2011) *Basic Res Cardiol* 106:617–633 [78]	LV dilation, fibrosis, and ventricular arrhythmias	
	Plakoglobin		Zebrafish KO	Martin et al. (2009) *Dev Biol* 327:83–96 [79]	decreased heart size, bradycardia, cardiac edema	Interference with Wnt/b-catenin signaling
			Murine KO	Kirchhof et al. (2006) *Circulation* 114:1799–1806 [80]	VT, RV dilation and dysfunction	

Costameres	Talin	HCM	Murine Tln1cKO	Manso et al. (2013) *J Biol Chem* 288:4252–4264 [81]	Blunted hypertrophy, less fibrosis, and hypercontraction to pressure overload	Blunted ERK1/2, p38, Akt, and Gsk3 after mechanical stress
	Filamin C	DCM	Medaka zebrafish K1680X	Fujita et al. (2012) *Dev Biol* 361:79–89 [82]	Enlarged heart, myocardiac wall rupture, detachment of myofibrils from sarcolemma, Z-disk destruction	Disrupted structural integrity of cardiac and skeletal muscles for support against mechanical stress
Nuclear envelope	Lamin A	DCM	Murine N195K	Ho et al. (2013) *Nature* 497:507–511 [83]	Nucleocytoplasmic shuttling of Mlk1	Modulation of actin polymerization via Mlk1
			Murine KO	Sullivan et al. (1999) *J Cell Biol* 147:913–920 [84]	Degeneration and condensed vacuolated cytoplasm, mineralization, and atrophy	Emerin dislocation

increased force generation, ATP hydrolysis, and actin–myosin sliding velocity, showing that the hypertrophy is not a compensatory response to diminished contractile function. However, models of HCM also show abnormal Ca^{2+} cycling in cardiomyocytes before overt histopathologic changes occurred in the myocardium and delayed myocardial relaxation that occurs before the onset of hypertrophy, suggesting that diastolic dysfunction is a direct consequence of HCM mutations [27]. Hearts from models of HCM progressively accumulate myocardial fibrosis in the same manner as human patients, and fibrosis is considered to be a cellular substrate for cardiac arrhythmias and sudden cardiac death in humans.

60.4.2 Dilated Cardiomyopathy

Animal models of DCM mostly resemble human mutations in genes encoding cytoskeletal and sarcomeric/Z-disk proteins and present with ventricular dilation and thinning of the ventricular walls correlated with loss of heart muscle mass. In addition, functional changes in non-myocytes induce fibrotic scars that stiffen the heart tissue and impede normal cardiomyocyte contractility. Novel DCM mechanisms such as mutations in RNA-binding motif protein 20 (*RBM20*), impaired Z-disk assembly, sensitivity to apoptosis and abnormalities in myofibrillogenesis under metabolic stress, protein folding, inhibition of protein aggregation, and degradation of misfolded proteins have been explored.

60.4.3 Restrictive Cardiomyopathy

RCM is the least common but most lethal form of cardiomyopathy where impaired ventricular relaxation due to increased stiffness of the myocardium and pressure in the ventricles overcomes the changes in myofibrillar arrangement and cardiomyocyte gross abnormalities [85]. Primary impaired cardiac relaxation manifested by a decreased LV end-diastolic dimension (LVEDD) and an increased end-diastolic dimension in both atria with preserved cardiac ejection fraction (EF) was emphasized in transgenic mice with overexpressed mutant cardiac troponin I (cTnI-p.Arg193His) as a result of disturbed sensitivity of myofilaments to Ca^{2+} cycling in cardiomyocytes [86, 87]. In recently reported knockin myopalladin ($Mypn^{QWT/Q526X}$) mice, carrying a human RCM-causative nonsense MYPN-p.Gln529X mutation, authors nicely paved the molecular mechanisms of disturbed Z-disk mechanosensitive pathways in the development of familial RCM due to persistence of mutated myopalladin peptide [62].

60.4.4 Arrhythmogenic Right Ventricular Cardiomyopathy

Many models of ARVC with mutations in genes encoding desmosomal proteins such as desmoplakin 2 (DSP2) [74], plakophilin 2 (PKP2), desmocollin (DSC),

desmoglein (DSG), plakoglobin (JUP), and non-desmosomal (laminin receptor 1, *CYPHER*/ZASP, and ryanodine receptor 2) proteins have been developed. Structural and functional alterations include progressive, diffuse, or segmental loss of cardiomyocytes, probably due to cardiomyocyte apoptosis or necrosis and replacement with fibrotic and adipose tissue [88]. Fibro-fatty tissue primarily is seen in the right ventricle (RV), with common LV involvement in later stages of the disease [77], causing impaired blood pumping and arrhythmias. Embryonic lethality at E11.5 day is observed in PKP2 knockout mouse due to abnormal heart morphogenesis and stability, suggesting crucial roles of desmosomes in cardiac development and function [75].

60.4.5 Left Ventricular Noncompaction Cardiomyopathy

Animal models of LVNC demonstrate spongiform ventricular myocardium with deep trabeculations [89, 90]. Although mechanisms responsible for pathology are poorly understood, numerous animal studies suggest that defects in cellular growth and differentiation secondarily affect a process of ventricular compaction leading to LVNC phenotype. The critical pathways in establishing normal myofibrillogenesis, cardiomyocyte polarity, and ventricular compaction involve the Notch-NRG1-ErbB and/or Notch-BMP10-noncanonical Wnt signaling cascades [90–93]. Interestingly, FKBP12-deficient mice exhibited multiple structural anomalies that recapitulated syndromic forms of LVNC with ventricular septal defect or VSD [94, 95].

Conclusion

More than 600 cardiomyopathy-associated genetic mutations are described as a cause of different types of cardiomyopathies. A favorable strategy in developing vast animal models of various types of cardiomyopathies in humans improved our understanding of genetic impact on heart function and molecular mechanisms responsible for cardiomyopathies and cardiac dysfunction. It is clear that mutant proteins in cardiomyocytes due to mutations in coding genes can perturb cardiac function whether the prime distress occurs in the contractile apparatus or neighboring cellular complexes, yet persistent cellular stress leads to tissue, organ, and organism level pathology and pathophysiology. Current research focuses on ways to target the disease-causing and pathophysiologic pathways therapeutically before irreversible cell and tissue reorganization and degeneration occurs in the heart. Scientific approaches such as cell transplantation, gene therapy, small interfering RNAs, and microRNAs that specifically target these specific pathways endure innovative treatment options of inherited cardiomyopathies. Moreover, a wide range of innovative technologies and techniques in animal modeling, including recently developed CRISP/CAS9 and TALEN approaches [96], will lead to advances in our knowledge on the etiology, pathophysiology, and therapeutics of inherited cardiomyopathies.

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Part XVIII

Arrhythmias

Clinical Presentation and Therapy of Arrhythmias

61

David J. Driscoll

Contents

61.1 The Normal ECG

The normal cardiac rhythm originates from the sinus node that is located at the junction of the superior vena cava and the right atrium (Fig. 61.1). The cells of the sinus node exhibit spontaneous depolarization which is the reason that those cells function as the primary pacemaker of the heart. Depolarization of these cells activates the cells of the right atrium. Depolarization of the cells of the atria produces the "p-wave" of the ECG. The electrical activity of the atria reaches the ventricle by

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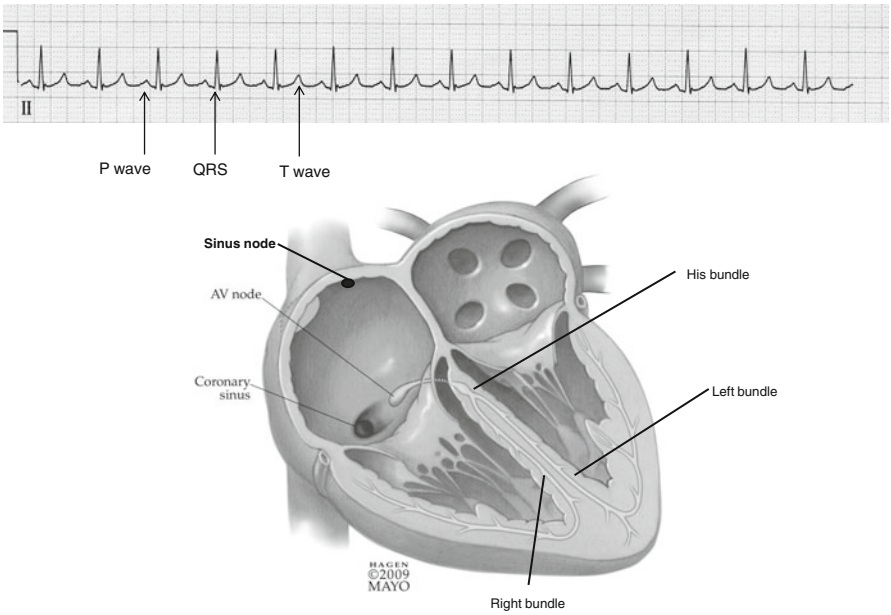

Fig. 61.1 Cardiac diagram depicting the electrical conduction system. Normal ECG

traveling through the atrioventricular node (AV) and the bundle of His. Conduction through the AV node is relatively slow and this accounts for the "PR" interval which is the isoelectric period on the ECG between the end of the p-wave and the q-wave of the QRS complex. The QRS complex represents the depolarization of the cells of the ventricles, and the T-wave represents the repolarization of the cells of the ventricles.

61.2 Introduction

Arrhythmias can be classified based upon the site of origin of the arrhythmia (supraventricular or ventricular), the heart rate of the arrhythmia (tachyarrhythmia or bradyarrhythmia), the function or dysfunction of the AV node (atrioventricular block or dissociation), or the underlying mechanism of the arrhythmia (reentry or bypass tract, automatic focus, ion channel defect).

Tachyarrhythmias originating from above the ventricles are termed supraventricular tachycardias and include atrial tachycardia, AV node reentry tachycardia, and tachycardia resulting from an accessory pathway (e.g., Wolff-Parkinson-White syndrome, Fig. 61.2).

Atrial fibrillation and atrial flutter, technically, are supraventricular arrhythmias but usually are simply referred to as atrial fibrillation and atrial flutter. Tachyarrhythmias originating from the ventricles are termed ventricular tachycardias.

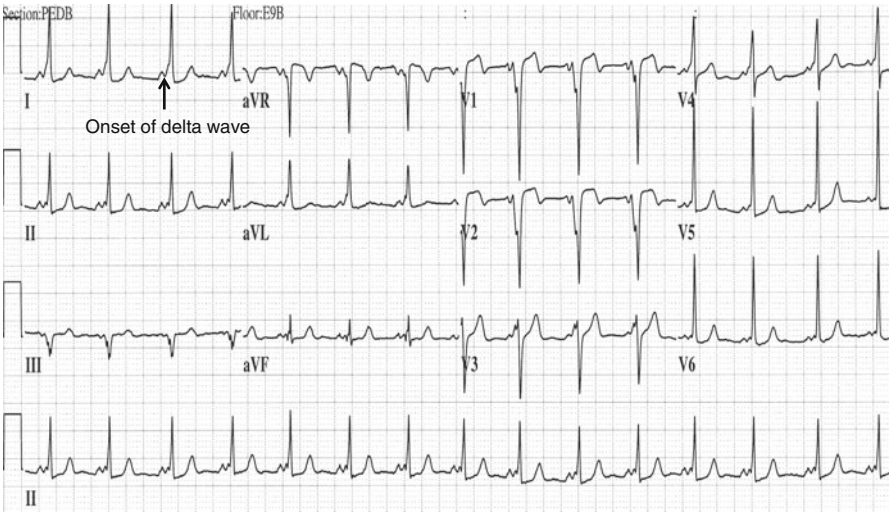

Fig. 61.2 This is an example of Wolff-Parkinson-White syndrome. Note the short PR interval and the delta wave (Reproduced or adapted from Driscoll D (2006) *Fundamentals of Pediatric Cardiology*. Lippincott Williams & Wilkins, with permission of the author and publisher)

Bradyarrhythmias (heart rate is too slow) can result from sinus node disease or AV node disease. In the latter case, the heart rate is slow because conduction through the AV node is abnormal. These bradyarrhythmias include first, second, and third degree atrioventricular block. In first degree atrioventricular block, the PR interval is prolonged but the heart rate is normal. In second degree block, some, but not all, of the atrial beats are conducted to the ventricles, and in third degree block, none of the atrial beats are conducted to the ventricle.

There are a number of arrhythmia syndromes that can be inherited, and these frequently can be associated with syncope, seizures, and sudden death. Many of these conditions result from mutations in genes encoding critical ion channels in the heart. These so-called channelopathies, a term coined by Dr. Michael Ackerman, occur in approximately 1 of 1000 persons.

61.3 Long QT Syndrome

Long QT syndrome (LQTS) is one of these (Fig. 61.3). When first described, two forms were recognized and termed Jervell and Lange-Nielsen syndrome (autosomal recessive) and Romano-Ward syndrome (autosomal dominant). Many mutations in 16 LQTS susceptibility genes have been identified. Most of these are due do to mutations in *KCNQ1* causing LQT1, *KCNH2* causing LQT2, and *SCN5A* causing LQT3. Historically, the hallmark of and diagnostic marker for LQTS was a prolonged QT interval on the electrocardiogram. However, with the advent of gene testing, it is clear that 40–50 % of patients who are genotype positive for a mutation

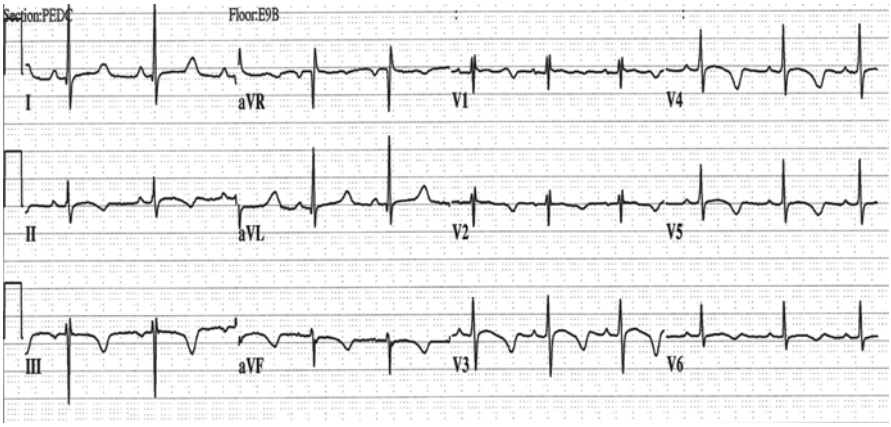

Fig. 61.3 This is an example of QT prolongation. Note that the T-wave is more distant from the preceding QRS than is normal (Reproduced or adapted from Driscoll D (2006) *Fundamentals of Pediatric Cardiology*. Lippincott Williams & Wilkins, with permission of the author and publisher)

in one of the pathologic mutations have a *normal* QT interval on the electrocardiogram.

Indeed, there is considerable overlap of the corrected QT interval (QTc) between normal individuals and patients with LQTS. The QTc is derived from Bazett's formula in which the QTc is equal to the QT interval divided by the square root of the RR interval. In general, a QTc greater than 480 ms in postpubertal females or greater than 470 in postpubertal males increases the probability of LQTS. These values represent the 99th percentile for QT intervals. However, if there is no personal history of LQTS-triggered spells or family history, this increased probability of LQTS still measures <10th percentile chance. Guidelines of the American Heart Association and American College of Cardiology indicate that a QTc $\geq$ 450 ms in adult males and $\geq$ 460 in adult females is defined as a "prolonged QTc." The conundrum is that about 50 % of patients with genetically proven LQTS will have a QTc less than these cutoff values, and 20 % of normal individuals would exceed these cutoffs.

61.4 Channelopathies

It is now recognized that these, as well as a number of other conditions, with or without eponyms, are due to inherited abnormalities of ion channels. In addition to Jervell and Lange-Nielsen and Romano-Ward syndrome, there are Anderson-Tawil syndrome, Timothy syndrome, drug-induced torsades de pointes, short QT syndrome, catecholaminergic polymorphic ventricular tachycardia (CPVT), Brugada syndrome, progressive cardiac conduction disease or Lev-Lenegre disease, and familial atrial fibrillation.

61.4.1 Andersen-Tawil Syndrome

Andersen-Tawil syndrome (ATS) includes ventricular arrhythmias, periodic paralysis, and dysmorphic features. It results from mutations in *KCNJ2*. It is a unique channelopathy representing a link between cardiac and skeletal muscle excitability.

61.4.2 Timothy Syndrome

Timothy syndrome includes lethal arrhythmias, webbing of the digits, immune deficiency, hypoglycemia, and cognitive disorder. The first Timothy syndrome-associated mutation was a *de novo* mutation, annotated as Gly406Arg in exon 8A of the *CACNA1C* gene.

61.4.3 Short QT Syndrome

Short QT syndrome (SQTS) is defined as a QTc $\leq$ 330–340 ms. It results from a gain of function in KCNH2, KCNQ1, and KCNJ2. Patients with short QT syndrome may present with sudden death, syncope, palpitations, and/or atrial fibrillation. Many patients have a family history of premature sudden death.

61.4.4 Catecholaminergic Polymorphic Ventricular Tachycardia

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is associated with sudden death or syncope during exercise. It results from a mutation in the *RYR2-encoded* cardiac ryanodine receptor or calcium release channel and much less commonly from homozygous or compound heterozygous mutations involving calsequestrin encoded by *CASQ2*.

61.4.5 Brugada Syndrome

The electrocardiographic features of Brugada syndrome include cove-type ST elevation in the right precordial leads with or without and rSr' pattern. It is associated with sudden death. There are now over 15 Brugada syndrome susceptibility genes, but the most common genetic cause stems from loss-of-function mutations in *SCN5A*, accounting for about 20 % of the disease.

61.4.6 Lev-Lenegre Disease

Lev-Lenegre disease causes atrioventricular block and, in many cases, is familial. Lev's disease tends to occur in older individuals and was thought to result from age-related fibrosis of the conduction system. Lenegre disease tends to occur in younger

individuals. Indeed, both (i.e., Lev-Lenegre disease) may be due to a mutation on chromosome 3p21. The cardiac sodium channel, SCN5A, is encoded on chromosome 3p21. Several SCN5A mutations have been associated with AV conduction block. It has been proposed that in Lenegre's disease, haploinsufficiency combined with aging leads to a progressive decline in conduction.

61.4.7 Wolff-Parkinson-White Syndrome

Wolff-Parkinson-White (WPW) syndrome, characterized by a short PR interval and a delta wave, is associated with supraventricular tachycardia (Fig. 61.2). Although typically sporadic, it can be familial.

61.5 Diagnosis

In many cases, the type of arrhythmia can be ascertained from the surface electrocardiographic tracing. In rare cases, an intracardiac electrophysiologic study may be necessary to precisely define the type and underlying mechanism of the arrhythmia. In general, supraventricular arrhythmias are more benign than ventricular arrhythmias.

61.6 Treatment

The treatment of any arrhythmia depends upon an accurate definition of the type and mechanism of the specific arrhythmia. The spectrum of treatment includes observation, drug therapy, electrical or pharmacologic cardioversion, pacemaker insertion, radiofrequency ablation, automatic internal cardiac defibrillator, cardiac surgery, and in very unusual circumstances, cardiac transplantation. It is important to determine if an arrhythmia or an arrhythmia syndrome is potentially inheritable since, in those cases, it is critical to screen family members for the condition. It also is important to search for potentially inheritable arrhythmias in family members of subjects who have unexplained early or sudden death, drowning deaths, and other deaths that occurred in unusual circumstances.

61.7 Outcome

Outcome of treatment of arrhythmias depends upon the type of arrhythmia and whether it is an isolated arrhythmia or is associated with congenital heart disease or myocardial disease.

Human Genetics of Arrhythmias

62

Erik Schulze-Bahr and Sven Dittmann

Contents

Abstract

Inherited forms of cardiac arrhythmias mostly are rare diseases (prevalence <1:2,000) and either considered to be "primary electrical heart disorders" due to absence of structural heart abnormalities or "cardiac ion channel disorders" due to the myocellular structures involved. Precise knowledge of the electrocardiographic features of these diseases and their genetic classification will enable early disease recognition and prevention of cardiac events including sudden cardiac death.

The genetic background of these diseases is complex and heterogeneous. In addition to the predominant "private character" of a mutation in each family, locus heterogeneity involving many ion channel genes for the same familial

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arrhythmia syndrome is typical. Founder mutations or mutational hot spots are uncommon. Moreover, phenotypes may vary and overlap even within the same family and mutation carriers. For the majority of arrhythmias, the clinical phenotype of an ion channel mutation is restricted to cardiac tissue, and therefore, the disease is nonsyndromic.

Recent and innovative methods of parallel DNA analysis (so-called next-generation sequencing; NGS) will enhance further mutation and other variant detection as well as arrhythmia gene identification.

62.1 Introduction to Inherited Forms of Cardiac Arrhythmias or Primary Electrical Heart Diseases: Disorders of Cardiac Ion Channel Genes

The field of cardiac arrhythmias and pathophysiologic insights into arrhythmogenesis has dramatically matured, driven, in the last two decades, by the in-depth understanding of inherited forms of arrhythmia. Components of cardiac ion channels (for survey, see Fig. 62.1) have been identified that are the clues to cardiac action potential generation and inherited forms of primary electrical heart diseases (Tables 62.1 – 62.3).

Many identified mutations are "private" (i.e., family specific). Pinpoint protein regions of the native ion channel that cause ion channel dysfunction have been identified and may allow modification by drugs. Pathophysiological and common disease pathways can be recreated in patient-derived cellular models (human-induced pluripotent stem cells; hiPSCs) and transdifferentiated cardiomyocyte-like cells that share the patient's genetic setting and are subjected to comprehensive biomedical research.

Nearly every inherited arrhythmia is genetically heterogeneous. For some primary electrical disorders (PEDs) of the heart, more than 10 different genes or associated loci are known (e.g., long QT syndrome, atrial or ventricular fibrillation). In addition, there is significant allelic heterogeneity (e.g., > 300 different LQTS mutations). In contrast, in some PEDs the mutation detection rate ("sensitivity of a genetic test") is still low (e.g., atrial or ventricular fibrillation, 10–20 %) raising the issue of clinical phenotypic conditions. Therefore, precise knowledge and recognition of the genetic forms of PEDs are essential, including differentiation from non-genetic forms. In the light of existing genetic heterogeneity, but also of unforeseen genetic complexity in known disease genes, next-generation sequencing (NGS) technologies will improve modern genetic diagnostics. Together with sufficient pathogenicity variant prediction, this parallelized gene analysis (e.g., several hundreds of genes for a distinct phenotype in a single analysis run) will further replace DNA analysis depending on Sanger sequencing approaches. However, apart from delineating the genomic complexity of monogenic cardiac disorders, it is likely that upon NGS analyses, cardiovascular genes not being previously linked to the patient's phenotype will be addressed and novel genes may be identified.

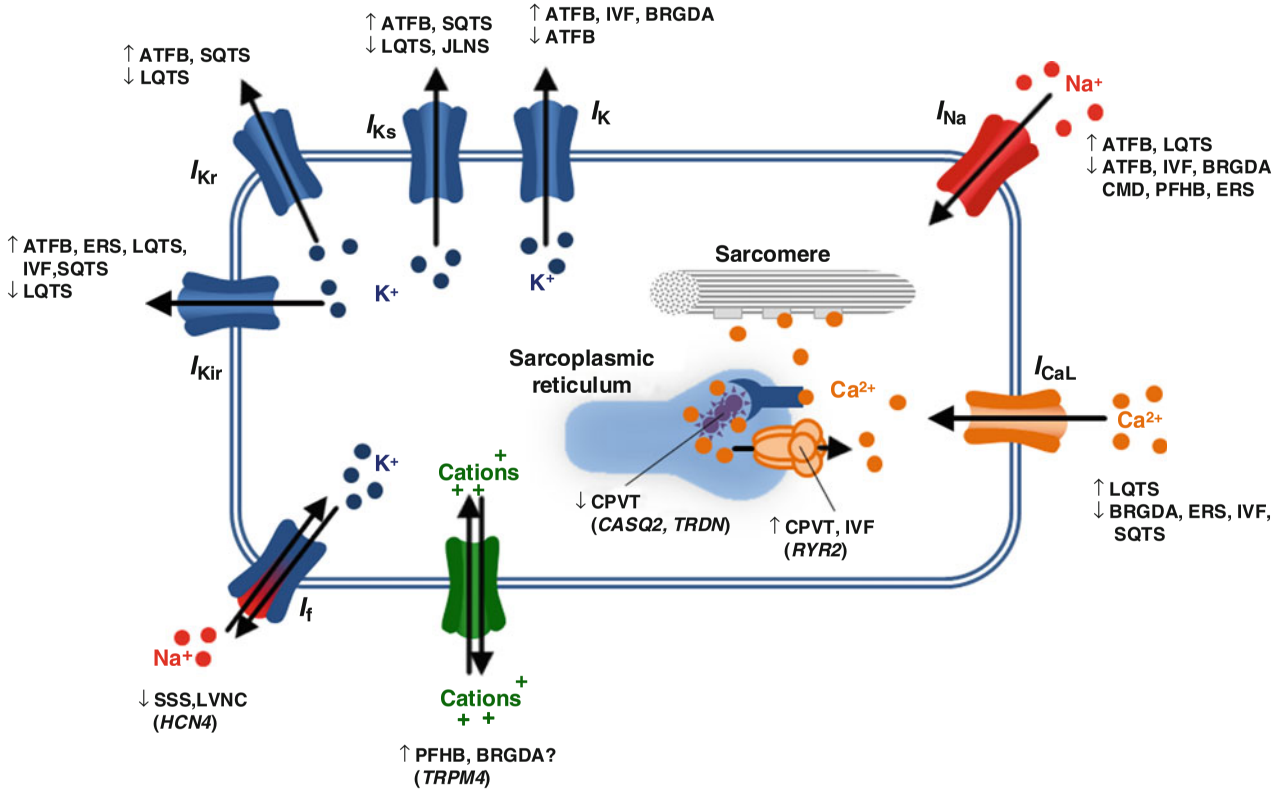

Fig. 62.1 Major cardiac ion channels and associated primary electrical disorders. Abbreviations: *I* current, ↑ current increase (gain-of-channel function), ↓ current decrease (loss-of-channel function), I_K potassium current, I_{Na} sodium current, I_{Ca} calcium current, *LQTS* long QT syndrome, *SQTS* short QT syndrome, *IVF* idiopathic ventricular fibrillation, *PFHB* progressive familial heart block, *ATFB* atrial fibrillation, *BRGDA* Brugada syndrome, *SSS* sick sinus node syndrome, *ERS* early repolarization syndrome, *JLNS* Jervell and Lange-Nielsen syndrome, *LVNC* left ventricular noncompaction cardiomyopathy. Gene symbols are in italic

Importantly, additional confirmatory research steps are required to establish a relationship between a novel gene and the phenotype.

62.2 Inherited Forms of Ventricular Arrhythmias

Familial forms of arrhythmias were described carefully several decades ago [e.g., 1–6] and were essential to elucidate the genetic basis of arrhythmias. These clinical observations are still important today, since in the era of human molecular genetics, these disorders have been subject to systematic gene investigations and identification. The rapid technological improvements of molecular genetic approaches – currently as whole-exome sequencing by NGS – and the detailed knowledge of the human genome [7, 8] have replaced genetic linkage and many candidate gene analyses (see Chap. 18). NGS certainly will speed up gene identification [9–13], as, for example, has been shown for familial forms of ventricular fibrillation due to a *CALM1* gene mutation [14].

In the following sections, selected types of familial arrhythmias and their genetic bases are discussed. Two international consensus documents are available as guidelines for diagnosis and indications for genetic testing [15, 16].

62.3 Congenital Long QT Syndrome (LQTS)

LQTS is characterized by prolongation of the QT interval, typically measured in a baseline 12-lead or exercise ECG (recommended speed, 50 mm/s). These values have to be corrected for heart rate (by using Bazett's formula to drive the corrected QTc value). A QTc of >450 ms (males) or >460 ms (females) is indicative for a LQTS but there is overlap with the normal population. The presence of interventricular conduction delay such as complete right or left bundle branch block may limit the use of the QT interval. The QT interval also can be affected by many drugs. In contrast to congenital LQTS, drug-induced QT prolongation ("acquired LQTS") is often not genetic, and only in 10–15 % of case can the drug be considered to have unmasked "hidden LQTS" [17]. For many physicians, recognition of LQTS and accurate QT interval measurements are still difficult [18]. Due to cascade family investigations and systematic genetic testing, it now has become clear that there are many LQTS mutation carriers without symptoms [19]. These asymptomatic but LQTS mutation-positive patients still have congenital LQTS, even though the term "syndrome" might be misleading. The overall risk for cardiac events or arrest might be lower and mainly is determined by the degree of QT interval prolongation and exposure to risk or event-triggering factors [20, 21].

Since 1995, 13 genes have been associated with congenital LQTS. A significant portion of mutations (sensitivity approx. 35 %) can be identified in the *KCNQ1* gene (subform LQT1; chromosome 11p15.5) encoding the α-subunit of the slowly activating, delayed outward rectifying K+ channel I_{Ks} (Kv7.1). These subunits form together with additional ß-subunits (e.g., *KCNE1*; LQT5) tetrameric channels.

Mutations in *KCNQ1* mainly result in decreased potassium outward current ("loss of function"). In addition to digenic inheritance [in 3–5 %, 22], other severe forms are the autosomal recessive Jervell and Lange-Nielsen syndrome (JLNS) with additional severe, bilateral sensorineural hearing loss. The second key gene for LQTS is the *KCNH2* gene on chromosome 7q36.1 that encodes the pore-forming α-subunit of the rapidly activating delayed rectifier potassium channel I_{Kr} (Kv11.1; human ether-a-go-go-related gene (*hERG*)) and plays an essential role in the final repolarization of the ventricular action potential as well as in drug-induced proarrhythmia [23]. "Loss-of-function" mutations in *KCNH2* (sensitivity, 30 %) lead to LQT2, whereas opposite effects by a "gain of function" shorten the QT interval (so-called short QT syndrome, SQT1). Another LQTS subform, LQT3, is related to cardiac sodium channel defects by *SCN5A* gene (chromosome 3p22.2) mutations. The gene encodes the α-subunit from cardiac isoform of the voltage gated sodium channel (Nav1.5); the main electrophysiological mechanism is a "gain of function" (either due to abnormal channel gating or impaired trafficking) with pronounced inward sodium (I_{Na}) current, and mutations occur in 10–15 % of LQTS patients. In addition to the molecular and mechanistic differences in the major LQTS subforms, patients differ clinically by differences in the pharmacological response, phenotypic disease modification (e.g., repolarization pattern in surface ECG), and disease severity [e.g., 20, 21].

Concerning additional but mainly rare LQTS subforms (sensitivity <1 % or less), mutations in other ion channel genes or regulatory subunits have been found. These ion channel subunits are encoded by *KCNE2* (K+; LQT6), *KCNJ2* (K+; LQT7; Andersen-Tawil syndrome), *KCNJ5* (K+; LQT13), and *SCN4B* (Na+; LQT10). Other genes for LQTS, sometimes based on a well-characterized single case report with a proven functional basis of the mutant, are *ANK2* (ankyrin-B, LQT4), *AKAP9* (A-kinase anchor protein; LQT11), *CACNA1C* (Ca2+; LQT8; also Timothy syndrome), *CALM1* (calmodulin 1), *CALM2* (calmodulin 2), *CALM3* (calmodulin 3), *CAV3* (caveolin 3; LQT9), and *SNTA1* (syntrophin alpha 1; LQT12) (Tables 62.1 – 62.3).

A mutation analysis is essential not only in patients with evident LQTS but also in asymptomatic family members with normal QTc interval (i.e., <440–460 ms) who have a tenfold increased risk of cardiac events in comparison to non-carriers [20]. The type of mutation and the location of the mutation within the particular channel domains have been proposed to impact on the underlying biophysical defect and, subsequently, on severity of the disease [24, 25]. However, the clinical utility of such information has not yet been validated. The obvious variability of clinical presentation among carriers of the same LQTS mutation, even in the same family, initiated studies to investigate the role of single-nucleotide polymorphisms (SNPs) for disease modulation. So far, rare SNPs alleles have been investigated in LQTS patients and in the general population and have been proposed to influence repolarization [26–29]. These statistically promising approaches already have been partly replicated. Further studies are needed to show the impact on clinical decision-making upon SNP genotyping, since the overall effect of minor SNPs alleles on the QTc interval usually is small (<5 ms).

Table 62.1 Potassium channel genes (*KCNx*) and inherited forms of arrhythmias

Gene	Disease (OMIM)	Protein, current	Sensitivity A: >10 % B: 1–10 % C: <1 %	Inheritance	Channel dysfunction
KCNA2	BRGDA	ß-Subunit (Kvß3) I_{to}	C	AD	Gain of function, I_{to} ↑
KCNA5	ATFB7	Major subunit (Kv1.5) I_{Kur}	B	AD	Loss of function, I_{Kur} ↓ Gain of function, I_{Kur} ↑
KCND3	BRGDA	Accessory subunit (Kv4.3)	C		Gain of function, I_{to} ↑
	ATFB	$I_{to,f}$	C		Gain of function, I_{to} ↑
KCNQ1	LQT1	α-Subunit	A	AD	Loss of function, I_{Ks} ↓
	JLN1		A	AR	Loss of function, I_{Ks} ↓
	SQT2	(Kv7.1)	C	AD	Gain of function, I_{Ks} ↑
	ATFB3	I_{Ks}	C	AD	Gain of function, I_{Ks} ↑
KCNH2	LQT2	α-Subunit (Kv11.1)	A	AD	Loss of function, I_{Kr} ↓
	SQT1	I_{Kr}	C	AD	Gain of function, I_{Kr} ↑
KCNE1	LQT5	ß-Subunit	C	AD	Loss of function, I_{Ks} ↓
	JLN2	$I_{Ks/Kr}$	A	AR	Loss of function, I_{Ks} ↓
	ATFB		C		Gain of function, I_{Ks} ↑
KCNE2	LQT6	ß-Subunit	C		Loss of function, I_K ↓
	ATFB4	I_K	C		Gain of function, I_K ↑
KCNE3	BRGDA6	ß-Subunit	C		Gain of function, I_K ↑
	ATFB	I_K	C		Gainof function, I_K ↑
KCNE5 (KCNE1L)	IVF	ß-Subunit	C		Gain of function, I_{to} ↑
	ATFB	I_K	C		Gain of function, I_{Ks} ↑
KCNJ2	Andersen-Tawil	Major subunit	A	AD	Gain of function, I_{K1} ↑
	LQT7	(Kir2.1)	C	AD	Gain of function, I_{K1} ↑
	SQT3	I_{K1}	C	AD	Gain of function, I_{K1} ↑
	ATFB9		C	AD	Gain of function, I_{K1} ↑
KCNJ5	Andersen-Tawil	Major subunit (Kir3.4) $I_{K,ACh}$	C	AD	Loss of function, $I_{K,ACh}$ ↓
	LQT13		C	AD	Loss of function, $I_{K,ACh}$ ↓
KCNJ8	ERS, IVF	Major subunit (Kir6.1) $I_{K,ATP}$	C		Gain of function, $I_{K,ATP}$ ↑

Abbreviations: *BRGDA* Brugada syndrome, *LQTS* long QT syndrome, *JLNS* Jervell and Lange-Nielsen syndrome, *SQTS* short QT syndrome, *ATFB* atrial fibrillation, *IVF* idiopathic ventricular fibrillation, *ERS* early repolarization syndrome, sensitivity: mutation detection rate per gene, *AD* autosomal dominant, *AR* autosomal recessive, (-)

Table 62.2 Sodium channel genes (*SCNx*) and inherited forms of arrhythmias

Gene	Disease (OMIM)	Protein, current	Sensitivity A: >10 % B: 1–10 % C: <1 %	Inheritance	Channel dysfunction
SCN5A	ATFB10	α-Subunit $I_{NaV1.5}$	A	AD	Loss of function, $I_{Na}\downarrow$
					Gain of function, $I_{Na}\uparrow$
	BRGDA1		A	AD	Loss of function, $I_{Na}\downarrow$
	CMD1E		B	AD	Loss of function, $I_{Na}\downarrow$
					Gain of function, $I_{Na}\uparrow$
	IVF		C		Loss of function $I_{Na}\downarrow$
	LQT3		B	AD	Gain of function, $I_{Na}\uparrow$
	PFHB1A (PFHBI)		A	AD	Loss of function, $I_{Na}\downarrow$
	SSS1		B	AD, AR	Loss of function, $I_{Na}\downarrow$
	MEPPC			AD	Gain of function, $I_{Na}\uparrow$
	ATRST1		C		
SCN10A	BRGDA	α-Subunit $I_{NaV1.8}$	C	AD	Loss of function, $I_{Na}\downarrow$
SCN1B	BRGDA5	ß-Subunit I_{Na}	C	AD	Loss of function, $I_{Na}\downarrow$
	ATFB13		C		Loss of function, $I_{Na}\downarrow$
SCN2B	BRGDA	ß-Subunit I_{Na}	C	AD	Loss of function, $I_{Na}\downarrow$
	ATFB14		C		Loss of function, $I_{Na}\downarrow$
SCN3B	IVF	ß-Subunit I_{Na}	B		Loss of function, $I_{Na}\downarrow$
	BRGDA7		C		Loss of function, $I_{Na}\downarrow$
	ATFB16		C		Loss of function, $I_{Na}\downarrow$
SCN4B	LQT10	ß-Subunit I_{Na}	C	AD	Gain of function, $I_{Na}\uparrow$
	ATFB17		C		

Abbreviations: *BRGDA* Brugada syndrome, *LQTS* long QT syndrome, *ATFB* atrial fibrillation, *ATRST* atrial standstill, *IVF* idiopathic ventricular fibrillation, *CMD* cardiomyopathy, dilated, *MEPPC* multifocal ectopic Purkinje-related premature contractions, *PFHB* progressive familial heart block, sensitivity: mutation detection rate per gene, *AD* autosomal dominant, *AR* autosomal recessive, (-)

Another experimental approach to study the effect of mutant LQTS channels is the use of reprogrammed cardiac-like myocytes that take the advantage of patient's tissue (usually skin fibroblasts) with identical genetic content including the LQTS mutation [30–32]. The cellular LQTS phenotype by using patient-derived cells has been successfully recapitulated in several LQTS subtypes [33–39] and very recently has been able to show a gene-dosage response for autosomal recessive LQTS [40].

62.4 Brugada Syndrome

Brugada syndrome (BRGDA) is a clinical and genetic overlap syndrome and currently is diagnosed by the presence of a pathognomonic ECG (so-called type 1 Brugada ECG) [15]. The ECG criteria have been modified recently [41], and in addition to them, BrS patients may have early repolarization abnormalities (J waves)

and/or type 1 ECG in the inferior leads [42]; these patients are considered to have a worse prognosis. Conditions mimicking BRGDA (e.g., hypothermia, pectus excavatum, drug-induced BRGDA) have to be excluded carefully [43, 44]. In an international effort (FINGER registry), among 1,029 BRGDA patients, aborted SCD occurred in 6 % and syncope (otherwise unexplained) occurred in 30 %. The majority of patients were asymptomatic (64 %), and during a median follow-up of 31.9 (14–54.4) months, only 5 % of cardiac events occurred indicating a lower than initially expected rate of cardiac events (7.7 % in patients with aborted SCD, 1.9 % in patients with syncope, and 0.5 % in asymptomatic patients). Cardiac risk was related to previous symptoms and a spontaneous type 1 ECG, whereas genotype, gender, family history, or inducibility of ventricular tachyarrhythmia during electrophysiological study was not informative [45].

In 15–20 %, mutations in the cardiac sodium channel gene *SCN5A* (sodium channel, voltage gated, type V, alpha subunit; BRGDA1) are responsible for BRGDA. This gene encodes for the α-subunit of the cardiac voltage gated sodium channel Nav1.5. Typically and in contrast to LQT3 mutations, a "loss of function" results in decreased inward current (I_{Na}). Currently, there is a debate whether BRGDA is solely a disorder of disturbed depolarization or early repolarization (phase 2). Of note, some *SCN5A* mutations may cause overlap syndromes with LQT3 [46] and, more often, with atrioventricular conduction disturbances. The disease is age dependent and progressive in nature which is comparable with other forms of progressive familial heart block (PFHB), e.g., due to *TRPM4* [47] or *LMNA* gene mutations.

Similar to BRGDA1, loss-of-function mutations in the L-type calcium channel genes (*CACNA1C*: calcium channel, voltage dependent, L type; BRGDA3; *CACNB2B*: calcium channel, voltage-dependent, ß-2 subunit; BRGDA4; and *CACNA2D1*: calcium channel, voltage-dependent, α-2/δ subunit 1) result in decreased inward currents (I_{Ca}) and BRGDA. However, these patients typically have early repolarization and/or short QT intervals [48, 49] (Tables 62.1).

Other BRGDA genes associated with cardiac sodium channel formation or regulation are rarely found (<1 %) in other BRGDA subforms [50]. So far, mutations in *SCN1B* (sodium channel, voltage gated, type I, ß-subunit, BRGDA5) and in *SCN3B* (sodium channel, voltage gated, type II, ß-subunit, BRGDA7) are reported for BRGDA, together with a loss of function and decreased inward currents. Mutations in the *GPD1L* gene (glycerol-3-phosphate dehydrogenase 1-like, BRGDA2) also resulted in similar reduction of the inward I_{Na} current via *SCN5A* interaction and reduced membrane trafficking [51]. Recently, another α-subunit of a sodium channel, *SCN10A* (sodium channel, voltage gated, type V, alpha subunit), was identified as a putative gene for BRGDA [52]. However, since this sodium channel is primarily expressed in neuronal cells, it remains unresolved how these loss-of-function mutations in *SCN10A* are linked with the cardiac phenotype. Due to the close chromosomal proximity of *SCN5A* and *SCN10A*, a genomic interaction on *SCN5A* expression might be a clue to the pathogenesis [53, 54]. Of note, cardiac sodium channel gene expression is related to other factors such as *HEY2* (a helix-loop-helix transcriptional repressor) [54], connexin 43, or other cell adhesion molecules [55, 56] that might modulate cardiac conduction in congenital or acquired settings.

Table 62.3 Calcium channel (*CACNx*) and cation channel genes and inherited forms of arrhythmias

Gene	Disease (subform)	Protein	Sensitivity A: >10 % B: 1–10 % C: <1 %	Inheritance	Dysfunction
CACNA1C	Timothy syndrome	α-Subunit CaV1.2	A	AD	Gain of function, I_{CaL} ↑
	LQT8		C	(-)	Gain of function, I_{CaL} ↑
	BRGDA3		B	AD	Loss of function, I_{CaL} ↓
	SQT4		C	AD	Loss of function, I_{CaL} ↓
	ERS, IVF		B	AD	Loss of function, I_{CaL} ↓
CACNA1D	SANDD	α-Subunit CaV1.3	C	AR	Loss of function, I_{CaL} ↓
CACNA2D1	BRGDA10	α-,δ-Subunit	B	AD	Loss of function, I_{CaL} ↓
	ERS, IVF		B	AD	Loss of function, I_{CaL} ↓
	SQT6		C	AD	Loss of function, I_{CaL} ↓
CACNB2	BRGDA4	ß-Subunit	B	AD	Loss of function, I_{CaL} ↓
	ERS, IVF		B	AD	Loss of function, I_{CaL} ↓
	SQT5		C	AD	Loss of function, I_{CaL} ↓
RyR2	CPVT1	Ryanodine receptor 2 (RYR2)	A	AD	Gain of function, diastolic $[Ca2+]_i$ ↑
CASQ2	CPVT2	Calsequestrin 2	C	AR	Loss of function, diastolic $[Ca2+]_i$ ↑
TRDN	CPVT5	Triadin	C	AR	Loss of function
HCN4	SSS2	Cardiac pacemaker (cation) channel	B	AD	Loss of function, I_f ↓
TRPM4	PFHB1b	Cardiac cation channel (Purkinje cells)	A	AD	Gain of function, ↑

Abbreviations: *SSS* sick sinus syndrome, *BRGDA* Brugada syndrome, *LQTS* long QT syndrome, *SQTS* short QT syndrome, *IVF* idiopathic ventricular fibrillation, *ERS* early repolarization syndrome, *PFHB* progressive familial heart block, *SANDD* sinoatrial node dysfunction and deafness, sensitivity: mutation detection rate per gene, *AD* autosomal dominant, *AR*, autosomal recessive, (-)

Candidate gene approaches in patients with BRGDA led to additional publications of rare variants (sensitivity <1 %) in additional ion channel genes. Examples are the *KCNE3* gene (K+; BRGDA6), *KCND3* (K+), and *KCNJ8* (K+). Of note, a mutation in *HCN4* (hyperpolarization-activated cyclic nucleotide-gated potassium channel 4; BRGDA8), the gene responsible for cardiac pacemaker current (I_f), and mutations in the *TRPM4* gene (transient receptor potential cation channel, subfamily m, member 4) [57, 58] and in *MOG1*, another modulator of sodium channel gene expression and function, have been found in some patients with BRGDA.

62.5 Catecholaminergic Polymorphic Ventricular Tachycardia

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is a potentially malignant and heritable arrhythmia syndrome characterized by bidirectional or polymorphic VT during physical or emotional stress [59]. The baseline ECG is usually normal, but during exercise (heart rate >120–140 bpm), typically monomorphic and later polymorphic or bidirectional premature ventricular beats (VES) occur. Some patients may have these during emotional rather than physical stress. Consequently, patients may experience syncope or cardiac arrest during a sustained polymorphic or bidirectional ventricular tachycardia or due to ventricular fibrillation (VF) that occurred secondarily. There still are differential diagnoses to be considered (e.g., cardiomyopathies, digitalis intoxication, VES at resting conditions). Initial reports indicated significant mortality rate is 30 % below the age of 40 when left untreated. However, upon genotyping, it became clear that the initial cases were biased and that genotype-positive patients with CPVT might have also milder courses.

The majority of CPVT cases (mutation sensitivity, 50–60 %) were caused by heterozygous mutations in *RYR2* gene (ryanodine receptor 2, CPVT1) [60] that encodes the sarcoplasmic reticulum (SR) Ca2+ channel called ryanodine receptor because of its affinity for binding the alkaloid ryanodine. It is a large, 560 kDa protein that localizes in the membrane of the sarcoplasmic reticulum in areas facing the T tubules where the L-type calcium channels are located. This large gene (103 exons) typically is investigated in a stepwise analysis (3–4 levels) if a NGS facility is not available. Mutations in *RYR*2 but also in the *CASQ2* gene (CPVT2) [61] result in a nonphysiologic, diastolic calcium release from the sarcoplasmic reticulum (SR) ("leak") and the development of triggered arrhythmias. In summary, *RYR2* gene mutations are associated with a gain of function, whereas mutations in other CPVT genes are due to a loss of function. So far, all reported *RYR2* mutations are missense mutations [62]. Genotype-phenotype correlations in CPVT1 have not been established to date. In contrast to the autosomal dominant forms, recessive forms are known in the *CASQ2* gene that encodes calsequestrin 2, a protein that binds free calcium inside the SR and modulates the RyR2 function by acting as a luminal calcium sensor, and in the *TRDN* gene (triadin, CPVT5). Triadin forms a complex with the cardiac ryanodine receptor and calsequestrin and junctin proteins. Because of this function, triadin is part of the calcium influx from the sarcoplasmic reticulum and regulates concordantly Ca2+ uptake into the SR (Table 62.3).

For these two genes, but also for all CPVT subforms apart from CPVT1, the mutation frequency (sensitivity) is quite low (<1 %). Other genes for CPVT are genes encoding calmodulin (*CALM1* (CPVT4) and *CALM2*), or the CPVT3 locus (gene unknown so far) in a large Arabian family mapped the disease locus on chromosome 7p22-p14 [63, 64].

Using patient-derived cells, human-induced pluripotent stem cell technology enabled investigation of CPVT models in cardiac-like myocytes [65, 66] as in LQTS (described above). Consistently with *in vitro* models, patient-derived cardiac-like

myocytes demonstrated delayed afterdepolarization that was pronounced during adrenergic stimulation and also led to triggered activity [66, 56]. These particular cells were used for pharmacological rescue of the cellular phenotype using flecainide (similar as in clinical and experimental observations [67]), thapsigargin, and CaMKII inhibitors [68] showing the principle capability of these components for a disease-directed and/or personalized medicine.

62.6 Other Uncommon Forms of Inherited Arrhythmias

62.6.1 Idiopathic Ventricular Fibrillation

The term "idiopathic ventricular fibrillation" (IVF) is used when a cardiac arrest remains unexplained despite extensive cardiac and non-cardiac investigations; this includes evaluation of known cardiac, respiratory, metabolic, and toxicological etiologies that may lead to cardiac arrest. Ideally, cardiac investigations include all invasive and noninvasive modalities including cardiac biopsy in case of suspected cardiomyopathy. Ideally, the event of VF occurring should be documented for definite diagnosis. In 1992, the hypothesis was advanced that concealed inherited forms (e.g., cardiomyopathies, LQTS, CPVT) of arrhythmogenic disorders could underlie IVF [69]. It is likely that a subset of sudden cardiac death (SCD) victims without a known cause (so-called sudden unexpected death syndrome (SUDS) or sudden infant death syndrome (SIDS)) have underlying IVF, in particular when a postmortem cause remains unestablished.

Familial forms of IVF have been described [10, 70, 71]. So far, candidate (ion channel) gene approaches have been taken in IVF patients and have focused on genes predisposing to other inherited arrhythmia syndromes. A single study reported on a founder haplotype on chromosome 7q36 involving the *DPP6* gene locus [70]. However, a causal gene variant at this locus and in *DPP6* (VF2) remains unidentified, even in the view that the protein might act as an action potential regulator of I(to) currents in human Purkinje fibers and thereby predispose to early repolarization features such as is known in IVF [72]. The majority of IVF patients, however, do not have a positive family history. Of note, case reports of IVF with ion channel gene mutations have been described, e.g., in *SCN5A* (VF1) in calcium channels genes (*CACNA1C*, *CACNA2D1*); in potassium channels *KCNQ1*, *KCNE5*, and *KCNJ8*; and in other genes (like *RYR2*, *SCN3B*, *TNNT2*, and *CALM1*). The overall mutation sensitivity for IVF is around 10–15 %.

62.6.2 Short QT Syndrome

Short QT syndrome (SQTS) is characterized by a very short QT interval, absence of the ST segment, and symmetric, tall T waves on the ECG (<330 ms, or <360 ms and symptoms). There is a predisposition to atrial and ventricular fibrillation, due to the shortness of myocellular refractoriness [73]. Therefore, SQTS is highly lethal with

SCD often being the first manifestation of the disease. Genetic studies have identified mutations in genes encoding potassium channels that initially had been described for LQTS (i.e., *KCNH2*, *KCNQ1*, and *KCNJ2*) and now have been shown to harbor opposite cellular effects on ionic currents (i.e., gain of function and increase). Mutations in genes encoding the CaV1.2 L-type calcium channel subunits (*CACNA1C*, *CACNB2*) cause either short QT syndrome or an overlapping phenotype that combines an abbreviated QT interval and a Brugada ECG phenotype and are characterized by a cellular loss of channel function. A recent study identified mutations in only 14 % of SQTS cases despite familial disease in almost of half the cases [74].

62.6.3 Atrial Fibrillation

Atrial fibrillation (ATFB) is the most common type of cardiac arrhythmia and a leading cause of cardiovascular morbidity, particularly stroke. It affects 1–2 % of the general population and has an age-dependent occurrence. Typically, ATFB is a multifactorial arrhythmia due to a series of factors affecting atrial chamber size, function, and myocellular function. A number of studies have demonstrated that AF, and in particular lone or early-onset ATFB ("idiopathic forms"), has a substantial genetic component. These are expected to compromise 10–20 % of all.

Gene mutations in patients with lone and familial AF, although rare, have now been recognized for many years. Presently, mutations in >15 genes have been associated with ATFB. However, the complexity of monogenic ATFB is illustrated by the recent finding that both gain- and loss-of-function mutations in the same gene can cause ATFB. Also, somatic gene mutations (e.g., in connexin 40/*GJA5*) have been noted, but appear to be uncommon [75]. To date, cardiac ion (K+, Na+) channel genes are mainly affected, e.g., *KCNQ1* (ATFB3), *KCNE2* (ATFB4), *KCNA5* (K+; ATFB7), and *KCNJ2* (ATFB9), or the following sodium channels: *SCN5A* (ATFB10) and the ß-subunit genes *SCN1B* (ATFB13), *SCN2B* (sodium channel, voltage gated, type II, ß-subunit; ATFB14), *SCN3B* (ATFB16), and *SCN4B* (ATFB17). Other genes for genetically determined AF include the protein transporter ABCC9 (ATP-binding cassette; ATFB12), *GJA5* (gap junction protein, alpha-5; ATFB11) which form intracellular channels, the main component of the nuclear pore complex *NUP155* (nucleoporin 155-KD; ATFB15), and *NPPA* (natriuretic peptide precursor a, ATFB6). There are additional linked loci where no genes were identified so far. These regions are 10q22-q24 (ATFB1), 6p16-p14 (ATFB2), 4q25 (ATFB5), and 16q22 (ATFB8) (Tables 62.1 and 62.2). So far, none of the ATFB gene loci explained a significant fraction of genetic variance (sensitivity <5 %) [17]. Therefore, genetic investigations in lone, early-onset, or familial AF are restricted to selected individuals (class IIb indication).

Genome-wide association studies (GWAS) have indicated that plenty common single-nucleotide polymorphisms (SNPs) have a role in the development of ATFB. Following the first GWAS discovering the association between *PITX2* and ATFB, several new GWAS reports have identified SNPs associated with susceptibility of ATFB. To date, nine SNPs have been associated with AF [76–80]. The exact

biological pathways involving these SNPs and the development of ATFB are now starting to be elucidated and have to bridge the identified quantitative trait loci with transcriptional changes of associated genes in cardiac tissue [81].

Conclusion

Many cardiovascular disorders – including distinct cardiac arrhythmias – have a genetic background and occur in a familial setting. The majority of these belong to the group of rare ion channel diseases, since their prevalence usually is low (< 1:2,000). Often, a widespread genetic heterogeneity and complexity (5–15 specific disease-causing genes) is known and a "private" (family-specific) mutation is associated with a variable phenotypic manifestation. It is expected that NGS technologies will enhance diagnostic power and disease gene identification and thereby improve patient care. Early recognition of patients at risk will be useful to prevent sudden cardiac death.

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Molecular Pathways and Animal Models of Arrhythmias

63

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Abstract

Arrhythmias account for over 300,000 annual deaths in the USA, and approximately half of all deaths are associated with heart disease. Mechanisms underlying arrhythmia risk are complex; however, work in humans and animal models over the past 20 years has identified a host of molecular pathways linked with both arrhythmia substrates and triggers. This chapter will focus on select arrhythmia pathways solved by linking human clinical and genetic data with animal models.

63.1 Introduction

Over the past two decades, our understanding of arrhythmias and arrhythmia-associated pathways has increased with the advent of genetic research in congenital arrhythmias. These findings, when combined with clinical and animal data, have redefined the ability to diagnose and treat congenital and acquired forms of heart disease. Traditionally, congenital cardiac arrhythmias have been described as "channelopathies" in the context of the affected protein, most often an ion channel. It also has become relevant to address ion channel-associated gene products that support the structure and function of ion channels, as their role in the pathophysiology of arrhythmias is more relevant than previously recognized. Channelopathy mechanisms illustrated in large and small animal models have provided insight to the "trigger and substrate" basis of arrhythmias.

63.2 Ventricular Arrhythmias

63.2.1 Brugada Syndrome

Two primary pathogenic hypotheses have been suggested for Brugada syndrome involving early repolarization and late activation of ventricular myocytes. These two etiological theories are not mutually exclusive and have often demonstrated a mechanically heterogeneous disease. An important breakthrough in the ability to define the molecular pathways underlying arrhythmia disorders is the application of electrophysiology to single cardiomyocytes. This approach, pioneered for investigating neuronal membrane currents, utilizes glass micropipettes to measure the currents moving through a small membrane "patch." The technique can be utilized to measure populations of myocyte currents or modified to measure the movement of currents through single channels. Currents implicated in the arrhythmogenesis of Brugada syndrome include the fast inward sodium current (I_{Na}), transient outward potassium current (I_{to}), and the L-type calcium current ($I_{Ca,L}$).

To date, variants in twelve genes have been identified as pathogenic for Brugada syndrome [1]. Most notably, *SCN5A*, which codes for the alpha-subunit of the $Na_v1.5$ voltage gated Na^+ channel (I_{Na}), accounts for approximately 20% of Brugada

syndrome patients [1, 2]. Several studies attribute conduction abnormalities in Brugada syndrome to Na^+ channel defects, the majority of them indicating a loss-of-function of $Na_v1.5$ [3, 2]. *SCN5A+/−* mice replicate phenotypes found in Brugada syndrome patients including atrial, atrioventricular, and ventricular conduction defects. Further, *SCN5A+/−* mice show reduced right ventricle expression of $Na_v1.5$ leading to reduced current, reduced upstroke velocity, and reduction of maximum right ventricle I_{Na}. These findings link mechanistic pathogenesis of Na^+ channels and the shortened epicardial action potential (AP) that gives rise to transmural current flow during the plateau phase of the action potential, prolonged QT interval, and subsequent ST segment elevation in precordial leads as classically observed in ECGs of Brugada syndrome patients.

Dysfunction of calcium channels in canine and rabbit cardiomyocytes has been associated with the pathogenesis of Brugada syndrome. A synergistic relationship between I_{Na} and $I_{Ca,L}$ is essential to an appropriate action potential in ventricular epicardium; therefore, disruption of this synergism may permit the I_{to} current to limit the action potential amplitude, allowing a second upstroke and an extrasystole resulting in polymorphic ventricular tachycardia. In subsequent studies with Brugada syndrome patients, an association between loss-of-function variants in calcium channel subunits and shorter than normal QT interval with ST segment elevation was identified [4]. Action potential heterogeneity was observed in canine ventricular cardiomyocytes with calcium channelopathy, displaying a deep phase 1 notch that inactivated the I_{Ca} allowing dispersion of repolarization, phase 2 reentry, and polymorphic ventricular tachycardia. On ECG, these canine models showed prolonged QT intervals, ST elevation, and developed T-wave alternans, supporting a close genotype-phenotype relationship with calcium channel dysfunction and Brugada syndrome.

63.2.2 Long QT Syndrome

As with many inherited arrhythmogenic diseases, the long QT syndrome (LQTS) phenotype involves ion channel proteins but can be precipitated via several different avenues of cellular dysfunction. Prolonged QTc intervals are derived from delayed repolarization of the ventricle due to either reduction in repolarizing currents or increase in depolarizing currents. This implicates loss-of-function mutations in K^+ channel subunits or gain-of-function mutations in Na^+ and Ca^{2+} channel subunits and their respective regulatory proteins.

A host of variants have been discovered in *KCNQ1*, most of which act to reduce I_{Ks} channel trafficking to the cell membrane, reduce I_{Ks} responsiveness to β-adrenergic signaling, or alter channel gating which all act to prolong the action potential duration producing a prolonged QT interval. I_{Ks} activation is governed by sympathetic stimulation, namely, protein kinase A phosphorylation mediated by the targeting protein Yotiao. This pathway ensures rapid repolarization and a shortening of the AP during episodes of increased heart rate. Interruption of I_{Ks} and Yotiao association, as demonstrated in *KCNQ1* Gly589Asp and *KCNQ1*$^{-/-}$ mouse models,

renders I_{Ks} insensitive to β-adrenergic stimulation, slowing repolarization and producing a prolonged QTc [5]. These findings support the hypothesis that LQTS1 patients with dysfunctional I_{Ks} channels are unable to appropriately respond to β-adrenergic stimulation and thus develop prolonged action potential durations due to an inability to initiate repolarization and subsequently trigger arrhythmogenesis. Several LQTS models develop early afterdepolarizations or action potential prolongation recovery by an increased L-type calcium channel and late I_{Na} currents resulting in oscillations of the membrane potential that can produce premature action potentials [5]. While in a state of decreased I_{Ks} and bradycardia, murine, rat, rabbit, and human data have shown that induction of early afterdepolarizations into the action potential compromises membrane repolarization, prolonging the QT interval and subsequently triggering polymorphic ventricular tachycardia [5]. Additionally, canine and murine models have displayed amplification in transmural dispersion of repolarization heterogeneity, when modeled after reduced I_{Ks} as in LQT1. Some models suggest that ventricular M cells (middle layer of ventricular myocytes) are integral to the prolongation of ventricular repolarization due to their naturally low volume of I_{Ks} and resultant prolonged QT interval [5]. Although implications of a coupled pathogenic mechanism involving both *KCNQ1* variants and I_{Ks}-deficient M-cell physiology have been made, these interactions are still unclear. Beyond potassium channel alpha subunits, human arrhythmia may result from dysfunction in potassium channel accessory or beta subunits including *KCNE2*. *Kcne2−/−* mice display defects in action potential and QT interval duration as well as defects in multiple repolarizing potassium currents [6, 7].

While loss-of-function $Na_v1.5$ variants can cause Brugada syndrome, gain-of-function variants at the intracellular link between domains III/IV (DIII/IV) cause persistent I_{Na} during the action potential plateau phase, resulting in delayed repolarization and prolonged QT. Mice homozygous or heterozygous for the $Na_v1.5$ ΔKPQ deletion display aberrant $Na_v1.5$ inactivation, persistent I_{Na}, and prolonged action potential durations resulting in spontaneous ventricular arrhythmias [8]. *Scn5a*Δ/+ mice showed paradoxical transient AP prolongation upon abrupt acceleration of pacing rate that favors the occurrence of early afterdepolarizations, demonstrating rate dependent arrhythmogenesis. This observation was due to the increased I_{Na}, causing sudden increase of sodium and calcium load so that during each action potential, the increased SR calcium release activated a larger Na/Ca exchange current, favoring early afterdepolarizations and reentry arrhythmias [8]. These outcomes were confirmed by a second study using *Scn5a*Δ/+ mouse hearts, during which prevention of ventricular tachycardia was observed by decreasing dispersion of repolarization and suppressing early afterdepolarizations. Additionally, cardiomyocytes from transgenic Asn1325Ser mice showed delayed inactivation and generated a late persistent current, prolonged action potential, and early afterdepolarizations resulting in the LQTS phenotype. Finally, models using induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) harboring the *Scn5a*1798insAsp/+ variant demonstrated increased persistent I_{Na}, prolonged action potential duration, and early afterdepolarizations at low pacing rates [1]. Interestingly, these phenotypic findings were replicated in iPSC-CMs from a human

patient with a similar *SCN5A* 1795insAsp/+ mutation at the COOH terminus of $Na_v1.5$, evoking small sustained currents similar to ΔKPQ variants.

Loss-of-function mutations to ankyrin-B (encoded by *ANK2*) in cardiomyocytes demonstrate a central role in the arrhythmogenesis of congenital LQTS4 or "ankyrin-B syndrome." In myocardium, ankyrin-B associates with Na/Ca exchanger (NCX) and Na/K ATPase (NKA) at the cell membrane [9]. Postnatal investigation of ankyrin-B−/− mice showed significant defects in expression and membrane localization of NCX and NKA. Surviving heterozygous ankyrin-B+/− mice displayed the same defects resulting in dysfunctional NKA channels and intracellular Na^+ accumulation, generating inhibition of NCX function, reduction of Ca^{2+} extrusion, and increase in intracellular Ca^{2+} [9]. Mohler et al. demonstrated that sequestration of intracellular Ca^{2+} into the sarcoplasmic reticulum created an increase in cardiomyocyte capacitance, leaving the myocardium susceptible to delayed afterdepolarizations upon catecholaminergic stimulation due to spontaneous Ca^{2+} release [1, 9]. Furthermore, diastolic Ca^{2+} waves are initiated by dysfunctional cytoplasmic RyR2 sparks in ankyrin-B+/− mice. These waves resulted in a transient inward Ca^{2+} current by NCX, producing delayed afterdepolarizations, triggering an action potential, extrasystoles, syncope, and even death [9]. Supporting this, human and mouse studies of *ANK2* loss-of-function mutations indicate polymorphic ventricular tachycardia immediately following increases in sympathetic tone as with exercise or stress (see Fig. 63.1); however, sinus bradycardia and QT prolongation are demonstrated primarily in patients with severe *ANK2* mutations [9].

63.2.3 Short QT Syndrome

The majority of short QT syndrome (SQTS) variants result from a gain-of-function of potassium channel subunits; however, phenotypic plasticity has been noted in some forms of SQTS [3]. Penetrance in probands and murine models has

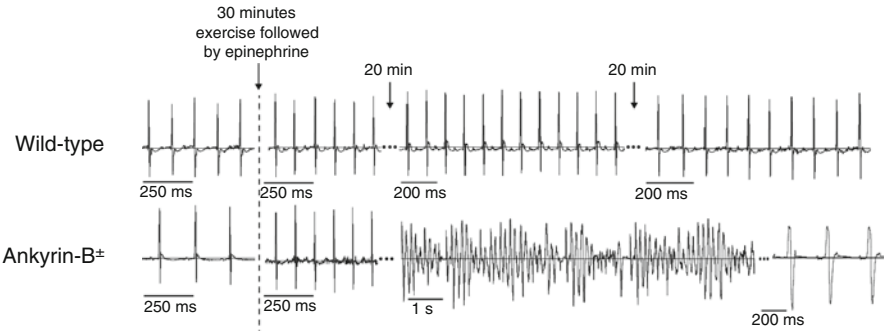

Fig. 63.1 Polymorphic ventricular arrhythmia phenotypes in mouse model. Representative ECG recordings from wild-type and ankyrin-B± mice at baseline and following exercise and treatment with adrenergic agonist. Center recording for ankyrin-B± mouse illustrates typical example of arrhythmia lasting minutes from this mouse model (Reprinted from DeGrande et al. [18], with permission from Elsevier)

demonstrated varying degrees of severity in QT shortening because of the variant's effect on associated subunits of the potassium channel. While a host of variants have been identified (i.e., SQTS4 with *CACNA1C*), this section will focus on SQT1 and SQT3.

Gain-of-function variants in I_{Kr} have been closely associated with SQTS1, namely, through the Asn588Lys missense variant. Under normal physiologic conditions, HERG allows timely repolarization of the ventricles and returns to resting membrane potential; however, Asn588Lys channels have been shown to increase the amplitude of I_{Kr} and shorten the action potential duration resulting in arrhythmogenesis [10]. The introduction of a positive charge in the channel pore increases the energy requirement for channel inactivation, leading to greater repolarization earlier in the action potential and shorter refractory periods. This change leaves the ventricles susceptible to premature stimulation. Of note, changes in extracellular potassium concentrations do not produce a change in channel conductance, indicating a disruption in K^+ permeability as the primary pathogenic mechanism. Corderio et al. produced a mouse model showing that arrhythmogenesis may begin in SQTS1 when the shorter ventricular refractory period produced by short action potential durations is combined with the longer Purkinje cell action potential duration in the ventricles, producing a heterogeneous repolarization and resultant polymorphic ventricular tachycardia [10].

Accelerated repolarization and shortened QT intervals also are associated with variants in *KCNJ2*, resulting in gain-of-function of the Kir2.1 channel protein. The Kir2.1 channel sustains resting membrane potentials, modulates cardiac excitability, and participates in determining the length of the QT interval by way of an inward rectifying current, I_{K1}, during the repolarization phase of the action potential [11]. SQTS3 is characterized by an asymmetric T wave specific to an Asp172Asn Kir2.1 mutation demonstrating slow ascending and rapidly descending limbs [12]. The highly conserved 172 position is integral for normal channel blocking, but its function can be disrupted with an acidic to neutral residue change as seen in Asp172Asn, allowing an increased outward current shortening repolarization [12]. Guinea pig myocytes with overexpressed Kir2.1 channels produced an increased I_{K1} density, duplicating the gain-of-function pathogenic mechanism as seen in SQTS3. Moreover, increased outward current in mice overexpressing Kir2.1 showed an increased rate of change in membrane potential during the last phase of the action potential, producing a shortened repolarization and subsequent QT as seen in SQTS [12]. These changes were arrhythmogenic in both the ventricles and atria of transfected mice, inferring that the same mutation may increase risk for both ventricular and atrial fibrillations in an individual with SQTS3.

63.2.4 Catecholaminergic Polymorphic Ventricular Tachycardia

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is found in autosomal dominant, recessive, and idiopathic forms. The most common genetic mechanism for CPVT is autosomal dominant gain-of-function variants in the *RYR2* gene

responsible for the assembly of the RyR2 or ryanodine receptor [3]. This protein controls the release of Ca^{2+} from the sarcoplasmic reticulum by way of L-type Ca^{2+}channel signaling and thus is central to the timely stimulation of sarcomeres that produce heart muscle contraction. Mutations in RyR2 and associated proteins can cause premature channel opening and Ca^{2+} release leading to delayed afterdepolarizations, bidirectional ventricular tachycardia, and ventricular fibrillation upon β-adrenergic stimulation [1]. Although about 70 RyR2 variants have been identified, the mechanisms by which CPVT originates from these mutations can be largely attributed to the effects on RyR2 luminal Ca^{2+}sensitivity, binding to association proteins, sarcoplasmic reticulum Ca^{2+} load, and lowered initiation threshold [1]. Several RyR2 Arg4496Cys knockin mouse models show that β-adrenergic stimulation induces CPVT Ca^{2+} waves by increasing store overload-induced Ca^{2+} release (SOICR), producing a higher Ca^{2+} and lower sarcoplasmic reticulum Ca^{2+} levels during resting state, which are susceptible to the Arg4496Cys lowered Ca^{2+} wave initiation threshold. Additionally, Purkinje cells in this model were more vulnerable to changes in intracellular Ca^{2+} leading to early and delayed afterdepolarizations, inferring a significant role in CPVT arrhythmogenesis [1]. The heterogeneity of a single RyR2 mutation was demonstrated by Loaiza et al. showing altered RyR2 cytosolic Ca^{2+} sensitivity, protein kinase A phosphorylation, and luminal Ca^{2+} modulation in Val2475Phe mice [11]. Similarly, Val2474Ser mice developed atrial fibrillation as a result of increased SOICR and lowered induction threshold in atrial cardiomyocytes. A final CPVT murine model involved a reduced binding affinity and decreased RyR2 association to FKBP12.6 that normally serves to stabilize the RyR2. Dissociation of these proteins in FKBP12.6$^{-/-}$ mice was shown to destabilize the closed state of RyR2, allowing uncontrolled Ca^{2+} release and inducing ventricular tachycardia. While it is clear the pathogenic mechanisms of specific CPVT mutations are varied, Loaiza et al. asserted that the production of delayed afterdepolarizations may be a point of convergence for RyR2 mutations and the CPVT phenotype [11]. Finally, animal models of human CPVT arrhythmias have yielded new information on the mechanisms underlying disease. For example, human mutations in calsequestrin 2, a sarcoplasmic reticulum calcium-binding protein, result in CPVT. Unexpectedly, *Casq2*$^{-/-}$ mice display defects in the sarcoplasmic reticulum size, as well as defects in calcium release and polymorphic arrhythmia [13].

63.2.5 Early Repolarization Syndrome

Increase of ventricular I_{to}, I_{KATP}, or $I_{K,Ach}$ current and decrease of $I_{Ca,L}$ and I_{Na} current are suspected in the arrhythmogenesis of early repolarization syndrome (ERS); however, the exact mechanism is still unknown. ERS shares a close phenotype with Brugada syndrome as evidenced by its intermittent concomitant presentation in some patients, with diagnosis differentiating primarily on the area of the heart affected. Genotypes of ERS demonstrate heterogeneity and variants have been identified in six genes.

The *KCNJ8* Ser422Lys variant produces a gain-of-function of the Kir6.1 subunit causing decreased ATP sensitivity and increased I_{KATP}. This, along with an outward imbalance of potassium currents facilitated by increased I_{to} during bradycardia or $I_{K,Ach}$ during vagal influence in epicardium, can facilitate a shift in the transmural voltage gradient between endocardium and epicardium resulting in phase 2 reentry, amplification of the J wave, and polymorphic ventricular tachycardia. In whole cell patch clamp studies, left inferior ventricular epicardium demonstrated a higher susceptibility to ERS due to higher concentration of Kir6.1 and higher levels of I_{to} when coupled with known ERS mutations including *KCNJ8*.

Pathogenesis of ERS has also been attributed to loss-of-function variants in L-type calcium channels leading to a decreased inward Ca^{2+} current (I_{Ca}). Burashnikov et al. found that mutations in any one of three subunits of the L-type calcium channel (LTCC), α1, β2, and α2δ, were found in patients with ERS; however, the β2 subunit was found in higher frequency associated with ERS [14]. Pharmacological loss-of-function in LTCC was modeled in canine hearts using verapamil, an LTCC blocker, and resulted in a net outward shift of current balance during the early action potential phase, allowing phase 2 reentry and induction of ventricular arrhythmia. The same model demonstrated further loss of left ventricular epicardial action potential dome and early repolarization morphology when verapamil and acetylcholine were infused together, inferring a parasympathetic role in arrhythmogenesis of ERS. This result indicates the need for future investigation into the implications of bradycardia and circadian rhythms with regard to the arrhythmogenesis of ERS.

63.3 Atrial Arrhythmias

Human atrial arrhythmias may result from defects in atrial size, alterations in atrial fibrosis, or defects in atrial excitability. Animal models have been important in defining the pathways underlying these disparate phenotypes. While too many to detail in depth, protein classes with described mouse models linked with atrial fibrillation include models of G-protein-coupled receptor signaling, altered ion channel proteins and protein complexes, altered calcium homeostasis, defects in proteins involved in cardiac conduction (i.e., connexin 40), as well as defects in transcription factors, cytokines, and growth factors [15].

Conclusion

Identifying the pathogenic mechanisms of congenital cardiac arrhythmias has profound implications on the recognition, diagnosis, and treatment of these conditions. Importantly, studies in the last 10 years have revealed a pivotal subclass of arrhythmogenic mutations affecting non-ion channel proteins, expanding the mechanistic substrates of "channelopathies," and therefore prompting augmented study in this area. The use of animal models has afforded an accurate framework for the investigation of human arrhythmias, providing improved insights into the clinical presentations of these diseases. Further understanding of genotype-phenotype relationships in the congenital arrhythmia syndromes will allow

clinicians to more accurately screen, risk-stratify, and apply personalized therapy to patients across the globe. While mice, rabbits, and guinea pigs have served as primary models for human arrhythmia, atypical models including zebra fish offer exciting promise for our understanding of human disease [16]. Furthermore, computational approaches continue to offer high throughput and rapid evaluation of cardiac disease susceptibility [17].

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Perspective

An Integrated Future for Congenital Heart Disease: Leveraging Knowledge

The fate of children with congenital heart disease (CHD) has been dramatically altered over the last 50 years and that fate is poised once again to undergo paradigm-shifting advances. Clinical breakthroughs involving novel surgical and catheter-based approaches, as well as advances in cardiopulmonary bypass imaging, and pre- and postoperative management have combined to result in major improvements in survival for many forms of CHD, although many complex forms of CHD remain challenging. However, in comparison to the revolutionary changes in surgical approach observed in the 1960s, 1970s, and 1980s, there have been considerably fewer fundamental changes in our approach to CHD over the last 20 years. During this same period, the emergence of ongoing sequelae among the growing number of CHD patients surviving surgical palliation has revealed the limitations of our knowledge and interventions.

In contrast, there has been an explosion in our understanding of the genetic and developmental basis for CHD at the molecular level over the last two decades, providing the foundation for more tailored and informed approaches to intervention, prevention, and outcomes management. Although this knowledge is only recent and has yet to lead to significant changes in clinical care, one can begin to envision the path that will inevitably emerge over the coming decades that will lead to fundamental shifts in our approach to CHD. This textbook elegantly integrates knowledge of clinical anatomy with developmental biology, molecular biology, and human genetics in a way that marries the more recent basic advances with the rich history of clinical advances. Here, I will briefly synthesize how our greater foundation of knowledge and emerging technologies can be leveraged with clinical medicine to alter the natural history of CHD.

The rich knowledge of the molecular biology underlying cardiac developmental events has led to a new understanding of CHD based more on etiologic origins during development rather than the ultimate anatomic findings or region affected at birth. For example, transposition of the great arteries and truncus arteriosus may both be

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conotruncal defects, but have vastly different embryologic origins. Similarly, a subset of DORV may be related to abnormal neural crest development, while a different subset may be due to abnormalities in the process of cardiac looping. The recognition that discrete pools of cardiac progenitor cells contribute to specific regions or structures and are selectively regulated by independent gene networks provides a molecular basis for understanding the origin for many forms of CHD. As we begin to leverage the power of modern human genetics and search for genetic variance that leads to CHDs, it becomes imperative to properly classify CHD based on the etiologic origin, as those are the ones that would presumably share a genetic cause.

The revolutionary advances in DNA sequencing over the last few years have begun to reveal an increasing role for genetic variants in the etiology of CHD. While ~25 % of CHD can now be explained by genetic mutations, it is likely that this number will continue to increase as we are able to broadly sequence DNA from larger populations of CHD, along with their parents. Furthermore, a correlation of genetic variants to longer-term outcome variables should lead to identification of those at greatest risk for complications even after palliation. To date, the majority of genes that contribute to disease involve signaling and transcriptional events that alter gene expression in early cardiac progenitors, altering their fate and morphogenesis. A deeper interrogation of the precise mechanisms and gene networks by which these factors function will be essential to truly understand the cause and effect relationship with disease. Recent advances in single cell biology and genome-wide epigenetic studies, coupled with computational and systems biology approaches, promise to provide a comprehensive understanding of the cellular basis for CHD.

The ability to connect genetic variance with disease mechanism has only recently emerged and was facilitated by a combination of stem cell technology and novel tools for efficient gene editing in human cells. The limitations of deeply analyzing the consequences of disease mutations in relevant human cells have been overcome by induced pluripotent stem cell (iPSC) technology, which involves induction of pluripotency from adult somatic cells such as dermal fibroblast or peripheral lymphocytes. Subsequent differentiation of such iPSCs from patients with disease into cardiomyocytes, smooth muscle, or endothelial cells results in vast quantities of cells carrying disease-causing variants that can be interrogated for functional consequences. Efficient gene-editing techniques can correct or introduce relevant mutations, facilitating direct cause and effect studies in human cells, while also allowing rapid introduction of human mutations in mice for more three-dimensional morphogenetic studies. By accurately modeling the defects underlying human disease, such models can then be utilized for drug discovery to identify agents that could alter the fundamental defect, particularly for those conditions where there may be ongoing postnatal consequence to a congenital defect. For example, the age-dependent calcification observed in patients with bicuspid aortic valve is likely related to the primary molecular defect leading to the morphologic abnormality and could potentially be prevented by appropriate intervention postnatally.

As the population of survivors with CHD grows and ages, we are beginning to see numerous comorbidities that may share etiologies with the cardiac morphologic defect. For example, poor neurodevelopmental outcomes have been observed in

many with CHD as they reach school age and beyond. Growing evidence suggests that the same genetic variants that lead to a cardiac defect may also affect neurologic development or outcomes, rather than cyanosis or cardiopulmonary bypass being the primary culprit for the neurologic deficits. The potential to mitigate this effect is attractive, as significant neurologic development continues postnatally. Similarly, heart failure is a rapidly expanding challenge in CHD survivors despite optimal surgical intervention. Novel technologies based on an understanding of cardiac developmental biology may allow regeneration of new heart muscle through conversion of existing fibroblasts within the heart, improving cardiac function. Other conditions such as septal defects may also benefit from the ability to create new muscle from existing cells if neighboring cells could be coaxed to expand and "fill-in" the defect. Furthermore, the recognition of fundamental differences in the developmental and molecular origins of right and left ventricular cardiomyoctes may allow conversion of the failing systemic right ventricle to a more left ventricle-like physiology, potentially mitigating the need for cardiac transplant in such conditions.

An ultimate, but long-term, goal of integrating a deep understanding of the etiology of CHD with clinical knowledge is to identify measures that might actually prevent the emergence of CHD even in those that are genetically predisposed. This would require knowledge of those genetically at risk, the mechanisms by which those genetic variants predispose to disease, and identification of methods to safely modify the pathways affected by the variants through the mother. This paradigm has been best demonstrated by the observation that maternal folic acid supplementation has dramatically lowered the incidence of neural tube defects. The recognition that most CHD is caused by heterozygous mutations that only reduce gene dosage slightly provides hope that relatively small changes in dosage of gene networks may be sufficient to overcome the disease threshold, ultimately preventing disease even in the presence of gene mutations.

Although we are poised to translate modern knowledge of CHD into novel therapies in the coming years, this will only occur in the setting of integrated teams that understand the clinical, developmental, molecular, and genetic underpinnings of the disease process. The unique organization of this textbook promotes such an environment and will be a valuable tool in this critical effort. I am highly optimistic that over the next two decades, major leaps in addressing CHD will emerge from our deep understanding of biology, and those leaps will change the face of what remains a leading cause of childhood morbidity and mortality.

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