Gustav Steinhoff *Editor*

Regenerative Medicine - from Protocol to Patient

1. Biology of Tissue Regeneration

Third Edition

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Foreword: Regenerative Medicine: From Protocol to Patient

Third Edition

The vision to unravel and develop biological healing mechanisms based on evolving molecular and cellular technologies has led to a worldwide scientific endeavour to establish *regenerative medicine*. This field involves interdisciplinary basic and (pre) clinical research and development on the repair, replacement, regrowth or regeneration of cells, tissues or organs in congenital or acquired diseases. Stem cell science and regenerative biology is prompting the most fascinating and controversial medical development of the twenty-first century. It can be envisaged that this development will establish completely new molecular and cellular techniques for medical diagnosis and therapy. The early rush of scientific development was initiated more than one hundred years ago by the physiology of blood regeneration (Hall and Eubanks 1896) and successful vascular surgical techniques for organ transplantation (Carrel and Guthrie 1905). However, the clinical realization of allogenic blood transfusion lasted until the discovery of the blood group antigens (Landsteiner and Levine 1928) and successful routine allogenic organ and bone marrow transplantation towards the end of the last century.

Similar to the field of allogenic cell and organ transplantation, it seems that *regenerative medicine* again condenses mankind's visions, hopes and fears regarding medicine: Hopes of eternal life and effective treatment of incurable disease, as well as fears of the misuse of technology and uncontrolled modifications of life are polarizing the scientific field. The development and public acceptance of new ethical and regulatory guidelines is a necessary process to support further clinical development. Nevertheless, the vision of a new medicine using the regenerative power of biology to treat disease and restructure the organism is setting the aims for scientific, technological and medical development. Viewing the great expectations to restructure and regenerate tissues, organs or even organisms, the current attempts of both scientists and physicians are still in an early phase of development.

The field of *regenerative medicine* has developed rapidly over the last 20 years with the advent of molecular and cellular techniques. This collection of volumes on *Regenerative Medicine: From Protocol to Patient* aims to explain the scientific knowledge and emerging technology, as well as the clinical application in different organ systems and diseases. The international leading experts from four continents describe the latest scientific and clinical knowledge in the field of *regenerative medicine*. The process of translating the science of laboratory protocols into therapies is explained in sections on basic science, technology development and clinical translation including regulatory, ethical and industrial issues.

This collection is organized into five volumes: (1) *Biology of Tissue Regeneration*; (2) *Stem Cell Science and Technology*, (3) *Tissue Engineering, Biomaterials and Nanotechnology*, (4) *Regenerative Therapies I.;* and (5) *Regenerative Therapies II. Biology of Tissue Regeneration (Volume 1)* focuses on regenerative biology with chapters on the extracellular matrix, asymmetric stem cell division, stem cell niche regulation, (epi)genetics, immune signalling, and regenerative biology in organ systems and model species such as axolotl and zebrafish.

Stem Cell Science and Technology (Volume 2) provides an overview of the classification of stem cells and describes techniques for their derivation, programming and culture. Basic properties of differentiation states, as well as their function are illustrated, and areas of stem cell pathologies in cancer and therapeutic applications for these cells are discussed with the emphasis on their possible use in *regenerative medicine*.

Tissue Engineering, Biomaterials and Nanotechnology (Volume 3) focuses on the development of technologies, which enable an efficient transfer of therapeutic genes and drugs exclusively to target cells and potential bioactive materials for clinical use. The principles of tissue engineering, vector technology, multifunctionalized nanoparticles and nanostructured biomaterials are described with regards to the technological development of new clinical cell technologies. Imaging and targeting technologies, as well as the biological aspects of tissue and organ engineering are described.

Regenerative Therapies I (Volume 4) gives a survey of the history of regenerative medicine and clinical translation including regulation, ethics and preclinical development. Clinical state-of-the-art, disease-specific approaches of new therapies, application technologies, clinical achievements and limitations are described for the central nervous system, head and respiratory systems. Finally, *Regenerative Therapies II (Volume 5)* contains state-of-the-art knowledge and clinical translation of regenerative medicine in the cardiovascular, visceral and musculoskeletal systems.

These volumes aim to provide the student, the researcher, the healthcare professional, the physician and the patient with a complete account of the current scientific basis, therapeutical protocols, clinical translation and practised therapies in *regenerative medicine*. On behalf of the sincere commitment of the international experts, we hope to increase your knowledge, understanding, interest and support by reading the book.

After the successful introduction of the first edition in 2011, this publication has been developed and expanded for the third edition into five volumes.

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Chapter 1
Extracellular Matrix and Tissue Regeneration

Zygmunt Mackiewicz, Yrjö Tapio Konttinen, Emilia Kaivosoja, Vasily Stegajev, Hanoch Daniel Wagner, Jaakko Levón, and Veli-Matti Tiainen

Abstract Extracellular matrix (ECM) is an important component of stem cell niche areas, which provide residence, regulate stem cell pool size and control stem cell mobilization. ECM is a complex interlinked composite of collagenous molecules, non-collagenous molecules and water-rich mucopolysaccharide ground substance. Cells are integrated to their matrix via integrin and non-integrin receptors, which control adhesion, migration, division, growth, anoikis, transdifferentiation and other cellular behaviour. ECM safeguard cells and tissue architecture and strength, but also growth factor deposits, which proteinases as signalling scissors can release in a site- and process-specific manner. Selected processes, like wound healing, cartilage and heart ECM, and tumor growth are used to exemplify participation of ECM in tissue regenerative processes.

Keywords Extracellular matrix • Stem cells • Regeneration

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1.1 Introduction

Bone marrow-derived mesenchymal stem/stromal cells (MSC) are nurtured in specialized niche areas by coordinated action via contact with soluble factors, extracellular matrix components and stem or feeder cells, which regulate the size and mobilization of the stem cell pool. MSCs can be recruited from their protected niche locations, be locally activated, or artificially harvested from various anatomical locations to be implanted for distant tissue regeneration. ECM fills the space between cells (interstitial matrix) and is present in tissue (basement membrane) and cellular (synovial lining) interfaces. ECM is a composite of fibrous collagen molecules and non-collagenous proteins embedded in water-rich mucopolysaccharides. Small leucine-rich proteoglycans (SLRP) regulate collagen fibrillogenesis, but also cross-link fibres and cells, and form deposits of growth and differentiation factors (Iozzo and Schaefer 2015). Elastins with its associated molecules, like fibrillin and fibulin, and adhesive glycoproteins laminin and fibronectin are important non-collagenous ECM proteins (Oasmanagic-Myers et al. 2015). Large aggregating proteoglycans or lecticans, like aggrecan, and hyaluronan form the ground substance. Scaffolds or derivatization of scaffolds with ECM molecules are used to regulate proliferation and differentiation of progenitor and stem cells.

Cells are integrated to ECM via at least 24 different non-covalently coupled heterodimeric cellular integrin receptors, which also form an important link in the architecture of cellular actin cytoskeleton. They allow migration and outside-in and inside-out signalling acting together with soluble factors and cell-cell contacts. Discoidin domain receptors, Lutheran Lu/B-CAM complex and α/β-dystroglycan complex are non-integrin matrix receptors. Glycan binding receptors, in particular extracellular C-, R- and I-type lectins and galectins play roles in cell-matrix adhesion and signalling, including MSC recruitment, bone marrow stem cell niche and adult collagen remodelling.

Proteinases modulate the composition of the ECM and are divided based on their catalytic mechanism to secrete neutral serine and metalloendoproteinases (and amino- and carboxypeptidases), and to mostly intralysosomal acidic cysteine and aspartic endoproteinases (Van Doren 2015; Klein et al. 2015; Kessenbrock et al. 2015). They mediate tissue degradative events in normal remodelling and pathological tissue destruction, but are increasingly recognized as signalling scissors.

Matrikines are peptides released by partial proteolysis of extracellular matrix macromolecules, which can participate in regulation of cell activities. There are known the “natural” matrikines, which unchanged participate in signaling from the extracellular ambience and “cryptic” matrikines (matricryptins) that require proteolytic processing to reveal the ligand from ECM protein. Unlike traditional soluble growth factors, most matrikines are characterized by low binding affinity to their receptors. Some of these peptides modulate proliferation, migration, protease production and apoptosis (Maquart et al. 2005; Maquart and Monboisse 2014; Ricard-Blum and Salza 2014; Wells et al. 2015).

Classical examples of processes which reflect stromal stem cell function and some general and some site specific challenges comprise wound healing and tumor growth.

1.2 Stem Cell Niche

Stem cells niche refers to a local microenvironment able to home, house, interact, maintain and mobilize one or more stem cells interacting with them and regulating their fate (Gattazzo et al. 2014; Rezza et al. 2014). The cellular "host" of the niche probably represents a cell, which produces such extracellular matrix (ECM) components, which the stem cell itself may not be able to produce or organize to a niche, but to which it adheres via integrin and non-integrin matrix receptors. Interactive participation of the nurturing "host" cell and stem cell in the process and their responsiveness to external stimuli, such as stem cell mobilizing pro-inflammatory cytokines, makes the niche dynamic. The niche regulates stem cell proliferation and differentiation during the embryonic development but maintains stem cells in a quiescent state in adults, and helps them to get activated upon tissue injury and to disclose the potential of the stem cells to undergo also asymmetric cell divisions (Doe and Bowerman 2001). This may be determined by the orientation of the cytokinesis of the stem cell division, which is in part regulated by the composition of the niche and by integrin mediated anchorage. If the division occurs in a plane parallel to the niche cell-stem cell contact surface, the proximally located parent cell is likely to remain in contact with the niche whereas the distally located daughter cell is displaced from it. This maintains the stemness and the size of the stem cell pool and produces progenitor cells, which loose contact with the niche and their stemness and leave the niche to migrate and/or transit via circulation to a new location to terminally differentiate to specialized cells, respectively (Ellis and Tanantzapf 2010).

Interactive niche-stem cell factors can be classified to three categories: (1) soluble factors (growth factor, nutrients, electrolytes, etc.), (2) direct cell-cell interactions and (3) ECM-stem cell/niche cell interactions. MSC integrin receptors for interstitial type I collagen $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_{10}\beta_1$ and $\alpha_{11}\beta_1$, may play a role in this respect, but MSC have also been described to contain e.g. α_3, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins (Docheva et al. 2007), which may help the stem cells to home, anchor, structure, divide and leave the niche as well as otherwise to perform their other functions as stem cells. However, in spite of the knowledge of the integrins present in MSCs, the actual integrin receptors relevant for the niche cells and for the MSCs are poorly known at present, but several redundant integrin receptors are probably involved (Ellis and Tanantzapf 2010).

Integrin chain specific antibodies coupled to paramagnetic micro-beads and various controlled culture substrate stretching devices have been developed to test the effect of integrin-mediated mechanical forces on cellular phenotype and function

(Sasaki et al. 2007; Pommerenke et al. 1996). Mechanotransduction may help to shape the stem cell niche and regulate the stem cell function (Kuo and Tuan 2008).

Understanding the effects of the above mentioned niche factors on chromatin remodelling and gene expression is essential for proper control of tissue engineering. A delicate balance protects stem cells from depletion, but at the same time prevents excessive, cancer cell-like proliferation. Stem cell niche constitutes the basic unit of stem cell physiology the same way as osteons, chondrons and salivons do in bone, cartilage and salivary gland tissue, respectively.

Niche has implications also for cancer cells and hematopoietic cells. Self-renewing cancer stem cells may reside and renew in cancer stem cell niche composed of a specialized vascular bed of endothelial cells, some sort of mesenchymal cells and ECM components (Nie 2010). Bone marrow derived MSCs in their bone marrow stem cell niche may via their immunosuppressive properties be involved in cancer progression and metastasis (Fulawka et al. 2014). MSCs of bone marrow may also provide the cellular support structure in the niche for hematopoietic stem cells (Battiwalla and Hematti 2009).

To regulate the size of the stem cell or progenitor cell pool, stem cells have a capability pendulate between asymmetric and symmetric cellular divisions (Kfoury and Scadden 2015). Probably the stem cell pool is expanded during embryogenesis, whereas asymmetric divisions allow rapid generation of progenitors upon high demand in various regenerative processes. The process of expansion of the stem cell pool must be controlled at some critical checkpoints to prevent cancer, whereas to latter is also strictly regulated to prevent precocious depletion of the stem cell pool; the cells can probably switch back and forth between these two different modes of proliferation (Oskarsson et al. 2014; Lane et al. 2014). The size of the stem cell pool is probably diminished upon aging via senescence or apoptosis (Jung and Brack 2014).

In spite of their predicted existence, it is a challenge to identify stem cells in their natural surrounding unanimously (Boulais and Frenette 2015). In tissues they cannot be recognized by their ability to differentiate along various specialized cell lineages and demonstration of a palette of markers used for cultured or cloned stem cells by using flow cytometry are not easily adaptable to histological tissue sections at the single cell level in static cytometry.

When cell detatch from ECM it become homeless and die via programmed death mechanism called anoikis (Paoli et al. 2013). Cancer cells develop anoikis resistance.

1.3 Mesenchymal Stromal Cells

Mesenchymal stem cells (MSCs) can be recruited from their protected niche, locally activated, or artificially harvested from various anatomical locations to be implanted as such or in tissue engineering constructs to tissue defects to facilitate repair by expantion, differentiation or perhaps mostly by orchestration of the more simply

programmed resident or immigrant repair cells during tissue regeneration (Sharma et al. 2014; Ikebe and Suzuki 2014; Kfoury and Scadden 2015).

Bone, cartilage and other tissue defects can be repaired by differentiated autologous cells or tissues isolated from the donor site(s) for transplantation, but often it is impossible to obtain enough autologous cells or tissues for such repair procedures and harvesting them from e.g. iliac bone or non-weight bearing femoral head cartilage can be complicated and painful. Allogeneic and xenogeneic cells are better available for clinical use, but immunosuppressive treatment is necessary to overcome immunological. Usage of foetal cells might provide a low immunogenic option (O'Donoghue and Fisk 2004) but raises ethical issues. Induced pluripotent stem cells have huge potential in the field but only the first clinical trials are ongoing.

Tissue injury triggers cellular mechanisms, which regulate homing and engraftment of circulating and local stem cells to fill the void and to regulate the sequential and ordered healing cascades. To avoid scar tissue formation and promote true regeneration with functional tissue, various natural or synthetic scaffolds seeded with stem cells have raised interest. They could be used to augment healing in critical size defects, non-union, non-healing and otherwise clinically threatening tissue defects.

Autologous MSCs have potential to proliferate via symmetric cell divisions and then to differentiate into specialized phenotypes via asymmetric cell divisions. Differentiation is regulated by a dynamic and complex extracellular microenvironment that contains a lot of biophysical and biochemical information, including soluble cues (i.e. growth factors and cytokines), cell – cell contacts, cell – ECM contacts, and physical forces. In addition to the extracellular microenvironment, the chemical and physical properties of a biomaterial control stem cell fate (Kaivosoja et al. 2012).

Vision of MSCs as precursors for resident cellular components of various specialized tissues is often oversimplified. MSCs can prolong the survival of skin and cardiac allografts, ameliorate the course of acute graft-versus-host disease and experimental autoimmune encephalomyelitis, orchestrate tissue repair (Zhao et al. 2010; Das et al. 2013).

MSCs participate in the healing of different tissues damage, however, they are also recruited by cancer cells to aid tumor growth and progression (Sun et al. 2014; Chang et al. 2015).

1.4 Extracellular Matrix

ECM fills the space between cells (interstitial matrix) and is present at tissue (basement membrane) and cellular (synovial lining) interfaces. It is synthesized, maintained and modulated by cells to adapt the growth, evolution, aging, changing mechanical and developmental needs, to meet the reparative needs after micro- and macrodamage to renew and regenerate and to produce new editions of instructive

outside-in signalling matrix in heathy and tumour tissue (Mouw et al. 2014; Bonnans et al. 2014; Picup et al. 2014; Wong and Kumar 2014). ECM is a composite of fibrous collagen molecules and non-collagenous proteins embedded in water-rich mucopolysaccharides. Small leucine-rich proteoglycans (SLRP) regulate collagen fibrillogenesis (fibre thickness), but also cross-link fibres and cells and form deposits of growth and differentiation factors. Elastin with its associated molecules, like fibrillin and fibulin, and adhesive glycoproteins laminin and fibronectin, are important non-collagenous proteins. Apart from SLRPs, large aggregating proteoglycans or lecticans, like aggrecan, and hyaluronan form important components of the ground substance. Scaffolds or derivatization of scaffolds with ECM molecules are used to regulate proliferation and differentiation of progenitor and stem cells. ECM components are widely used for therapeutic purposes (Kular et al. 2014).

Extracellular vesicles – microvesicles are not structural part of ECM. They represent transport mechanism of intercellular communication, usually in ECM milieu. Two types of microvesicles are known: membrane-derived vesicles (EVs) and exosomes. EVs can transfer mRNA and microRNA to target cells, release of apoptotic bodies. The role of microvesicles in tissue regeneration is important (Rani et al. 2015).

1.4.1 Extracellular Matrix: Collagens

ECM fills the void between cells (interstitial matrix) and between tissue interfaces (basement membrane), providing by its toughness structure and physical support to tissue-typical multicellular but dynamically generated (organogenesis) and maintained (remodelling) architecture as well as adhesion substrate and an instructive editable matrix, which literarily is decisive for cellular survival and for the regulation of multiple aspects of cellular behaviour (Aszodi et al. 2006). For most soft (skin, fat, fasciae, muscles, tendons, blood vessels, brain, peripheral nerves etc.) and hard or semi-hard (bone, cartilage, cornea etc.) connective tissues collagen nanofibres form a three dimensional and highly organized scaffolded backbone (Mienaltowski and Birk 2014), whereas the more hydrophilic and permeable ground substance largely composed of proteoglycans and glycoproteins occupies the interfibrillar spaces enabling transfer and filtration of nutrients, oxygen, metabolites and bioactive factors as solubilized, in granules or in EVs. Linker proteins bind these two major components to extensive networks, which provide dynamic and adjustable biomechanical strength, associated with flexibility, to such cell-matrix composite structures. Due to the high biomechanical and instructive demands imposed to the ECM, it undergoes almost continuous mechanotransduction and remodelling by replacing damaged and degenerated tissue elements with new intact and properly organized, to adapt to the varying local functional needs (Aszodi et al. 2006).

Human body contains altogether 28 different collagen types, which are classified to nine different subtypes, including fibrillar collagens, which form the bulk of the interstitial stromal collagens (Table 1.1). Structurally all collagens are characterized

Table 1.1 Classification and types of collagens based on the collagen chain encoding genes, their distribution in tissues and disorders caused mutations

Classification	Type	Own chains	Gene	Exons	Chromo-some[a]	Distribution in tissues	Disorders caused by mutations in genes
Fibrillar collagens	I	αl	*COL1A1*	51	17q21.33–q22	Bone, tendon, ligament, skin	Osteoporosis, scars, Ehlers-Danlos syndrome, osteogenesis imperfecta
		α2	*COL1A2*	52	7q22.1		
	II	αl	*COL2A1*	54	12q13.11	Cartilage, intervertebral disc, vitreous humor	Several chondrodysplasias
			COL2A2	54			Osteoarthritis
	III	αl	*COL3A1*	51	2q24.3–q31	Co-expressed with collagen I in vasculature and skin	Ehlers-Danlos syndrome (type IV), granulation tissue, Dupuytren's contracture
			COL3A1	51	1		arterial aneurysms
	V	αl	*COL5A1*	66	9q34.2–q34.3	Co-expressed with collagen I in lungs, cornea, bone and placenta	Ehlers-Danlos syndrome (types I and II)
		α2	*COL5A2*	54	2q14–q32		
		α3	*COL5A3*	66	19p13.2		
	XI	αl	*COL11A1*	68	1p21	Co-expressed with collagen II	Chondrodysplasias, non-systematic hearing loss, osteoarthritis
		α2	*COL11A2*	66	6P21.1		
		αl(II)					
	XXIV	αl	*COL24A1*	57	1p22.3	Co-expressed with collagen I in bone and cornea	Not known
	XXVII	αl	*COL27A1*	61	9q32	Co-expressed with collagen II in cartilage and epithelia	Not known
	XXVIII	αl	*COL28A1*	32	7p21.3	Peripheral nerves	Not known

(continued)

Table 1.1 (continued)

Classification	Type	Own chains	Gene	Exons	Chromo-some[a]	Distribution in tissues	Disorders caused by mutations in genes
3D network	IV	αl	*COL4A1*	52	13q34	Most basement membranes	Alport syndrome, Goodpasture's syndrome
(BM-collagens)		α2	*COL4A2*	47	13q34	Glomerular and alveolar BM	(COL4A3, COL4A4, COL4A5)
			COL4A2	47	8		Alport syndrome with diffuse oesophageal leiomyomatosis
		α3	*COL4A3*	52	2q34–q37		(COL4A5, COL4A6)
		α4	*COL4A4*	48	2q35–q37		
		α5	*COL4A5*	51	Xq22		Lethality at 14 weeks, progressive glomerulonephritis, renal failure[b]
		α6	*COL4A6*	46	Xq22		Alport syndrome
Microfibril (Beaded filaments)	VI	α1	*COL6A1*	36	21q22.3	Wide tissue distribution, associated with type I collagen, not bone	Bethlem myopathy, Ulrich myopathy
		α2	*COL6A2*	36	21q22.3		
		α3	*COL6A3*	41	2q37		
Anchoring fibril	VII	α1	*COL7A1*	118	3p21.31	Squamous epithelium BM zone	Epidermolysis bullosa
			COL7A1	118	9		
Hexagonal lattice	VIII	α1	*COL8A1*	5	3q12.3	Many tissues, Descemet's membrane of cornea	Corneal endothelial dystrophy
		α2	*COL8A2*	2	1p34.2		
	X	α1	*COL10A1*	3	6q21–q22	Hypertrophic cartilage	Schmid metaphyseal chondrodysplasia
			COL10A1	3	10		

FACITs	IX	α1	*COL9A1*	38	6q12–q14	Associated with type II fibrils in cartilage and cornea	Epiphyseal dysplasia, intervertebral disc disease, osteoarthritis
		α2	*COL9A2*	32	1p32		
			COL9A2	32	4		
		α3	*COL9A3*	32	20q13.3		
	XII	α1	*COL12A1*	65	6q12–q13	Associated with type I fibrils in perichondrium, ligaments and tendons	Disruption of periodontaland skin matrix structures[b]
	XIV	α1	*COL14A1*	44	8q23	Associated with type I fibrils in many tissues	Not known
	XVI	α1	*COL16A1*	67	1p35–p34	Associated with type II in hyaline cartilage and withmicrifibrils in skin	Not known
	XX	α1	*COL20A1*	35	20q13.33	Associated with type I fibrils in sternal cartilage, cornea and tendon	Not known
	XXI	α1	*COL21A1*	28	6p12.3–p11.2	Associated with type I fibrils in vessel walls	Not known
FACIT-like	XIX	α1	*COL19A1*	51	6q12–q14	Rare BM zones, in developing muscles	Abnormal muscle layer in the oesophagus[b]
	XXII	α1	*COL22A1*	63	8q24.23	Associated with microfibrils at tissue junctions	Not known
	XXVI	α1	*EMID2*	13	7q22.1	Testis and ovary	Not known

(continued)

Table 1.1 (continued)

Classification	Type	Own chains	Gene	Exons	Chromo-some[a]	Distribution in tissues	Disorders caused by mutations in genes
Transmembrane	XIII	α1	*COL13A1*	41/42	10q22	Many tissues at a low level	Fetal lethal, cardiovascular and placental defects, tumor formation[b]
			COL13A1	42	10		Progressive muscular atrophy[b]
	XVII	α1	*COL17A1*	56	10q24.3	Skin and intestinal epithelia	Epidermolysis bullosa
	XXIII	α1	*COL23A1*	20	5q35.3	Heart, lung and brain metastatic tumor cells	Not known
	XXV	α1	*COL25A1*	35	4q25	Neurons	Not known
Multiplexins	XV	α1	*COL15A1*	42	9q21–q22	Many BM zones	Mild myopathy, cardiovascular defects[b]
			COL15A1	40	4		
	XVIII	α1	*COL18A1*	43	21q22.3	Endothelial and epithelial BM zones	Knobloch syndrome
			COL18A1	43	10		Vascular abnormalities in the eye[b]

[a]The chromosomal locations and the exones were collected from the Entrez Gene data base

[b]In transgenic mouse models; BM-basement membrane

Modified from Jälinoja (2007), Cosgrove et al (1996), Reichenberger et al. (2000), (Myllyharju and Kivirikko 2001), Fukai et al. (2002), Sund et al. (2001), Kvist et al. (2001), Ricard-Blum (2011) and Eklund et al. (2001)

by the archetypical Gly-X-Y repeat sequences, in which X is often proline and Y either hydroxyproline or hydroxylysine. After synthesis of collagen α-chains, regularly repeated glycins with their minimal side chains (-H) allow three individual collagen chains to wind up around each other into triple helical collagen monomer, tropocollagen, with globular amino- and carboxyterminal propeptide ends. The collagen superhelix domain is, due to its structure, very resistant against non-specific proteinase-mediated degradation. After processing and removal of the globular propeptides individual collagen monomers spontaneously non-enzymatically assemble side by side to near one quarter overlapping supramolecular stacks with the typical cross-striation visible in electron micrographs. Fibre thickness for type I collagen is regulated in part by type III collagen and for type II collagen (Wiliusz et al. 2014) in part by collagens IX embedded in part inside the collagen type II fibre and type XI located on the surface of collagen type II fibre. These still relatively loose fibre stacks mature by covalent cross-linking in a process which involves specific cross-linking enzymes, hydroxyproline and hydrolysin and ascorbic acid without which develops a disease leading to spontaneous bleedings and known as scurvy. The characteristics of cross-linking are importent in healthy tissue and pathology (Snedeker and Gautieri 2014). Collagen network provides substrate for attachment of ground substance and cells and provides the framework for deposition of various bioactive factors.

Purified native or processed allogeneic and recombinant human collagens, especially type I, are composed of nanosize biodegradable biofibres with potential for use in plastic and cosmetic surgery, drug delivery and tissue engineering in form of sheets, pellets, plugs, sponges and other products (Chattopadhyay and Raines 2014). Collagen sponge seeded with bone marrow-derived MSCs can develop healing tissue which to its biomechanical strength to 75 % matches that of the corresponding healthy tissues (Juncosa-Melvin et al. 2006).

Basement membranes support epithelia (Choi et al. 2015) and endothelia casting them to their spatial shapes, such as simple sheets, tubes or relatively sophisticated tubuloacinar, tubuloalveolar and vascular structures. Basement membrane also surrounds some individual cells, like adipocytes, Schwann cells and skeletal muscle cells. Basement membrane components are found in the intercellular cementing substance between fibroblast-like type B and macrophage-like type A synovial lining cells joining them to form synovial lining or intima of the joint cavity, which in spite of its sheet-like structure does not have an actual sheet-like basement membrane.

Basement membrane is a 100–300 nm thick barrier with perforations in the order of ~50 nm permitting free bidirectional movement of small molecules whereas the movement of cells and larger molecules is controlled (Kruegel and Miosge 2010). Certain cells, like the neutrophils, do cross the BM with great efficiency. Chemoattractants and proteolytic events play a role in this process.

It has been thought that type IV collagen polymer network serves as the base platform upon which that laminin network is deposited but laminin polymers may actually serve as a template for the subsequent assembly of the BM (Li et al. 2005; McKee et al. 2007). Collagen IV and laminin forms ternary complexes linked

together by nidogen-1 and -2 (Fox et al. 1991; Kohfeldt et al. 1998). A heparan sulphate/chondroitin sulphate proteoglycan, perlecan is also found as an integral part of this network and is important for its integrity and as a local storage of growth factors (Gohring et al. 1998).

The type IV collagens were first identified by Kefalides in 1966 (Kefalides 1966). Depending upon its location it is synthesized either by fibroblasts, paranchymal cells, epithelial cells, endothelial cells, or by various other cells that are surrounded by the BM. The collagen type IV genes in human encoding its different α-chains are arranged head-to-head in three pairs. Gene encoding type IV collagen α1, α2 (*COL4A1, COL4A2*) and α3, α4 *(COL4A3, COL4A4)* is located on chromosome 13, and on chromosome 2, respectively, while the gene encoding for type IV collagen α5, α6 (*COL4A5, COL4A6*) is located on chromosome X. A common ancestral gene may have been duplicated three times resulting in six evolutionary related genes (Zhou et al. 1994). Sequences and characteristic exon-intron organizations divide them into α1-like group (*COL4A1, COL4A3, COL4A5*), and α2-like group (*COL4A2, COL4A4, COL4A6*). A unique feature of the type collagen IV gene pairs is that they share bidirectional promoters.

All type IV collagen *a* chains are ~1400 amino acids long. They are composed of a ~15-residues long N-terminal 7 S domain, collagenous segments consisting of Gly-X-Y repeats, which are interrupted by 22 short non-collagenous sequences (which provide flexibility and serve as cell-binding sites) and a ~230-residue long C-terminal non-collagenous NC1 domain (Kalluri 2003). Three type IV collagen wind up to a triple helical tropocollagen. According to the currently known combinatorial rules three distinct trimers are formed, α1α1α2, α3α4α5, and α5α5α6 (Boutaud et al. 2000; Hudson et al. 2003; Khoshnoodi et al. 2008).

The collagen monomers further self-assemble to supramolecular networks. First, dimers are formed by head-to-head association of two protomers via their trimeric NC1 domains so that bonding NC1 hexamers are formed. Four dimers then join at their N-terminal cystine- (disulfide bonds) and lysine-rich (lysine- hydroxylysine bonds) containing regions to form a heavily glycosylated 7 S-tetramer. This knot is relatively resistant to collagenase activity and can be isolated from bacterial collagenase treated basement membranes at a sedimentation coefficient 7 S (Risteli et al. 1980). α1α1α2(IV) and α3α4α5(IV) trimers form independent networks, while α1α1α2/α5α5α6 (IV) molecules form combined aggregates (Borza et al. 2001).

Type IV collagen composition of the basement membrane seems to affect cell adhesion, proteolytic susceptibility and ability to interact with other BM components (Kalluri 2003). The α1(IV) and α2(IV) are ubiquitously found throughout the human body, while the other 4 α chains have a more restricted tissue distribution. The α3α4α5(IV) network is found in the glomerular and some tubular basement membranes of the kidney, cochlea, eye, lung and testis, whereas the α5α5α6(IV) trimer is located in the skin, oesophagus, Bowman's capsule of the kidney and smooth muscle cells. A temporal regulation of type IV collagen α-chains expression is seen for instance in the glomelular BM of human kidney. During early embryonic development (day 75), the genes which encode α1(IV) and α2(IV) chains are expressed. As the development proceeds the expression of genes encoding the α3(IV), α4(IV) and α5(IV) chains starts while the levels of α1(IV) and α2(IV)

chains gradually decrease. This switch in gene expression during developmental is critical for maturation of the glomerular BM.

Mutations in the genes encoding either α1(IV) or α2(IV) are embryonic lethal, while mutations in the genes encoding the α3(IV), α4(IV) or α5(IV) chains may lead to human diseases (Hudson et al. 2003; Hudson 2004). Mutation of the *COL4A5* gene results in Alport syndrome. This mutation being mostly inherited and results in glomerulonephritis and hearing loss. Goodpasture's syndrome is an autoimmune disease manifest by rapidly progressive glomerulonephritis and pulmonary hemorrhage. The Goodpasture antigen, which is usually the NC1 domain of the α3(IV) chain, is most exposed in the glomeruli and alveolar basement membranes.

Type IV collagen expression is suppressed by pioglitazone (Ohga et al. 2007; Ko et al. 2008) and methotrexate (Yozai et al. 2005). Tumstatin, the NC1 domain of α3(IV) chain, is an endogenous inhibitor of pathological angiogenesis and suppresses tumour growth via integrin αvβ3, because tumstatin binding inhibits focal adhesion kinase and some other signalling pathways, which inhibits endothelial cell proliferation and induces apoptosis (Maeshima et al. 2001, 2002).

Endothelial cells, basal cell layer keratinocytes, hepatocytes, carcinoma cells, melanoma cells, fibrosacroma cells and many other cells bind via adhesion receptors to multiple sites in the NC1 and/or the triple helix domains of type IV collagen.

1.4.2 Extracellular Matrix: Non-Collagen Proteins

Elastin is a highly elastic stretchable and recoiling strong and elastic fibrous protein of many connective tissue matrices of the body, including in particular large and medium size arteries, lung alveoli, skin and intervertebral discs. It yields under stress but stores energy upon stretching (Baldwin et al. 2013; Green et al. 2014). Polymorphic and soluble tropoelastin monomers is produced and secreted by smooth muscles cells in arteries and by fibroblasts. Around 65 kD size, glycine-, proline-, valine- and alanine-rich tropoelastin monomers are rapidly close to their cellular source covalently cross-linked by lysine oxidase to form elastic di-, tri- or tetrafunctional crosslinks, e.g. desmosine, isodesmosine. This leads to the formation of irregularly organized and randomly coiled amorphous and yellowish elastin-rich networks and sheets. In these structures elastin is surrounded by fibulin and fibrillin sheaths and a pathogenic mutation of fibrillin-1 is linked to Marfan syndrome with e.g. risk for dissection of the aorta.

Heterodimeric laminins glycoporteins form one of the two major non-collagenous networks in the basement membranes (Hohenester and Yurchenco 2013). Laminins affect tissue morphogenesis, maintenance and function by influencing proliferation, migration and differentiation (Jones et al. 2000; Halper and Kjaer 2014). The laminin network is via entacin or nidogen linked to the other major basement membrane network, which is composed of type IV collagen. Laminins (Aumailley 2013) are composed of five different α chains (of which α3 chain has two variants), three different β chains and three different γ chains, which according to currently known combinatorial rules can combine to 17 different trimeric laminin molecules

Table 1.2 Classification of laminins, their abbreviations according to current nomenclature with some alternative names and the genes encoding them

Laminin (LM)	Abbreviation and alternative names	Genes encoding the laminin chains
LM-α1ß1γ1	LM-111, Ln-1	*LAMA1, LAMB1, LMAC1*
LM-α2β1γ1	LM-211, Ln-2	*LAMA2, LAMB1, LAMC1*
LM-α1β2γ1	LM-121, Ln-3	*LAMA1, LAMB2, LAMC1*
LM-α2β2γ1	LM-221, Ln-4	*LAMA2, LAMB2, LAMC1*
LM-α3Aβ3γ2	LM-332/LM-3A32, Ln-5/5A	*LAMA3A, LAMB3, LAMC2*
LM-α3Bβ3γ2	LM-3B32, Ln-5B	*LAMA3B, LAMB3, LAMC2*
LM-α3Aβ1γ1	LM-311/LM-3A11, Ln-6	*LAMA3A, LAMB1, LAMC1*
LM-α3Aβ2γ1	LM-321/LM-3A21, Ln-7	*LAMA3A, LAMB2, LAMC1*
LM-α4β1γ1	LM-411, Ln-8	*LAMA4, LAMB1, LAMC1*
LM-α4β2γ1	LM-421, Ln-9	*LAMA4, LAMB2, LAMC1*
LM-α5β1γ1	LM-511, Ln-10	*LAMA5, LAMB1, LAMC1*
LM-α5β2γ1	LM-521, Ln-11	*LAMA5, LAMB2, LAMC1*
LM-α2β1γ3	LM-213, Ln-12	*LAMA2, LAMB1, LAMC3*
LM-α3β2γ3	LM-323, Ln-13	*LAMA3, LAMB2, LAMC3*
LM-α4β2γ3	LM-423, Ln-14	*LAMA4, LAMB2, LAMC3*
LM-α5β2γ3	LM-523, Ln-15	*LAMA5,LAMB2, LAMC3*
LM-α5β2γ2	LM-522	*LAMA5, LAMB2, LAMC2*

Modified from Patarroyo et al. (2002), Aumailley et al. (2005), Aumailley (2013), Tzu and Marinkovich (2008), Egles et al. (2007)

Table 1.3 Tissue distribution of laminin alpha, beta and gamma chains

α1	Early embryo, neuroretina, adult kidney proximal tubules, salivary and mammary glands
α2	Trophoblast, foetal skin and kidney, adult skin, skeletal and cardiac muscle, peripheral nerve, some capillaries, brain and other tissues
α3	Foetal skin, lung alveoli and bronchi, adult skin, alveoli, bronchi and most other epithelia
α4	Foetal skin and kidney, skeletal muscle, adult skin, cardiac and visceral smooth muscles, nerves, blood vessel endothelia, bone marrow and other tissues
α5	Foetal skin, lungs and kidney, adult skin, alveoli, bronchi, diverse epithelia, kidney, blood vessels, bone marrow, developing muscles and nerves, synaptic membranes
β1	Most tissues
β2	Foetal bronchi and alveoli, kidney, adult neuromuscular junction, blood vessels, kidney glomeruli
β3	Foetal skin and lungs, adult skin and most other epithelia
γ1	Most tissues
γ2	Foetal skin and lungs, adult skin, bronchi and most other epithelia
γ3	Kidney, lungs, reproductive tract, nerves and brain

Modified from Patarroyo et al. (2002), Tzu and Marinkovich (2008)

(Table 1.2). They have a tightly regulated tissue-specific localizations to be able to contribute to the heterogeneity and site-specific regulation of cells and tissues (Table 1.3). This latter aspect should be emphasized, because in spite of the fact that

the effect of soluble regulatory factors can by accident diffuse over and beyond their physiological limits to cause pathology, whereas solid regulatory molecules are from this point of view more site specific and safe.

One important in tissue regeneration adhesive fibrous glycoprotein either locally synthesized or precipitated from the circulation is fibronectin (Stoffels et al. 2013). In spite of local fibroblast-mediated fibronectin synthesis, it does not stain or stains only weakly at the base of chronic, non-healing ulcers (Herrick et al. 1992, 1996). This is probably due to rapid proteolytic degradation of newly synthesized extracellular fibronectin matrix in such inflammatory and proteinase-rich environment (Weckroth et al. 1996). Fibronectin, a major ECM protein, regulates non-canonical Wnt signaling during embryogenesis and in muscle regeneration. It modulates canonical Wnt signaling through modulation of β-catenin (Astudillo and Larraín 2014).

Cartilage oligomeric matrix protein (COMP) is a pentamer with 5 collagen binding "arms", found in cartilage, ligaments and tendons. COMP binds to free collagen type II and I molecules facilitating formation of banded fibres. It is not found in mature fibres, except at the tip/end of eventually growing fibres (Das et al. 2015).

1.4.3 Extracellular Matrix: Ground Substance

Ground substance is amorphous gel-like mass largely composed of proteoglycans, which are formed of an organizing protein core on the surface of the cell or in the interstitium, with attached linear hydrophilic glycosaminoglycan (GAG) bipolymers (mucopolysaccharides). GAGs are composed of 50–1.000 repeat disaccharide units and based on the structure of the disaccharide backbone, chemical bonding utilized between the sugar residues and side chain modifications, such as acetylation and sulphation.

GAGs are divided into (1) hyaluronates composed of D-glucuronate + GlcNAc linkage β(1–3), (2) heparin composed of L-iduronate α (1–4) N-sulfo-D-glucosamine-6-sulphate α(1–4) backbone with variable degrees of sulphation of the L-iduronate (2-O position) and/or glucosamine (3-O or 6-O position, in addition, the N-position of the glucosamine can be sulphated, acetylated or unsubstituted, located in mast cell granules, (3) heparan sulphate composed of D-glucuronate β (1–3) N-sulfo-D-glucosamine-6-sulfate β(1–4) with variable degrees of sulphation of the glucoronate (2-O position) and/or N-acetylglucosamine (3-O or 6-O position, in addition the N-position of the glucosamine can be acetylated, sulphated or unsubstituted). It contains fewer N- and O-sulphate groups and more N-acetyl groups than heparin, but it is heterogenous as it also contains heparin-like segments, found, *e.g.* in cell surface proteins, lung, basement membranes, heparin or heparin sulphate are found in extracellular perlecan (can alternatively contain chondroitin sulphate), cell surface syndecans and glypicans and a small leucin-rich proteoglycan (SLRP) known as prolargin (coded by the *PRELP* gene, standing for proline arginine-rich end leucine-rich repeat protein); SLRPs may in addition to proteoglycans

also contain O-linked oligosaccharides and sulphated tyrosine residues, and one member, integrin-binding chondroadherin, only contains O-linked short oligosaccharides, which form only 1 % of its molecular mass, (4) chondroitin sulphate (composed D-glucoronate β(1–3) N-acetyl-D-galactosamine β(1–4) backbone with variable degrees of sulphation of the glucoronate (carbon 2) and/or N-acetyl-D-galactosamine (carbon 4 in chondroitin-4-sulphate and/or carbon 6 in chondroitin-6-sulphate), *e.g.* cartilage, bone, tendons, ligaments, found in large aggregating proteoglycans or hyaluronan-binding lecticans (hyalectans), like aggrecan (forming 95 % of the proteoglycans in cartilage, bound to hyaluronan core), versican, neurocan and brevican, and some SLRP which contain either chondroitin and/or dermatan sulphate side chains, as found in decorin/small proteoglycan II (1 chain), biglycan/small proteoglycan I (2 chains) and epiphycan (2 chains) in the epiphysis), (5) dermatan sulphate (differs from chondroitin sulphate by also containing L-iduronate α(1–3) N-acetyl-D-galactosamine-4-sulphate β(1–4) disaccharides in its backbone with variable degrees of sulphation of the iduronate (carbon 2) and/or N-acetyl-D-galactosamine (carbon 4 and/or 6), *e.g.* skin, blood vessels, heart valves) and (6) keratan sulphates composed of D-galactose β(1–4) N-acetyl-D-glucosamine-6-sulphate β(1–3) with variable degrees of sulphation of the galactose (carbon 6) and/or N-acetyl-D-glucosamine (carbon 6), *e.g.* cornea, bone, cartilage, nucleus pul- posus, found in some SLRPs, like lumican, keratocan and mimecan (osteoglycin or osteoinductive factor) in the transparent cornea, integrin-binding osteoadherin (osteomodulin) in mineralized tissues and fibromodulin in the cartilage, all with 1–3 N-linked keratan sulphate chains and sulphated tyrosine residues). The sulphate content is highly variable and its molecular components are occasionally substituted with, *e.g.* fucose or mannose.

At the physiological pH most of their sulphate and carboxyl groups of these long molecules are negatively charged making these molecules viscous, highly charged, able to bind water and elastic. These molecules exert swelling pressure checked by the collagen fibres of the matrix. Proteoglycans occur as cell surface and interstitial molecules and provide a cell-friendly hydrogel-like but permeable surrounding for the cells. Perhaps the best recognized role of proteoglycans relates to their ability to bind and deposit growth factors, like most of the 22 now known fibroblast growth factors (FGFs), some of which bind less avidly and can have systemic, endocrine actions, hepatocyte growth factor (HGF), keratinocyte growth factor (KGF), vascular endothelial growth factors (VEGF) and endostatin derived from degradation of type XVIII collagen with anti-angiogenic properties. Due to local release and paracrine mode of action GAG-deposited growth regulating factors play important roles in tissue repair. Chondroitin sulphate sulphation motif epitopes are useful in the identification of articular cartilage progenitor cells.

Some hundred bottle brush-like aggrecan molecules, the prototype of lecticans, are in cartilage attached to a hyaluronan core via the globular G1 domain of the aggrecan core molecule located at the N-terminus. This binding to hyaluronan is enforced by a HA-binding link protein. G1 is via an interglobular domain combined with another globular domain, G2, followed by a long GAG-binding region, first one keratan sulphate-rich region (able to bind collagen) and then two chondroitin

sulphate-rich regions, and finally a third globular domain G3 (composed of a splice-dependent complement regulatory protein-like module and an epidermal growth factor (EGF)-like modules, and a constant C-type lectin-module) able to mediate binding to fibulin-1 and-2, fibrillin-1 and tenascin-R, but according to its lectin nature to cell surface glycolipids. Several of the G3 domain ligands are di- or oligomers and could therefore mediate cross-linking of the hyaluronan-lectican complexes to each other. Such cross-linking would be impaired upon age-related fragmentation of aggrecan and loss of the cross-linking G3 domains. Early arthritis is characterized by loss of metachromatic proteoglycan staining, which is due to proteolytic solubilisation of aggrecan by ADAMTS4 (a disintegrin and a metalloproteinase with a thrombospondin motif 4) and ADAMTS5, often at the sensitive interglobular domain. Versican is named for its versatile molecular structure, is produced by vascular smooth muscle cells, fibroblasts, keratinocytes and many other cells. In mesenchymal condensations and developing cartilage versican expression precedes aggrecan expression, which is found together with fibulins as in an attempt to organize the early matrix. Neurocan produced by neuronal cells and brevican produced by astrocytes (with the shortest core protein in this family, occurs also in a glycosylphosphatidylinositol-form) are mainly found in the nervous system and participate in glial scar formation and central nervous system repair (Fawcett and Asher 1999).

SLRPs decorin, fibromodulin and lumican reduce collagen fibre thickness of both type I and II collagens. Fibromodulin may also catalyze lateral growth of type I collagen, whereas perlecan with attached chondroitin sulphate can promote fibril logenesis of type II collagen.

Ground substance contains non-core protein bound and non-sulphated sixth GAG known as hyaluronan composed of D-glucoronate β(1–3) N-acetyl-D-glucosamine β(1–4) backbone and found in *e.g.* synovial fluid, articular cartilage, vitrous fluid of the eye ball. It can be 25–25,000 disaccharide units long and imparts high viscosity to hyaluronan containing body fluids. Due to its water binding ability a hyaluronan domain occupies some 1000-fold the volume of its dry state. GAG-derivatized chitosan membranes increased MSC growth rate about fivefold compared to tissue culture plastic or chitosan alone, but in a GAG-type and concentration-dependent manner. Effects of heparin, heparin sulphate, dermatan sulphate and chondroitin-6-sulphate are fibronectin-dependent, but those of hyaluronan and chondroitin-4-sulphate are fibronectin-independent (Uygun et al. 2009).

1.5 Integrin and Non-integrin Matrix Receptors

Integrins are 24 different non-covalently coupled heterodimeric cellular receptors composed of 16 α and 8 β chains, which form an important link in the integration of cellular actin cytoskeleton with the cellular surrounding, including the ECM. They do not only bind cells to matrix, but also allow exploration, migration and outside-in and inside-out signalling, which acts in concert with soluble and cell-cell signals in the regulation of cell behaviour. Discoidin domain receptors, Lutheran Lu/B-CAM

complex and α/β-dystroglycan complex form important collagen, laminin and/or other ligand binding matrix receptors. Lately lectins, glycan binding receptors, in particular extracellular C-, R- and I-type lectins and galectins have been shown to play roles in cell-matrix adhesion and signalling, including MSC recruitment, bone marrow stem cell niche and adult collagen remodelling.

1.5.1 Integrin-Type Matrix Receptors

Integrins form the major class of cellular receptors for ECM ligands (Heino and Käpylä 2009; Heino 2014), so much so that the other matrix receptors are often summed up as non-integrin receptors. Integrin receptors are heterodimers, which are composed of one of the 16 α and 8 β chains, which all cross the cell membrane only once. They combine in a non- covalent fashion along currently known combinatorial rules to altogether 24 different integrin receptors (Fig. 1.1). All integrin receptors are able to bind to at least two ligands, which leads to overlap and redundancy and cover many important components of the ECM. Alternative processing of α and β chains confers further diversity to the integrin receptor system.

The α-chain of the integrin receptor largely determines its ligand binding specificity, whereas the β-chain mainly participates in the assembly of integrins to specialized clusters known as focal adhesions, which mediate external physical stress from extracellular collagen, fibronectin, laminin and other matrix ligands to cellular actin cytoskeleton, i.e. integrate the cell to its matrix. Integrins are often grouped to subfamilies based on their ligand binding specificity, evolutionary relationships and topological restrictions *(e.g.* leukocyte integrins).

The binding force of one individual integrin-matrix ligand pair is minor compared to other more specialized anchoring transmembrane molecules, but the combined avidity of a myriad of integrins can resist considerable mechanical forces. At the same time this arrangement allows the cell to explore and respond to its extracellular matrix, to bind and to let go, i.e. enable dynamic cellular migration along solid substrates in a process known as haptotaxis. Integrins can form new bonds at the advancing edge of the cells, at the same time when integrin-ligand bonds dissociate at the retracting rear.

Integrins are not passive matrix binders but their expression and binding are actively regulated in a bidirectional inside-out and outside-in signalling, which qualitatively and quantitatively regulates integrin-mediated cell-matrix interactions. Binding to ECM delivers signals regulating migration, proliferation, growth, differentiation and apoptosis, often along the same signal transduction pathways which act in concert with various soluble chemotactic, growth and differentiation factors and their receptors. Thus, integrins both bind cell to its surrounding but also help the cell to respond to it. Integrins are not constantly active but their activity is regulated, in part via other integrins.

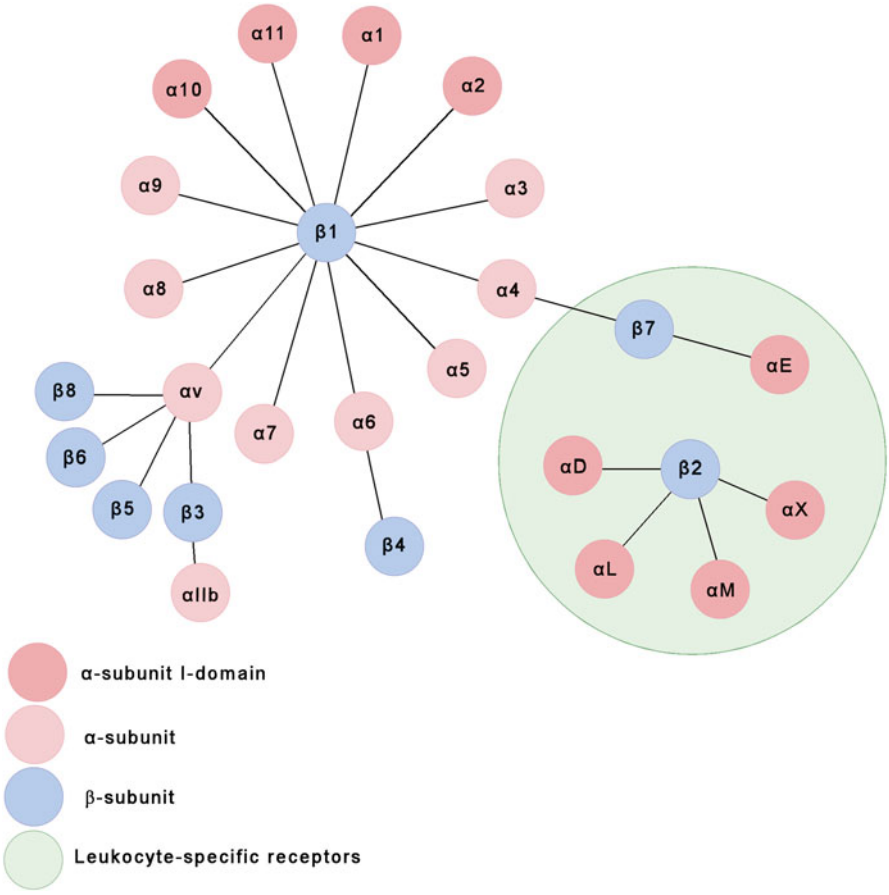

Fig. 1.1 Integrins are heterodimeric receptors composed of one of the 16 known α and 8 β chains. They combine in a non-covalent fashion along currently known combinatorial rules, which are shown in the figure, and which leads to the formation of altogether 24 different integrin receptors

1.5.2 Non-integrin Matrix Receptors

Discoidin domain receptors-1 and -2 (DDR1 and DDR2) mediate in its various isoforms cellular non-integrin binding to collagen and are tyrosine kinase receptors, which regulate cell adhesion, proliferation and ECM. DDR1 has been described in cells in brain, skin, colonic mucosa, kidney tubules, lungs and thyroid gland, whereas DDR2 has been found in heart and skeletal muscle, lung, brain and kidney. Cartilage collagen type II stimulates DDR2 and MMP-13 expression in chondrocytes, which parameters are linked to the severity of osteoarthritis (Sunk et al. 2007).

The Lutheran system Lu/B-CAM comprises Lutheran (Lu) and its alternatively spliced form, basal cell adhesion molecule (B-CAM). They are expressed by red

blood cells, over-expressed in sickle cells, but also expressed by vascular endothelial cells and epithelial cells. In normal cells they are polarized manner and in cancer cells they are over-expressed. They bind laminin α5 containing Lm-511, Lm-521 and Lm-523. Human embryonic stem cells synthesize laminin α1 and α5 chains together with laminin β1 and γ1 chains suggesting that Lms-111 and -511 may be important for the their cell-matrix contacts. Correspondingly, functional adhesions experiments suggested that in particular Lutheran blood group antigen and B-CAM together with Int α3β1 play an essential role for their adhesion to Lm-511, whereas Int α6β1 mediated adhesion to Lm-411 (Vuoristo et al. 2009). Such studies are important because one important role for the non-homologous feeder cell layer may be production of ECM, which is necessary for their interactions with stem cells and for stem cell proliferation and maintenance. It might be possible to culture stem cells without feeder cells and to replace stem cell-feeder cell communication by performing stem cell cultures on appropriate matrix substrate.

Alpha-dystroglycan is extracellular molecule, which binds laminin-α2, agrin and perlecan, whereas the associated transmembranous β-dystroglycan component links the dystroglycan complex intracellularly to dystrophin, which further mediates contact with the actin cytoskeleton. α/β-dystroglycan provides structural integrity and synaptic acetylcholine receptor organization in muscle and other tissues.

Lectins are sugar moiety specific carbohydrate binding non-integrin receptors mediating attachment and aggregation of cells via binding to and cross-linking (at least two sugar binding sites, referred to as carbohydrate-recognition domains) glycoproteins, glycolipids and other glycoconjugates (glycans). Some of them are cell membrane bound. If their glycan ligands locate in the extracellular matrix, they mediate cell-matrix recognition and interactions, but their main task seems to be recognition of various microbial pathogens. Due to their binding specificity, lectin-mediated binding can be regulated by blocking mono- or oligosaccharides, which are useful to study their sugar specificity and have potential as drugs and research tools.

Extracellular lectins include C-type (Ca^{2+}-dependent), R-type (ricin-like), I-type (immunoglobulin domain containing) and galectins (β-galactoside binding), but new extracellular and intracellular lectin families have been recently described. Selectins (endothelial E-, leukocyte L- and platelet and endothelial P-selectins) belong to C-type lectins, which participate in leukocyte recruitment (tethering and rolling). MSCs seem to lack the conventional P-selectin ligands, P-selectin glycoprotein ligand 1 (PSGL-1) and CD24. They may instead express some novel ligand because P-selectin on endothelial cells induces rolling and tethering of circulating MSCs. Chemokines attract and activate MSCs via chemokine receptors to express the very late activation antigen-4 (VLA-4 = Int $\alpha_4\beta_1$ receptor = CD49d/CD29), which firmly adheres the MSC to the vascular cell adhesion molecule-1 (VCAM-1, CD106) on endothelial cell, enabling transmigration to damaged tissues via diapedesis (Fox et al. 2007; Ruster et al. 2006). Selectins may also play a role in the homing to and maintenance of stem cells in the bone marrow stem cell niche. Endo180 on fibroblasts and macrophages, a member of R (ricin-like)-type lectin, contains

fibronectin-like domains, which can mediate binding to *e.g.* collagens I, II, IV and V. It forms a trimolecular complex with urokinase plasminogen activator (uPA) and its receptor (uPAR), but it is not known if its C-type and R-type lectin domains and glycan recognition sites are important in Endo180-mediated cell-matrix adhesion events. Endo180 is also a collagen internalisation receptor, which together with $\alpha_2\beta_1$ integrin receptors mediate specific binding, cellular uptake and delivery of collagens to intracellular, lysosomal degradation. In addition to its major role in the intracellular collagen degradation, endo180 seems to regulate the other major collagenolytic pathway, namely the extracellular and pericellular MMP-dependent collagen degradation pathway (Messaritou et al. 2009). I (Ig-like domain containing)-type lectins contain many members belonging to the siglec group (sialic acid-binding immunoglobulin superfamily lectins) or other I-type lectins. They have been described on various leukocytes, like macrophages, dendritic cells, B cells, neutrophils, eosinophils etc., but one of the best studied I-lectins is NCAM (neural cell adhesion molecule). NCAM can bind heparin/heparin sulphate containing cell surface and matrix proteins and chondroitin sulphate containing neurocan. It can also indirectly bind to collagen via heparin/heparin sulphate bridges (Angata and Brinkman-van der Linden 2002). Galectins (b-galactoside-binding) are expressed on many immune cells and participate in innate and adaptive responses by modulating T-cell apoptosis, proliferation, adhesion, chemotaxis and synthesis of cytokines and other mediators. They are also expressed on keratinocytes (galectin-7), lung (galectin-8) and adipocytes (galectin-12), where they play roles in skin healing, lung cancer and adipogenic signalling/adipocyte differentiation, respectively. Galectin-1 and -3 have been described to modulate cell-matrix interactions (Rabinovich et al. 2002) and galectin-9 to accelerate TGF-β3 induced chondrogenic responses (Arikawa et al. 2009).

Some broad-specificity scavenger receptors of class A, B and C may also bind components of extracellular matrix, *e.g.* CD36 belonging to scavenger receptor type on the surface of platelet can bind it to collagen. Hyaluronan can by bound by hyaluronan cell surface receptor CD44, which has several different isoforms and is found on the surface of chondrocytes and other cells.

1.6 Matrix Modulating Proteinases

Proteinases participate in normal tissue remodelling, but can cause tissue destruction when uncontrolled and excessively active. Proteinases are divided based on their catalytic mechanism to secretory neutral serine and metallo endoproteinases (and amino- and carboxypeptidases) and to mostly intralysosomal acidic cysteine and aspartate endoproteinases. Classification of the proteinases is based on their catalytic mechanisms, which is reflected in the key amino acids necessary for the catalysis. In practice, classification is often based on the use of class specific inhibitors.

1.6.1 Neutral Endoproteinases

Matrix metalloproteinase or MMP family consists of 22 members, subdivided in collagenases, stromelysins and other MMPs (archetypical MMPs); gelatinases; matrilysins; type I and II transmembrane "membrane type MMPs" (MT-MMP), GPI anchored MT-MMPs and secreted MMPs (furin-activatable MMPs).

MMPs have so an extended substrate specificity that they can in practice degrade any protein component of the ECM (Kessenbrock et al. 2015; Klein et al. 2015; Bonnans et al. 2014). MMP activity is regulated at the level of gene transcription (cis-regulatory elements and epigenetic mechanisms), translation (mRNA stability, translational efficiency and probably also micro-RNA-mediated), storage/secretion (*e.g.* pro-MMP8 is stored in neutrophils in the secondary or specific granules), focalization *(e.g.* MT1-MMP/TIMP-2/MMP-2 complexes), activation (of pro-MMP to MMP) and endogenous inhibitors (tissue inhibitor of metalloproteinases, TIMPs). MMPs are subjected to single nucleotide polymorphism, which can modulate their transcriptional efficiency and disease susceptibility. MMPs have a modular structure, which in archetypical MMPs includes a secretory signal sequence (pre-peptide), an activation peptide (pro-peptide), a catalytic Zn^{2+} containing domain, a hinge region and a hemopexin-like domain. In gelatinases the catalytic domain is flanked by a fibronectin-like domain and the MMP structure may contain a furin activation sequence (furin-activatable MMPs), a transmembrane domain (in transmembrane MT-MMPs), a cytoplasmic tail, a glycophosphatidylinositol (GPI) linker (and a GPI anchor), a cysteine array or an immunoglobulin domain, which regulate various aspects of MMP function, such a substrate specificity, activation and membrane-localization. Classification of MMPs is based on their domain arrangement (Fanjul-Fernandez et al. 2010). When neutral pH prevails in ECM only specialized proteinases, first described in the tadpole tail, collagenases, can degrade across the triple helix at ^{775}Gly-^{776}Ile(Leu), which is the specific initial cleavage site. At normal body temperature %- and ^{X}A-degradation fragments formed undergo helix-to-random coil transition to gelatines, which is simply denatured collagen.

MMPs can destroy old or excessive matrix to provide space for cells, such as during vascular invasion, fibroblast or osteoblast migration or chondrocyte alignment. Degradation of cell attachment substrates induces a special form of apoptosis in ECM-dependent mesenchymal cells, so called anoikis, but can also release suppressive effects and stimulate cellular proliferation and differentiation. Due to their effects on non-matrix proteins, such as cell surface molecules or heparin-bound matrix deposited factors and activation of latent pro-proteinase zymogens, MMPs can exert various anti-inflammatory and pro-healing effects. The relatively recently recognized fact that MMPs do not only degrade tissues, but also act as signalling scissors, may explain the failure of more or less generalized MMP-inhibitors (Steinmeyer and Konttinen 2006) in the treatment of tissue destructive diseases, such as cancer growth and metastasis.

As has been learnt from tissue engineering constructs, the pore size and interconnectivity have to be appropriate for the cells to migrate into wound healing scaffolds. With natural scaffolding substances such as fibrin and collagen this does not

pose much of a problem, because the cells are capable to widen proteolytically too tight pores. In contrast, if the matrix is too sparse, cells sense it and produce more matrix to create extracellular substrate for their integrin and non-integrin matrix receptors adequate for adhesion or directed migration. To at least slightly mimic this natural situation tissue engineering scaffolds are often constructed of bioresorbable (biodegradable) materials, which are hydrolyzed and actively degraded to be replaced by natural matrix. Matrix provides solid substrate along which the cells can migrate to assume their optimal positions in the matrix-cell composite in a process known as haptotaxis or contact guidance, guidance of cellular migration via extracellular matrix ligands instead of soluble chemotactic stimuli. This way extracellular matrix can regulated morphogenesis, would healing and vessel growth as well as pathological cancer cells.

Transmigration and invasion of MSCs requires coordinated action of selectins and glycoproteins, chemokines, integrins and adhesion molecules, cellular cytoskeleton and proteinases and their inhibitors, such as MT1-MMP, MMP-2, TIMP-1, TIMP-2 and TIMP-3 (Ries et al. 2007; Steingen et al. 2008).

Serine proteinase form the largest class of mammalian proteinases, which participate in coagulation, fibrinolysis, complement activation, kininogen metabolism and many other cascades as well as tissue remodelling and destruction. Important enzymes in tissue repair are elastase and cathepsin G in neutrophils and monocytes as well as mast cell tryptase and chymase. Neutrophil elastase is synthesized during the promyelocyte stage, stored in the primary or azurophilic granules and released from triggered neutrophils and activated macrophages. It degrades elastin, but also type III and IV collagens, cartilage proteoglycans, fibronectin and laminin. Elastase can activate pro-MMP-3 (pro-stromelysin-1) and degrades TIMPs. Cathepsin G is similarly stored and packaged in serglycin matrix in active form and can degrade matrix, activate some pro-MMPs and degrade TIMPs once released. Also plasminogen activators (tissue type and urokinase type), plasmin, plasma kallikrein are considered to take part in degradation of extracellular matrix (Takagi 1996).

Serine proteinases are inhibited α2 macroglobulin, which utilizes a bait sequence and entrapment, and by specific inhibitors of serine proteinases or serpins. Serpins comprise α_1-antitrypin (α_1-proteinase inhibitor, synthesized mainly in liver, the main inhibitor of elastase), α_1-antichymotrypsin (acute phase reactant, the main inhibitor of cathepsin G), antithrombin III, α_2-antiplasmin, plasminogen activator inhibitors (PAIs) and C1-inhibitor and protease nexins (*e.g.* uPA is inhibited by protease nexin-1). High expression of PAI-1 in MSCs seems to associate with a poor migration capacity (Li et al. 2009).

1.6.2 Acidic Endoproteinases

Cathepsins comprise in man 11 members, cathepsins B, C, F, H, K, L, O, S, W, X and Z. Asparate proteinase family has also many members, including cathepsin D and pepsins, which are produced by the chief cells in the stomach and known for their role in digestion. Acidic proteinases become activated by acid and are active in

phagolysosomes, in Howship's lacunae below the bone resorbing osteoclasts, in the stomach and extracellularly in acidic pH. They participate in the killing and digestion of microbes, ECM and autologous cellular components (autophagy or autophagocytosis). Apart from pH-dependent regulation of activation, cathepsins are inhibited by endogenous cysteine proteinase inhibitors, cystatins, *e.g.* the extracellular cystatin C. An acidic cysteine endoproteinase cathepsin K, the major cathepsin of bone resorbing osteoclasts, can cleave across the collagen triple helix at several sites and may play a role also in the extracellular degradation of matrix, not only in the Howship's resorption lacuna but also around loosening joint implants and other acidic locations (Ma et al. 2006). High levels of cathepsin B (a cysteine endoproteinase) and cathepsin D (an aspartate endoproteinase) are associated with a high migration capacity of MSCs (Li et al. 2009).

1.7 Wound Healing

Wound healing occurs in stages, which comprise haemostasis, inflammation, migration, proliferation and differentiation of fibroblasts and angioblasts, re-epithelialization and scar remodelling. Clot, early, intermediate and mature connective tissue matrices interact with the repair cells via integrin and non-integrin receptors so that chemokinetic, mitogenic and differentiation signals and lytic enzymes can be produces in organized waves following one another. Wound healing provides a good model for the study of regeneration and angiogenesis.

Skin wound healing encompass several stages which include haemostasis via vasoconstriction, adhesion and aggregation of platelets and activation of the external coagulation cascade to form a temporary blood clot and wound matrix filling the tissue defect and attracting blood leukocytes to the wound, and inflammatory protection of the wound site from microbial invasion by neutrophils and monocyte/macrophages, migrating from wound margins along the fibrin- and tenascin-rich temporary scaffold (Badylak 2002; Hodde and Johnson 2007; Agren and Werthen 2007), and removal of necrotic tissue and blood clot in a further proteolytic process, in part orchestrated by lymphocytes via chemokine- and cytokine-mediated mechanisms (Schultz et al. 2005). In addition, these leukocytes produce factors attracting and stimulating migration of fibroblasts to the lesional site via haptotaxis along fibrin, fibronectin and other components of the temporary wound matrix, fibroblast proliferation, fibroblast-mediated synthesis of subepithelial connective tissue dermal matrix or a more permanent wound matrix, and vascular endothelial cell in-growth and angiogenesis, to form so called granulation tissue. Next step is contraction of the open wounds via the action of specialized actin-rich myoepithelial cells, followed by re-epithelialisation by marginal epithelial cells in properly closed (or sutured) wounds and gradual remodelling of the early healing tissue (Clark 1995).

Cells can actively and dynamically assemble and disassemble matrix ligand-integrin receptor attachment areas as platforms to assemble cytoskeletal actin fibres to focal adhesion complexes, which in the subcytolemmal cytoplasm attract and

bind many adapter, linker and signalling molecules. Focal adhesions do not only temporarily anchor the cell to matrix, but they are also used for cellular migration via coordinated, directed and extracellularly guided contractions of the contractile cytoskeletal elements. Such phenomena play a role in migration of macrophages, fibroblasts and vascular endothelial cells to wound area and of epithelial cells from the wound margins to the subepithelial healing tissue to cover it again with an intact epithelium.

Dermal fibroblasts contain several integrins, including $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_v\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$. The collagen receptors ($\alpha_1\beta_1$, $\alpha_2\beta_1$) and the fibronectin receptors ($\alpha_5\beta_1$ $\alpha_3\beta_1$ and α_v-integrins) are expressed in the quiescent state and used for adhesion to matrix. It is not completely clear which b-subunits combine with the a_v to form the functional integrin heterodimers *in vivo*. When wounding occurs, quiescent fibroblasts are activated to migrate into the blood clot along collagen fibers or other molecules that cover or associate with the collagen fibers. There is evidence that in early wound-healing, fibroblast migration may be primarily mediated by fibronectin. Migrating fibroblasts express the primary fibronectin receptor $\alpha_5\beta_1$ and $\alpha_3\beta_1$ and in an experimental study migration was blocked with antibodies against $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins. Cells at the wound margin down-regulate the expression of the collagen-binding α_1 and α_2 integrins and express a_v integrins that can interact with multiple ligands, including fibronectin, vitronectin, fibrin and fibrinogen. It is not clear how these integrins are used for cell migration in vivo. However, there is some evidence that the composition of the ECM is one of the mechanisms that regulate integrin expression during wound-healing (Steffensen et al. 2001; Agren and Werthen 2007).

Unlike post-natal human skin wounds, which can lead to the development of chronic wounds, foetal skin wounds (<24 weeks gestation) and adult oral mucosal wounds heal rapidly without or only with minimal scarring. Therefore, an oral ulcer, which does not spontaneously heal within 2 weeks, has to be considered as oral cancer until shown otherwise. The reason for this effective healing without scar formation is that foetal and probably oral mucosal skin fibroblasts migrate more swiftly, produce and remodel ECM components faster and transform to wound closing myofibroblasts more rapidly than their adult counterparts (Irwin et al. 1998). Further, the composition of the provisional and mature wound matrices produced in foetuses and oral mucosa lesions differs slightly from that produced in adult wounds (Bullard et al. 2003).

Composition of the matrix signals to locally involved cells the current state of the wound healing and guides the clearance of necrotic and damaged tissues and synthesis of matrix components via outside-in signalling (Agren et al. 2000). Generally, ECM is pivotal in wound healing (Xue and Jackson 2015). This ECM-integrin signalling can synergistically utilize signalling pathways, which overlap with those used by soluble growth and differentiation factors and cell-cell signalling. If this well orchestrated cascade of event fails due to obliterating arteriosclerosis or diabetic macro- and microangiopathy, the risk for chronic ulcers increases due to inflammation, fibroblast senescence and uncontrolled proteolysis (Menke et al. 2007). On the other hand, exuberant, nodular and reddish hypertrophic or outright

keloid scarring may lead to contractures and aesthetic problems (Robles and Berg 2007). Growth factor therapies modulating cellular niches in the wound microenvironment are promising, particularly in the proliferative phase of wound healing (Zielins et al. 2015).

Endothelial cells contain at least nine different integrin receptors (Silva et al. 2008). They attach to vascular basement membrane through their laminin, collagen type IV and/or fibronectin binding β_1 integrin receptors, $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$ and $\alpha_9\beta_1$, but have also other specificities. This process is not passive, because various intracellular endothelial events modulate integrin-ligand binding in inside-out signalling. In addition, pericytes contain $\alpha_7\beta_1$ and $\alpha_8\beta_1$.

Especially cultured and sprouting vascular endothelial cells forming vacuoles and lumen *in vivo* express integrin receptors $\alpha_V\beta_3$ (the classical vitronectin receptor), a_vb_5 (vitronectin specific receptor) and up-regulate $\alpha_5\beta_1$ (the classical fibronectin RGD receptor), which bind them also to the provisional basement membrane matrix, which in addition to vitronectin may contain fibrinogen, von Willebrand factor, thrombospondin, fibronectin, thrombospondin or thrombin. Also a_ib_i and, a_2b_i are up-regulated. These integrin receptors as well as VEGFs, angiopoietins, FGF and transforming growth factor-β (TGF-β), are required for endothelial cell activation and angiogenesis because they regulate endothelial cell proliferation, migration, MMP activation and apoptosis (Brooks et al. 1994; Laurens et al. 2009). $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_5\beta_1$, $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrin inhibitors prevent angiogenesis (Nisato et al. 2003; Laurens et al. (2009). Endothelial cells align themselves actively along the matrix fibres, but also modulate it proteolytically to enable spouting towards the centre of the healing wound (Paweletz and Knierim 1989). In contrast, laminin binding integrin receptor α6β1 may promote endothelial cell differentiation and stabilization (Davis and Senger 2005).

Syndecan, a cell surface heparin sulphate proteoglycan, binds endothelial cells to heparin-binding domains of matrix fibrillar collagen, laminin, fibronectin, vitronectin and thrombospondin. It seems that when syndecan-1 comes into contact, probably via lateral interactions, with integrin receptors $\alpha_v\beta_3$ and $\alpha_v\beta_5$ they are clustered and activated (Beauvais et al. 2009). Various isoforms of the hyaluronan receptor CD44 mediate binding to hyaluronan, fibronectin and collagen and may also stimulate angiogenesis. It is expressed on vascular endothelial cells in granulation tissue.

Keratinocytes contain several integrins, including $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_8\beta_1$, $\alpha_9\beta_1$ and $\alpha_v\beta_5$ as well as the hemidesmosomal component $\alpha_6\beta_4$. To enable rapid migration, keratinocytes dissolve their hemidesmosomal complexes. Keratinocytes can then migrate on or through the provisional blood clot matrix or, in dermal wounds, under the clot in contact with dermal type I collagen-rich matrix, which require different and only partly overlapping set of integrins for cellular adhesion/migration, signalling and focalized proteolysis (Steffensen et al. 2001).

Venous, arterial and neuropathic leg ulcers form an increasingly important health problem leading to pain, complicating infections, amputations, and decreased quality of life. One strategy is to fill the persisting tissue defect with matrix biomaterial to push it through this bottleneck and to support fibroblast and endothelial cell adhesion, guided migration and local synthesis of ECM to fill the defect and to pro-

mote healing through the final stage of the natural wound healing process as described above. One biomaterial used for this purpose is Matrigel, a gelatinous basement membrane-like protein substrate composed of laminin and collagen matrix with growth factors deposited in it. Further, such relatively loose matrices exert mechanotranduction by transferring stains from ECM to cells embedded in or on it, which may stimulate matrix synthesis or induce directional sprouting (branching) and angiogenesis. This is an example how integrin and non-integrin matrix receptors modulate cellular behaviour. Another such proteins is amelogenin, an ECM protein that self-assembles into globular micron-size aggregates, which are able to provide provisional matrix for cell attachment and healing of chronic wounds. MSC based therapy is one of a new therapeutic method for non-healing wounds. MSC are self-renewal, of multidirectional differentiation ability and weak immunogenicity (Li et al. 2015).

Healing of other organs' aseptic damage has much in commom with such described in the skin (Thomopoulos et al. 2015; Ninan et al. 2015; Biggs et al. 2015; Klein and Guha 2014; Eming et al. 2014).

1.8 Cartilage Extracellular Matrix

Cartilage is composed of chondrons, which are organized in lines and connected by interterritorial matrix (Zhang 2014; Wilusz et al. 2014; Siebuhr et al. 2014). In bradytrophic, blood vessel-free cartilage tissue ECM plays pivotal structurel and metabolism regulating role (Gao et al. 2014). Hyaline articular cartilage ECM contains a fibrous network of type II collagen embedded in PG and glycoprotein-rich hydrophilic ground substance. Hydrophilic heparan sulphate- and keratan sulphate-rich proteoglycans assemble via link proteins to huge macromolecular complexes organized around a HA core (Allemann et al. 2001). Pericellular matrix contains a collagen type VI-rich basket for chondrocytes, but also contains proteoglycans, fibronectin and structure enforcing type II and IX collagen (Chattopadhyay et al. 2014). Matrix proteoglycans decorin, biglycan and fibromodulin form deposits of various soluble factors, such as TGF-β1, IGFs and BMPs, which upon proteolytic release couple matrix degradation with neosynthesis of cartilage matrix (van der Kraan et al. 2002; Sekiya et al. 2001). Cellular adhesion via integrin ligation regulates the activity of some growth factor receptors. Apart from paracrine factors, chondrogenesis by juvenile chondrocytes seems to acquire autocrine morphogens, which are inhibited by serum-derived growth factors. Articular chondrocytes may lose their capacity to proliferate and maintain cartilage homeostasis at the onset of puberty (Solchaga et al. 2001).

Superficial (tangential), middle (radial) and deep (hypertrophic) zone are separated by a tidemark from the calcified cartilage matrix lying on the bone. Cartilage contains many growth factors, like TGF-β and IGF-1, and chondrocytes released factors like HMGB-1, which together with the integrin and non-integrin mediated matrix contacts regulate the behaviour of the chondrocytes, matrix production and

composition. The superficial zone of hyaline articular cartilage contains tangentially orientated flattened chondrocytes aligned along the tightly packed type II collagen fibre layers, providing resistance to shear forces of the articular gliding pair and protection to the deeper layer of the cartilage. Middle zone is composed of obliquely oriented collagen fibre meshes resisting compressive forces and serving as a transition zone between the superficial and deeper layers subjected to compressive forces. Tidemark is the interface between the deep hypertrophic cartilage zone and the underlying calcified cartilage, which tidemark can in osteoarthritic cartilage be multiplied (Alford and Cole 2005) and contains deposits of High Mobility Group Box-1 (HMGB-1) (Heinola et al. 2010). HMGB-1 is a non-histone, DNA-binding protein, which regulates gene transcription, but released into the extracellular space from necrotic, activated or perhaps even apoptotic hypertrophic chondrocytes acts as an endogenous alarmin and a master cytokine.

Against earlier dogma, cartilage contains some mesenchymal stromal cells in its superficial layers. Hyaline cartilage defects criate a strong therapeutic problem because therapeutic options are very limited. Autologous chondrocyte transplantation as placed in bioresorbable natural and synthetic scaffolds are used for tissue engineering applications. Cell-based cartilage therapy has strong molecular bakground (Duan et al. 2015; Bornes et al. 2014). Modern scaffolds and tissue engineering for easier cartilage regeneration are currently developed (Lim et al. 2014; Demoor et al. 2014; Cheng et al. 2014; Iwamoto et al. 2013).

1.8.1 Culture on Different Scaffolds

The aim of cartilage tissue engineering scaffolds is manifold. Optimally they allow cell seeding, they mimic the 3D environment of the ECM, provide preferably temporary and resorbable structural support, an increased surface area-to-volume ratio promoting cellular adhesion, migration and differentiation and integrate with host tissues (Capito and Spector 2003; van Osch et al. 2009).

Cartilage regeneration requires chondrocytes or MSCs able to differentiate to chondrocytes in numbers high enough to enable production and maintenance of hyaline articular cartilage under the demanding physicochemical articular circumstances (De Francesco et al. 2015; Getgood et al. 2009). ECM/scaffold-cell contacts in solid or gel-like scaffolds composed of or containing agarose, alginate, carbon nanotubes, chitosan, chondroitin sulphate, collagens, elastin-like polypeptides, fibrin, gelatine, hyaluronan, polycaprolactone, polylactic acid (PLA), polyglycolic acid (PGA) and polylactide-co-glycolide copolymer (PLGA) are used to support such a development. All these scaffolds can be coated with adsorbed proteins or immobilized functional groups. We know of no studies that have evaluated chondrocyte function on type II collagen sponges spiked with cross-linked chondroitin sulphate and hyaluronate. On the other hand, before mass production and clinical use of any such scaffolds, a balance must be reached between bioactivity/-compatibility and production costs. Apart from its chemical composition, also the shear stress

forces, loading and microarchitecture of the cartilage play a role in mechanotransduction and guidance. Optimal pore size and interconnectivity may at the cellular level be rather similar in different species. Devitalized, porous chicken knee 3D scaffold has been reported to form a good framework for bovine neocartilage formation (Warden et al. 2004). Over 95 % porous artificial collagen sponges, produced from approximately 0.5 wt% collagen solutions, can by lyophilized and physically or chemically crosslinked to obtain 120–200 mm pore size and permissive interconnectivity (Kato et al. 1995). Crosslinking and porosity prevented formation of amorphous hydrogels to which the cells can not migrate (Kato et al. 1995). It is possible to follow expression of chondrocyte genes of aggrecan core protein and collagen type II and accumulation of cartilage matrix in such porous 3D sponges (Glowacki and Mizuno 2007; Yates et al. 2005). Collagen scaffold- autologous chondrocyte tissue engineering constructs improve histological repair over controls of articular cartilage defects in rabbit, sheep and other experimental models (Lu et al. 2001). MSCs seeded in a collagen gel developed first at 12 weeks hyaline-like repair tissue and subchondral bone, but 12 weeks later articular cartilage was thin and incompletely integrated with host tissues (Wakitani et al. 1994). In contrast, in 2D monolayer cultures chondrocytes soon dedifferentiate to fibroblast-like cells, which produce tough fibrous-type interstitial type I collagen-rich matrix rather than hydrated elastic cartilage matrix.

For cell culture foetal bovine serum, rich in various but poorly defined as to its growth factor content, is used to support cellular proliferation and growth *in vitro*. Due to its heterologous nature, this may cause adverse inflammatory or immunological reactions. This has stimulated attempts to develop well defined synthetic cell culture media, which may contain critical growth factors like Transforming Growth Factor-β. Feeder cells could perhaps be used with tissue engineering scaffolds containing appropriate ECM molecules. Reprogramming of already differentiated cells using gene transduction offer a third option to produce and guide stem cells along the desired cell lineage.

When the distance of a cell from the surface of a 3D scaffold increases, diffusion of nutrients and oxygen to cells in the implant centre is impaired. Bioreactors and solvent flow have been used to extend this distance. Perfusion also exposes cells to shear stress and hydrostatic pressure dependent on the flow rates and other conditions applied. Under such dynamic culture conditions both adherent stromal and non-adherent haematopoietic cells are more evenly distributed to the 3D implants and usually display improved viability and matrix deposition compared to static culture conditions (Chia and Wu 2015; Shanbhag et al. 2005; Nichols et al. 2009).

Scaffolds seeded with genetically engineered chondrocytes, transduced with bone morphogenic protein-2, produced at 6 months in a rabbit model hyaline-like repair tissue biochemically and biomechanically similar to native tissue, whereas empty collagen sponges was compressed and adhered to the underlying structures (Wakitani et al. 1994).

Chitosan is a bi-copolymer of glucosamine and N-acetylglucosamine. Its degradation products are non-toxic and can be used in the synthesis of articular cartilage (Guo et al. 2006). Chitosan is cationic and, due to its high charge density in acidic

solutions, forms water-insoluble complexes with a variety of polyanionic substances, including some growth factors. Chitosan/glycerol copolymer hydrogel (BST Cargel, Biosyntech, Quebec, Canada) mixed with blood and injected into a chondral defect following microfracture provided in a rabbit model better results than microfracture alone and results from an ongoing human trial are awaited (Hoemann et al. 2007).

Chondroitin sulphate can be used to enhance growth factor binding capacity/proliferation and biocompatibility/matrix deposition of collagen-based scaffolds (Veilleux and Spector 2005). The major GAG of native cartilage tissue is chondroitin sulphate as clarified above.

Chondrocytes growing in a fibrin scaffold produced IGF-1 and produce type II collagen rich matrix (Fortier et al. 2002).

Hyaluronan is a hydrophilic macromolecular component of the ECM. Chondrocytes use various isoforms of hyaluronan receptor CD44 to attach and read hyaluronan. This stimulates chondrogenesis of MSCs by itself and matrix formation in collagen scaffolds is enhanced by addition of small amounts of hyaluronan. Hyaluronan can be cross-linked by esterification, glutaraldehyde or otherwise to produce hylan implants for cartilage repair, but such modification of the natural molecule impairs its biocompatibility and can lead to formation of degradation products that can cause chondrolysis (Knudson et al. 2000).

We believe that natural 3D scaffold materials, such as collagen, chitosan, chondroitin sulphate and hyaluronan, have an edge over synthetic products because they are better provided with cues involved with cellular adhesion and signalling at the same time when resorption can be designed to lead to reciprocal release of matrix deposited growth factors, to couple scaffold degradation with matrix formation.

Synthetic scaffolds produced of PLA, PGA or PLGA are easy to mould and produce and it is possible to control the speed of their biodegradation (Capito and Spector 2003). Carbon nanotube composites support chondrocyte proliferation and deposition of ECM (Khang et al. 2008). Similarly, collagen fibre-mimicking electrospun polycaprolactone nanofibre scaffolds support MSC-chondrocyte differentiation (Li et al. 2005b).

The future of cartilage repair and restoration is promising (Sakata et al. 2015; Tang and Wang 2015; Goldring and Berenbaum 2015) and the role of ECM and scaffolds (Kon et al. 2015; Seo et al. 2014) in this process is pivotal.

1.9 Bone

Bones (skeleton) provide support and define the shape and form of the body. Relatively rigid bones together with synovial, fibrous, cartilagineous (and bony) articulations and mechanically well placed insertions of ligaments, tendons and joint capsules make locomotion and guidance of it possible. Some parts of the skeleton protect internal organs and bones themselves contain bone marrow with hematopoietic and mesenchymal stromal cells, and most of the calcium and phosphate

mineral deposits of the body. Bone represents one of the most dynamic remodelling tissues of the body (Gruber 2015; Hambli 2014) so that osteoclasts, some mononuclear cells and osteoblasts form temporary bone multicellular units (BMU), which undergo activation-resorption-formation (ARF) cycles. Bone lining cells and lacunar osteocytes cover the outer and inner bone surface and the whole bone is surrounded by periosteum containing a protective outer fibrous and an inner cambian cell layer containing progenitors activated upon fracture and fracture healing. MSCs seeded on scaffolds can be induced to bone to replace various types of bone defects.

According to mode of mineralization, bone is divided into intramembranous bone (direct mineralization, *e.g.* the flat bones of the skull) and enchondral bone (formation of a cartilage model precedes mineralisation of the matrix, *e.g.* the long bones of the extremities). Bone is further divided into compact cortical and spongy trabecular bone. The basic unit of cortical bone is an osteon or a Haversian system, which is 0.2 mm in diameter and typically several millimetres long. It is composed of concentric bone lamellae, the interfaces of which are marked by lacunar osteocytes organized into cellular rings interconnected with bone canaliculi around a central Haversian canal, which houses blood vessels.

In addition to bone matrix entrapped osteocytes, bone contains bone matrix forming osteoblasts on the surface of the osteoid (newly forming bone), bone resorbing multinuclear osteoclasts in resorption lacunae and lining cells covering the surface of resting bone (resting osteoblasts). Bone is typically covered by periosteum composed of an outer protective fibrous layer and an inner cambium layer containing osteoblast progenitors.

BMU is composed of a few osteoclasts, which form the cutting cone resorbing bone, followed by a closing cone, in which new bone is formed by osteoblasts on the wall of the cavity formed by the osteoclasts. The transition zone between the cutting and closing cone is covered by relatively poorly defined mononuclear cells, which seem to clean and prepare the bone surface after osteoclast-mediated bone resorption for the attachment and bone synthesis work of the osteoblasts. Osteoclasts resorb the bone only a week or 2, but the subsequent *de novo* bone synthesis in the closing cone takes a few (6–7) months.

BMU nicely reflects different phases in the bone remodelling cycle, referred to as activation-resorption-formation (ARF) cycle. Activation leads to the fusion of osteoclast progenitors belonging to the monocyte/macrophage cell linage to multinuclear osteoclasts, which during the resorption phase lyse bone tissue. This resorption phase is somehow, in part probably via release of matrix bound chemotactic, growth and differentiation molecules coupled to osteoblast-mediated bone formation. In adult human skeleton the loss of bone and the subsequent formation of bone balance each other so that a *status quo* remains. It is not quite known what activates the ARF cycle, but microfractures of old and strained bone, leading to osteocyte death and release of osteocyte factors, may initiate the cycle and in part direct the BMU-mediated ARF activity towards microfractures.

The special feature of bone tissue is that it is a composite consisting of type I collagen-rich organic matrix in which impure 50 nm long, 28 nm wide and 2 nm thick nano-size hydroxyapatite crystals (Cui et al. 2007) have precipitated as bone

mineral during primary (rapid mineralization of newly formed osteoid seams) and secondary (slowly increasing mineralization of already formed bone) mineralization (Rho et al. 1998; Veit et al. 2006).

Bone is an ingenious natural material, a composite which consists of solid cortical and porous trabecular, polymeric and ceramic, and lamellar (a few microns thick) and woven (fibrous) phases (Canty and Kadler 2005) organized from nano- to macroscale. Toughness and visco-elasticity of bone depend on its collagen matrix (Young's modulus 1–2 GPa, an ultimate tensile strength 50–1000 MPa), which also defines the shape and volume of a bone, whereas the hardness and mechanical strength (Young's modulus 130 GPa, an ultimate tensile strength 100 MPa; Park and Lakes 1992) but also brittleness are attributed to hydroxyapatite composed of calcium, phosphorus, hydroxyl ions containing trace amounts of fluoride, sodium, magnesium and other ions (Gray et al. 1995). Non-collagenous proteins (NCP) of bone regulate impregnation of hydroxyapatite crystals in the organic bone collagen matrix framework. High energy fractures cause fractures even in young patients, whereas pathological, low energy fractures occur more commonly in elderly osteoporotic individuals.

In bone growth and fracture healing bone can form *de novo* from MSCs as a result of osteoinduction. This involves several MSC p1 integrin receptors, including collagen receptors $\alpha_1\beta_1$ and $\alpha_2\beta_1$, fibronectin receptor $\alpha_5\beta_1$ and laminin receptor $\alpha_6\beta_1$, but also multi-specific $\alpha_v\beta_3$ and $\alpha_V\beta_5$ receptors, which bind *e.g.* vitronectin and osteopontin (Gronthos et al. 2001). Osteoconduction or bone in-growth, as tissue regeneration in general, can be guided by haptotaxis and physical barriers. Migration of MSCs through tissues can be aided by αvβ3, which can bind and, focalize, MMP-2 to the leading edge of the migrating cells (Karadag and Fisher 2006). A well fixed implant in bone seems at the light microscopic level to be in direct contact with the surrounding bone although high resolution electronmicro- scopic images disclose a thin fibrous interface tissue even in these so called osteointegrated implants (Hutmacher et al. 1998).

According to the law of Wolff, the orientation, density, crosslinking and mineralization of the bone trabeculae adjust according to the local mechanical needs, in which mechanotransduction process interactions between bone cells and bone collagen and NCPs play a role at the sensing and transducing interface (Taylor 2007).

Bone tissue engineering aims to repair/regenerate bone defects, such as congenital, iatrogenic and non-unions (Tevlin et al. 2015). To avoid immunological rejection, usually autologous osteoblasts or MSCs are seeded to biodegradable (more rarely to biostable) but temporarily supporting and void filling porous scaffolds, which hybrids are referred to as tissue engineering constructs (TEC) or products (TEP). Scaffold is in a few milliseconds dynamically coated by soluble plasma/interstitial proteins, which upon maturation is more or less replaced first by provisional and finally by more mature ECM synthesized by the inmigrating scaffold colonizing cells. The natural bone milieu, with its embedded growth and differentiation factors, supports this process. The purpose designed scaffold should retain its strength from a few weeks to several months depending on the purpose of its use.

Gradually during a year or two the bioresorbable scaffold should be hydrolytically degraded to water and carbon dioxide without causing a foreign body reaction. This loss of external implant support protects against stress shielding, bone weakening and pathological peri-implant fractures and allows bone formation and remodelling, which lead to the replacement of the artificial construct with natural living bone (Hutmacher 2000).

Both natural and synthetic materials have been tried in bone tissue engineering, including polymers, such as gelatine, agar, fibrin, collagen, brushite and demineralised bone matrix; synthetic bioresorbable polymers, such as polyglycolic-lactic acid (PGLA), poly-L,D-lactic acid (PLDLA) or polycaprolactone (PCL); and porous ceramics, such as bioglass, hydroxyapatite composites or various other calcium phosphate compounds, or naturally occurring ceramics, such as coral. Composites of natural and synthetic materials, such as collagen – PLA- hydroxyapatite and chitosan – hydroxyapatite composites, have been studied. Just like in chondrogenesis, also the architecture of the scaffold matters and both human MSCs (from various sources) and human embryonic stem cells form bone better in natural 3D than on artefactual 2D scaffolds.

Fracture repair is a sequential process that requires a coordinated action of cells, signalling molecules and extracellular matrices (Tevlin et al. 2015). The sequence of events is initiated by the blood clot (provisional healing fibrin mesh scaffold) and ends up with organized, mature and remodelling lamellar bone. Intermediate steps can include formation of granulation tissue, cartilage (in enchondral bone formation) and woven bone.

1.10 Extracellular Matrix in Heart Regeneration

Ischemic heart disease is a common cause for angina pectoris, myocardial infarction and chronic heart disease, which in cases refractory to medicinal treatment cause a great clinical problem. Endogenous ability of necrotic heart muscle tissue or fibrous heart valvular tissue is poor. Therefore, attempts have been made to treat these patients with cell therapies, guided tissue regeneration and tissue engineering constructs. Many different cell types (MSC, cardiac stem cell, endothelial progenitor cell, embryonic stem cells, multipotent adult stem cells, skeletal myoblasts or hematopoietic stem cell) and processes (transmigration/extravasation, migration and engraftment, angiogenesis, proliferation, apoptosis, stem cell produced paracrine factors, cardiomyogenesis, arrhythmias) have to be mastered in clinical trials before these therapies can be more widely applied. Interestingly, these cell based therapies do not seem to act so much as cell replacement therapies as to act via complicated orchestration of the repair and regeneration.

1.10.1 Integrins in the Cell Cycle Withdrawal of Cardiomyocytes

Integrins are expressed in all cells, including cardiomyocytes. They participate in multiple critical cellular processes as adhesion, extracellular matrix organization, signaling, survival, and proliferation (Israeli-Rosenberg et al. 2014). Integrins have been studied in the developing heart. Integrin P1A chain was present in the proliferating cardiomyocytes but decreased with 30 % after birth. Integrin P1D was found a little later, at the foetal day 18, increased 2 days after birth and remained then constant, which resulted in 1:1 ratio of these integrin chains in the adult heart. Adhesion to matrix via these P1 integrins may drive cardiomyocyte proliferation, but this effect is lost in the neonatal heart and upon P1A to P1D shift. The adult P1D form in particular plays a role in anchorage and transmission of mechanical load to heart tissue during heart beats (Sun et al. 2003). After birth P1 integrin binding partners, the *a* chains of the heterodimeric laminin integrin receptors, are α3 and α6, which are transiently increased at neonatal day two, while three different isoforms of α7 chains, part of the LM-111 receptor, increased gradually to the adult stage. Fibronectin receptor α5β1 did not change during foetal-to-neonatal transition. Because cardiomyocyte proliferation ceases upon terminal differentiation shortly after birth, it is assumed that the above mentioned integrin changes initiate cell cycle withdrawal, inhibit S-phase entry and block the cells in G1 phase (Maitra et al. 2000).

1.10.2 Heart Diseases

Angina pectoris refers to ischemia of the heart muscle, usually caused by stenosis of one or more of the coronary arteries impairing local blood delivery critically upon strenuous physical or mental activities, but it can also be caused by anaemia (universally diminished oxygen delivery) or hypertrophic heart diseases and/or rapid arrhythmias (increased oxygen consumption). Myocardial infarction (MI) upon rupture of the atherosclerotic plaque and subsequent intra-arterial blood clot (thrombosis) formation leads to ischemia, then injury and finally necrosis of cardiac tissue. One of the important clinical consequences of atherosclerotic heart diseases is chronic heart failure leading to dyspnoea, fatigue, swelling, increased jugular vein pressure etc.

MI triggers rapidly a process of repair to maintain the structural integrity of the heart. Signal transduction involves cell-cell signalling, but also soluble factors and ECM-cell signalling. Inflammatory leukocytes invade the injured area, angiogenesis ensues, and fibroblasts start to replicate. This early inflammatory phase of healing results in granulation tissue. In large transmural Q-wave MIs the entire heart may be engaged in the repair, which leads to diffuse and widespread fibrosis and remodelling also at sites remote from the actual infarct area. Postinfarction healing

is almost complete 6–8 weeks following MI, but the infarct scar, which was once considered to be relatively inert, is quite a dynamic remodelling tissue.

During the inflammation phase leukocyte produce pro-inflammatory cytokines, such as TNF-α and IL-1β, which up-regulate local synthesis, secretion and activation of matrix metalloproteinases (*e.g.* MMP-2 and -9). In this early phase, these cytokines play a protective and coordinating role so that anti – IL-1β impairs repair (Hwang et al. 2001). Similarly, glucocorticosteroids leads to poor healing. MCS can stimulate fibroblasts to produce MMP-2, MMP-9 and MT1-MMP, which reduces post-MI fibrosis (Mias et al. 2009). Still remaining cardiomyocytes hypertrophy.

Necrotic tissue is gradually phagocytosed and replaced with a collagen-rich scar (Deten et al. 2002). Diffuse fibrosis develops to enforce the heart and cells are via integrin and non-integrin matrix receptors integrated to this fibrous scaffold. Particularly β1 integrins seem to be associated with anchoring of the myocytes to matrix. Contractile myofibroblasts proliferate and angiogenesis ensue and, to a limited extent, progenitor cells proliferate (Deten et al. 2001; Sun et al. 2003).

MMPs are present in healthy myocardial cells and interstitium, but only in low concentrations and mostly as latent zymogens. They are readily activated within minutes of ischemia by free radicals and proteinases, such as plasmin and other MMPs. The ratio between MMPs and tissue inhibitors of metalloproteinases (TIMPs) shifts in favour for MMPs (Takawale et al. 2015; Creemers et al. 2001).

1.10.3 Cell Therapies

Cell therapies have emerged as a potential new mode of treatment in a variety of cardiac diseases, including acute MI, refractory angina pectoris and chronic heart failure (Patel et al. 2015; Poglajen and Vrtovec 2015; Cao et al. 2015; Menasche 2009). Attention has been drawn to selection of appropriate cells, their delivery via myocardial implantation *vs.* intracoronary or systemic infusion, and creation of a specific extracellular matrix niche to promote engraftment, survival and function of the transplanted cells. Transplantable cells can be embedded in fibrin or peptide nanofibres, which seem to enhance graft retention due to higher viscosity of the injectable graft and improved cellular viability, to create a 3D environment and to improve cellular cohesion, cell-to-cell and cell-to-matrix contacts and signalling. Biomaterial "shell" may protect cells from inflammatory and immune damage inflicted by host neutrophils, monocyte/macrophages, lymphocytes and other cells. ECM of transplanted cells could be supplied with agents promoting to homing, migration, engraftment, proliferation and differentiation of these cells (Fig. 1.2). Repopulation of the damaged zone with contractile or regulatory cells and beneficial modulation of matrix may help to normalize the hemodynamic load on the surviving cardiomyocytes and the potentially deleterious consequences of ventricular remodelling (Penn and Mangi 2008).

Injury and/or repair triggered induction of stem cell homing factors in myocardial tissue lead to homing of bone marrow derived and cardiac stem cells to the

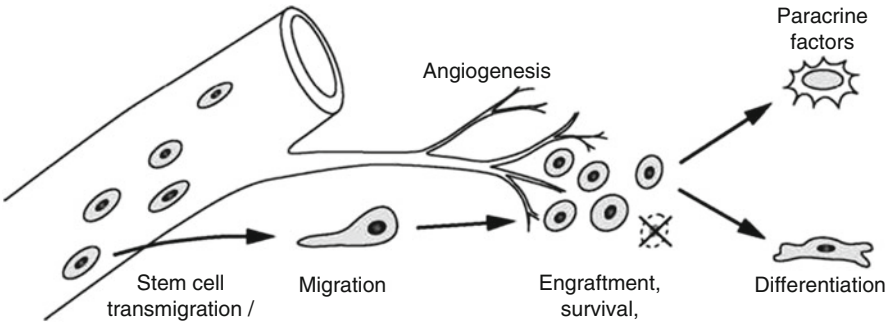

Fig. 1.2 As a result of tissue injury, MSCs have been mobilized from stem cell niches or injected into the circulation. At the site of the injury they transmigrate (extravasate) through the blood vessel wall to tissues or simply flow there with blood. They migrate in the interstitial tissues towards the injured area and engraft, but in spite of a growing distance from the source of oxygen and nutrients, they have to maintain their vitality to proliferate. Locally activated or injected stem and progenitor cells may participate in the process. The mainstream idea is nowadays that the stem cells produce locally factors and effects, which promote tissue repair and healing via paracrine mode of actions on a mix of some local resident or immigrant cells, but some of them may terminally differentiate to replace lost resident cells, according to the older view (Modified from Penn and Mangi 2008)

injured myocardium. Re-expression of laminin-1 (Lm-111) in the adult human heart revokes in part mechanisms, which were operative during organogenesis but are now engaged in heart repair. Lm-111 in the ischemic heart may help to create a niche permissive for epithelial-mesenchymal transition in the adult heart, whereas laminin-2 (Lm-211) seems to be essential for the maintenance of already existing cardiac muscle cells.

Binding of extracellular heart matrix ligands by integrins results in signal transduction across the plasma membrane that regulate cell shape, migration, growth, and survival, a process termed outside-in signalling.

Adhesion molecules and integrins (Wu et al. 2007; Ip et al. 2007) play a role in the mobilization, engraftment and migration of stem cells through injured myocardial tissue and in the modulation of their connective tissue microenvironment (Xiang et al. 2005; Shimazaki et al. 2008; Tamaoki et al. 2005). Several proteinases, which are potential targets for gene based modulation prior to stem cell transplantation, participate in these processes (Xiang et al. 2005). Inhibitors of MMP activation, such as plasminogen activator inhibitor-1 (PAI-1), affect leukocyte infiltration and remodelling of the left ventricle (Askari et al. 2003; Xiang et al. 2004). Up-regulation of PAI-1 decreased tissue-type plasmin (tPA) activity, which consequently diminished leukocyte infiltration and tissue degradation and decreased left ventricular dilation (Askari et al. 2003). Down-regulation of PAI-1 associated with increased tPA activity at the time of acute myocardial infarction increased engraftment of exogenously delivered CD34+ cells in the infarct zone due to enhanced vitronectin-dependent transendothelial migration from the blood stream (Xiang et al. 2004). This increase in stem cell engraftment after MI following PAI-1 inhibition

was recently shown to associate with a decrease in cardiac myocyte apoptosis and an improvement of heart function (Xiang et al. 2005).

Many attempts have been done to produce scaffolds that directly via their composition or indirectly via matrix remodelling enable myocardial grafts (Xiang et al. 2004, 2005). Particular interest has been paid to three factors: tenascin-C (Tamaoki et al. 2005), relaxin (Perna et al. 2005) and periostin (Litvin et al. 2006).

Tenascin-C is a provisional extracellular matrix molecule that is expressed during wound healing in various tissues, including myocardium following acute MI (Imanaka-Yoshida et al. 2001). Tenascin-C, up-regulated by angiotensin II (Nishioka et al. 2007), seems to be profibrotic. Although it is crucial for normal healing, its down-regulation associates with improved long-term outcome. Tenascin-C accelerates fibroblast migration and a-smooth muscle actin expression (myofibroblast formation). Increased serum tenascin-C indicates pathologically increased remodelling (Sato et al. 2006).

Relaxin is a hormone belonging to the relaxin superfamily, which also includes insulin-like peptides. Relaxin liberates NO and causes vasodilatation. Administration of relaxin decreased myonecrosis, cardiac myocyte apoptosis and leukocyte infiltration into the injured myocardium after an experimental MI (Perna et al. 2005). Chronic overexpression of relaxin in C2C12/RLX myoblasts increased local MMP-2, VEGF, vascular density and cardiac function compared to untreated and control animals treated with C2C12/GFP (green fluorescent protein) myoblasts alone (Bani et al. 2009).

Periostin is a secreted ECM protein, which regulates left ventricular remodelling, stem cell engraftment and differentiation in multiple heart diseases (Katsuragi et al. 2004). Periostin is found in cardiac fibroblasts and up-regulated in the ECM of the heart following MI. When periostin was injected into the infarct zone following acute MI, it caused adult cardiomyocytes to re-enter the cell cycle. This was associated with the activation of α_v, β1, β3 and β5 integrins and signalling via phosphatidylinositol-3-OH (PI3K) pathway. This was followed by improved ventricular remodelling and cardiac function, reduced fibrosis and infarct size and increased angiogenesis (Kuhn et al. 2007).

Matrix proteins, matrix receptors or modulators of matrix deposition offer molecular targets that could be genetically engineered or otherwise modulated prior to stem cell infusion or injection. Up-regulation could be achieved directly via introduction of encoding cDNA or indirectly via inducers, *e.g.* periostin.

Improved cellular survival in the inflammatory myocardial environment after MI poses a challenge because typically 90 % of all implanted and/or recruited cells die within a week (Laflamme and Murry 2005). Several principal strategies have been envisioned to improve stem cell intervention results and prognosis in MI, refractory angina pectoris or chronic heart failure. Because different stem cells tested, *e.g.* MSC, cardiac stem cell, endothelial progenitor cell, embryonic stem cells, multipotent adult stem cells, skeletal myoblasts or even hematopoietic stem cell, differ even the molecular details of these potential therapeutic strategies vary (Fig. 1.3; Penn and Mangi 2008). Cell surface receptors form potential targets for genetic engineering prior to stem cell delivery to target the cells correctly and effectively.

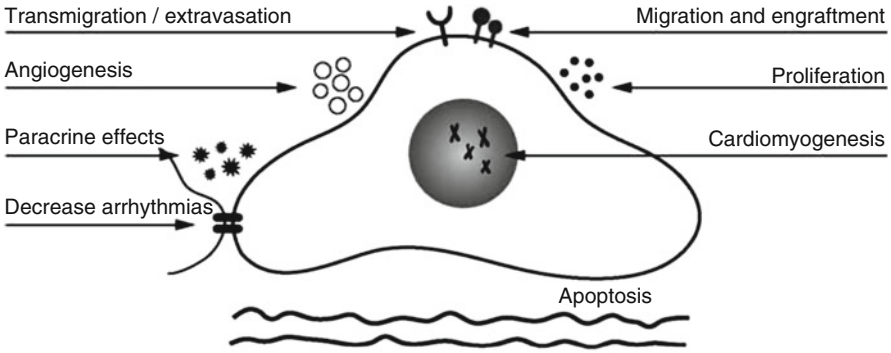

Fig. 1.3 Options to improve the beneficiail effects of mesenchymal stem cell therapies in heart disease include modulation of *1* – Transmigration/extravasation, *2* – migration and engraftment, *3* – angiogenesis, *4* – proliferation, *5* – apoptosis, *6* – stem cell producing paracrine factors (*e.g.* to help recruitment of endogenous resident stem cells), *7* – cardiomyogenesis and *8* – tendency to arrythmias (Modified from Penn and Mangi 2008)

Transmigration/extravasation of circulating stem cells could improve by up- regulation of receptors for stem cell homing factors (CXCR4/receptor for stromalcell derived factor-1). Transient integrin expression (e.g. β1 integrins) in delivered cells could improve stem cell migration and engraftment, whereas appropriate long-term integrin over-expression could alter stem cell differentiation towards the cardiac phenotype (Wu et al. 2006). Endothelial cell nitric oxide synthetase (eNOS)-mediated up-regulation of MMP-9 is estradiol dependent, suggesting a potential explanation for the better prognosis of MI in women (Iwakura et al. 2006). Because persistent ischemia leads to death of the transplanted cells, improved angiogenesis via angiogenic factors (VEGF, FGF-2, angiopoietins, TGF-β etc.) could enhance stem cell survival. Stem cell proliferation could be directly or indirectly be improved via growth and trophic factors (FGF-2, IGF-1, BMP-2 and VEGF) or signal transduces (constitutively active serine/threonine protein kinase P-Akt, which mediates growth factor-associated cell survival signals). To improve ECM-cell survival signals and to prevent anoikis, the composition of the ECM could be modulated as to its tenascin-C (to be decreased), relaxin (to be increased) and periostin (to be increased) content, but stem cells could also be embedded and added in epicardial collagen or laminin-1/Matrigel patches (Menasche 2007; Laflamme 2007) or the stem cell could be subjected anti-apoptotic gene transduction (of *e.g.* B-cell lymphoma 2=Bcl-2). Paracrine factors produced by stem cells can exert important effects, *e.g.* granulocyte-colony stimulating factor (G-CSF), stromal cell-derived factor 1 (SDF-1), monocyte chemoattractant protein-3 (MCP-3) and IL-8 promote are chemotactic and guide migration, VEGF, FGF-2 and HGF stimulate angiogenesis, FGF-2 stimulates proliferation, HGF reduces apoptosis, IL-10, TGF-P1 and HGF exert anti-inflammatory effects and HGF and IGF-1 activate neighbouring resident stem cells. Further, MSCs decrease production of collagen type III, collagen type I and TIMP-1 (Crisostomo et al. 2008) Cardiomyogenic stimuli, like 5-azacytidine, BMP-2,

FGF-4, HGF and transduction with Bcl-2 have tentatively been shown to induce differentiation of MSCs to cardiomyocytes, albeit this may on affect a small proportion of cells (Nesselmann et al. 2008). Finally, heart arrhythmias correlate with connexin protein expression (Mills et al. 2007). MSCs, which express connexins 40, 43 and 45 components of the gap junctions, have a significantly decreased arrhythmogenic potential, whereas skeletal myoblasts with a high arrhythmogenic potential do not express any connexin proteins *in vivo*. Transplantation of myoblasts engineered to express connexin 43 decreased significantly ventricular tachycardias compared to transplantation with control skeletal myoblasts (Roell et al. 2007).

1.11 Heart and Valve Tissue Engineering

All four cardiac valves, aortic, mitral, pulmonary and tricuspidal, are attached to a fibrous supporting skeleton of tendon like tissue via a flexible hinge region composed of loose connective tissue intermingled with some muscle tissue. They have a similar layered and strong architectural structure. Heart valves are covered by endocardium composed of valvular endothelial cells (VEC), which are in contact with blood and which form a thin haemocompatible surface. The connective tissue matrix of the heart valve consists of three layers, which contain valvular interstitial cells (VIC) embedded in it. On the outflow surface is a dense and strong collagen-rich layer, which provides structural strength and stiffness. Collagen fibres withstand high tensile forces when taut, but buckling occurs upon compression. In the central core of the valve is the middle layer composed of GAG-rich, loose and spongy connective tissue absorbing shear and compression forces during the heart cycles. On the inflow surface is a layer rich in elastin, which extends in diastole and recoils in systole. In the aortic and pulmonary valves these three layers are called *fibrosa, spongiosa* and *ventricularis*, respectively. Heart valves are mostly avascular and have relatively poor repair ability although they adapt to their functional requirements as is for example seen in so called Ross operation, when the autologous pulmonary valve is transplanted to replace the aortic valve subjected to high flow and pressure circumstances (and a homograft is used to replace the pulmonary valve).

Healthy heart valves maintain unidirectional blood flow and act as backflow valves via an extraordinarily dynamic functional structure with sufficient strength and durability to withstand acute stresses and chronic fatigue-induced changes. The diastolic pattern of collagen alignment in the plane of the valve tissue is virtually complete already early after valve closure because diastolic collagen realignment occurs when the back pressure increases from 0 to 4 mg Hg during the onset of cardiac diastole. Collagen crimp decreases rapidly as pressure is applied and is nearly completely (90 %) lost at a transvalvular back pressure of 20 mg Hg and only little further rearrangement occurs upon further increase of the pressure (Sacks and Yoganathan 2007).

Most VICs in the healthy valve are quiescent fibroblast-like cells, but they are highly plastic as shown by transition of their activation and matrix remodelling state

in response to mechanical loading, injury or disease. Valvuloplasty or replacement of damaged cardiac valves by bioprosthesis or mechanical prostheses enhances quality of life and is often life saving. Due to the risk of thromboembolic complications mediated via external coagulation cascade and in part integrin-mediated platelet aggregation and associated in particular with abiotic mechanical prosthetic valves, long-term anticoagulation is used in spite of inherent risks for hemorrhagic complications. Therefore, valvuloplasties or bioprostheses, produced for example from glutaraldehyde-fixed porcine atrio-ventricular valves or bovine pericardium, are used even in half or more of valve replacements. Tissue degeneration leads gradually in the course of time to a failure of the bioprosthesis. Cumulative damage and dystrophic cuspal mineralization, initiated already by the devitalized VICs in the installed bioprosthesis, are the major causal mechanisms. Due to chemical fixation with glutaraldehyde the VICs and fibroblasts in the bioprosthesis die. Lack of remodelling capacity leads to irreversible and cumulative valve damage and impaired valve survival.

Attempts have been done to produce tissue-engineered heart valves by culturing autologous cells derived from vascular wall or bone marrow on biodegradable synthetic valve-like polymer scaffolds. An alternative tissue-engineering strategy, called guided tissue regeneration (Mendelson and Schoen 2006; Brody and Pandit 2007) uses an implanted scaffold composed of natural biomaterial or de-cellularized valve designed to attract circulating endothelial and other precursor cells and to provide a suitable environment for their adherence, growth, and differentiation. Attempts to produce engineered heart tissue have been done by mixing cardiac myocytes from neonatal Fischer 344 rats with liquid type I collagen (component of the interstitial stroma), Matrigel (basement membrane-like component), and serum (to enable the normal protein adsorption) containing culture medium (Zimmermann et al. 2002). Collaboration between biologists, engineers and material scientists has been resulted in considerable progress in experimental 3D bioprinting and cardiovascular tissue engineering (Mosadegh et al 2015; Kheradvar et al. 2015; Chester et al. 2014).

Heart tissue engineering as a promissing cardiovascular therapy consider scaffolds, cell sources, signaling factors and ECM physiology (Alrefai et al. 2015; Cao et al. 2015; Jackman et al. 2015).

1.12 Tumour Extracellular Matrix

Cancer cells need to deal with extracellular matrix because they need space at the cost of the host tissue and without respect to normal tissue barriers pass them. At the same time, to be able to spread locally and to be able to form metastases after extravasation from blood or lymphatic vessels they need to be able to use host tissues as a platform for their integrin and non-integrin receptor-mediated motility. They do not only use their own proteolytic machineries but are able to trick the host cells to pave them the way through the interstitial tissues, basement membranes and cellular endothelial or lymphatic endothelial barriers. Generalized MMP inhibitors

have been a failure and the modern view of the proteinases regards them as often spatially strictly regulated signalling scissors, with important regulatory effects based on modulation of chemokines, cytokines, growth factors and their receptors.

In many cancers of epithelial origin epithelial mesenchymal transformation (EMT) is a central event. This term refers to dedifferentiation and detachment of the epithelial cells from the cell collective so that the detached cells become able to invade their immediate pericellular matrix, intravasate into the vascular or lymphatic circulation, only to later extravasate again at secondary metastatic tumour sites to establish a new tumour. This involves primarily cadherin switch from the epithelial E-cadherin to the mesenchymal (and neuronal) N-cadherin, but also integrin-mediated cell-ECM events play a role. Engagement of α1β1 and α2β1 integrin receptors with collagen, typical for many connective tissue-embedded mesenchymal cells, down-regulates E-cadherin (Koenig et al. 2006). If at the same time discoidin domain receptor-1 for collagen is engaged, N-cadherin is simultaneously up-regulated (Shintani et al. 2008). This cadherin switch and epithelial-mesenchymal transformation can via E-cadherin repressor Snail increase expression of αvβ3 integrin known for its presence in activated endothelial cells and for engagement in angiogenesis. This integrin is able to bind and focalize MMP-2 to the advancing edge of the cancer cells (Yilmaz and Christofori 2009), where also MT1-MMP is located. MT1-MMP can further focalize MMP-2 by using TIMP-2 as a bridge in ternary cell membrane-associated complexes. Integrins αvβ3 and αvβ8 bind pre-TGF-P1 (LAP-TGF-P1) released from degrading matrix depots, which TGF-P1 upon local activation further contributes to cancer progression.

Both integrins, growth factor receptors and their ligands and membrane- or integrin-bound MMPs, which tend to act together, can be rapidly regulated via clathrin-mediated endocytosis (CME), raft/caveolar endocytosis (RCE) and macropinocytosis, which can rapidly transfer such factors to strategic locations or remove them to lysosomal degradation or recycle them back to cell membrane to regulate anchorage dependent growth, growth factor sensitivity and effects, and invasion into matrix (Ramsay et al. 2007). The balance between different endocytosis pathways is delicate: engagement of the CME can promote the above mentioned TGF- P1 signalling, whereas endocytosis via RCE guides the same receptor-ligand complex towards lysosomal degradation (Di Guglielmo et al. 2003).

One key concept in modern cancer biology is the concept of cancer stem cells. It has been known that tumor tissues possess their own stem cells with specific niches (Fulawka et al. 2014; Bonnans et al. 2014; Wong and Kumar 2014). Cancers have been treated and prognoses assessed based on the TNM classification, where the size of the tumour (T), growth in the regional lymph nodes (N) and tumour metastases (M) play an important role. In some forms of cancer it has been noticed that most of the tumour cells actually represent relatively well differentiated tumour cells, which to a large extent have lost their “malignant stemness”. These tumour daughter cells cannot easily form metastasis and cannot be used to transfer cancer to cancer free animals. Among this multitude of cells reside a few cancer stem or parent cells, which maintain the tumour and send metastasis as described above. Because the immediate treatment responses and prognosis have been evaluated in

terms of the size of the tumour and its regress upon treatment, drugs with a cytotoxic effect on the relatively "benign" and almost terminally differentiated cancer have been positively selected even though they would have little effect on the cancer stem cells *per se*. This can form a serious bias for the drug screening and selection process between various singular or combination treatments. Drugs with a predominant effect on the relatively benign tumour cells lead to a promising initial response but recurrence occurs upon longer follow up because the cancer stem cells soon produce more cancer cells, increase the tumour size by rapid division and aggressively send metastasis. In contrast, attention should be paid to the control of the cancer stem cells: the initial response would be very modest, but gradually when the more differentiated cancer cells die of apoptosis or ischemic necrosis, the cancer would regress because the tumour cannot now be maintained by asymmetric divisions of the cancer stem cells.

Degradation of ECM is normally tightly controlled because insufficient degradation would prevent normal cell migration and tissue remodelling, whereas excessive degradation would result in loss of attachment and anoikis and pathological destruction of connective tissue. MMPs are usually produced, stored, secreted, focalized (compartmentalized) and finally secreted as latent pro-enzymes, the activation of which is tightly regulated. Pro-MMP activation often involves plasminogen-plasmin conversion, which is regulated by tissue (tPA) and urokinase (uPA) type plasminogen activators as well as plasminogen activator activated via other MMPs, *e.g.* MMP-3 activates pro-MMP-1. Active site zinc can be released via oxygen radicals and some MMPs are activated by furins prior to secretion. Finally, the activity of MMPs is regulated by the availability of and avidity for different substrates and inhibitors and the stability of the enzyme.

To be able to move around, cells focus integrin receptors and MMPs at the leading edge of the cell in broad and flat actin-organized lamellipodia, which can send long, stiff and rod-like actin bundle-rich sensory filopodia as sensing cellular organs ahead of the main front. Much of currently available information on the motility of the cells has been obtained from 2D cell cultures, but *in vivo* cells usually reside in a 3D surrounding and produce instead podosomes or, in case of cancer cells, somewhat homologous invadopodia. These cellular extensions combine inte- grin- and non-integrin mediated adhesion to the surrounding matrix and highly organized proteolytic machinery with an actin-rich cytoskeletal core as the driving dynamic force. Invadopodia-associated integrin receptors $\alpha 3\beta 1$, $\alpha 5\beta 1$, $\alpha 6\beta 1$, and $\alpha v\beta 3$ are suitable for attachment to laminin, fibronectin and vitronectin containing provisional matrix, focalization of the proteolytic, matrix degrading enzymes and signal transduction promoting the formation of invadopodia.

A key step in the regulation of MMPs is the conversion of the zymogen into the active proteinase. Because MMPs have the ability to act on extracellular matrix proteins, MMPs have been often implicated in tumour progression and metastasis as substances able to break down tissue barriers that otherwise restrain invasion (Coussens et al. 2002; Egeblad and Werb 2002; Jacob and Prekeris 2015). In cancer tissue several cell types, namely resident, inflammatory and tumour cells express

and regulate several different MMPs, which can either promote or restrain tissue destruction (Coussens et al. 2002; Egeblad and Werb 2002; Parks et al. 2004). Consequently, many pharmaceutical companies developed programs to target MMPs in cancer. Several drugs, designed to directly block the catalytic activity of MMPs, were tested even in phase III clinical trials, but none was effective (Coussens et al. 2002). The key shortcoming of the MMP inhibitor trials seems to be that they target the catalytic mechanism and lack MMP specificity (Coussens et al. 2002; Parks et al. 2004). They block the activity of all now known 24 MMPs, but also the activity of the related ADAMs (a disintegrin and a metalloproteinase) and ADAMTSs (a disintegrin and a metalloproteinase with a thrombospondin domain) as well as that of many other metalloenzymes. This is not an intelligent strategy because it is likely that the cells use several distinct mechanisms to balance and compartmentalize their metalloenzymes for remodelling, directed movement, pathological tissue destruction, including misguided pavement for invasion and metastasis for cancer cells. Further, MMPs play important roles as signalling scissors, anti-inflammatory and tissue protective roles because they can solubilise cell surface-bound cytokines, receptors, proteinases etc. More insight in the proMMPs activation and focalizaton is needed for selected blocking of the deleterious MMP-mediated processes at the same time when normal remodelling is retained.

1.13 Conclusion

It is concluded that ECM, although produced and degraded by cells, provides an important reference frame for cellular function as to structure and function. In particular, "immaterial" stimuli in form of biomechanical influences are mediated to cells via their matrix. Further, ECM it not a passive partner in cellular life. Part of its importance lies in the fact that the cells that produced it can die but the matrix script still stays after their apoptotic or necrotic death and disappearance from the scene. This is of particular importance in small and continuous scale in tissue remodelling and correction of the script and more drastically in tissue regeneration and particularly in repair when probably totally new textural information is produced: the progenitor and stem cells in particular can find important information from their predecessors and earlier cellular inhabitants of the matrix niche, from this rich source of information and growth factors so that the structures can be born and reborn over and over again, often in different and dynamic production- degradation phases in a process with a certain predetermined direction.

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Chapter 2
Stem Cell Niche

Chenhui Wang, Jun Chen, Pei Wen, Pei Sun, and Rongwen Xi

Abstract The adult stem cells, or tissue-specific stem cells, are essential for maintaining tissue homeostasis and commonly reside in specific local microenvironment named niche. The niche keeps stem cells in multipotent/unipotent state and prevents them from precocious differentiation, and in some cases positions them to undergo asymmetric division to produce differentiated progenies for tissue regeneration. The niches employ a variety of factors including cell adhesion molecules, extra cellular matrix, growth factors and cytokines in a tissue-specific manner to regulate the resident stem cells. Stem cells in turn may also contribute to niche integrity and function. Continuous elucidation of stem cell niche regulation at the cellular and molecular level would help understanding tissue homeostasis and disease mechanisms, and may also provide useful strategies for therapeutic application of stem cells.

Keywords Stem cell niche • Drosophila • Caenorhabditis • Asymmetric cell division • Stromal niche • Epidermal niche

2.1 Introduction

Unlike embryonic stem cells, which possess the innate self-replicating capacity (Ying et al. 2008), the maintenance of most adult stem cells, if not all, requires stimuli from specialized local microenvironment, or niche. Dynamic interactions between niches and stem cells govern tissue homeostasis and repair under physiological and pathological conditions throughout life. Deregulation of the stem cell niches has been implicated in many diseases, including aging, cancer and degenerative diseases (Voog and Jones 2010).

The stem cell niche hypothesis was initially put forward by Schofield, who proposed that the maintenance of stem cells requires association with a complement of cells, a 'niche' (Schofield 1978). However, it was not fully appreciated until studies in the model organisms, *Caenorhabditis elegans* and *Drosophila melanogaster*, demonstrated that the supporting stromal cells are important for the maintenance

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and self-renewal of germline stem cells. Subsequently, as new techniques and tools for characterizing stem cells in vivo are accessible, the stem cell niches are accompanyingly identified and characterized in many mammalian tissues. Because stem cells are usually regulated by both cellular niche cells and non-cellular components, the stem cell niche is currently defined as the local tissue microenvironment that houses and maintains stem cells (Morrison and Spradling 2008).

Studies on both invertebrate and vertebrate stem cell niches in a variety of tissues revealed some principles of their functions. The stem niche controls stem cell self-renewal and prevent their precocious differentiation by secreting signaling molecules or cell-surface ligands, and anchors stem cells in place by utilizing cell adhesion molecules or the extracellular matrix. The niche also frequently positions stem cells in a way facilitating their asymmetric cell divisions, so that after cell division, one daughter will remain in the niche to self-renew, while the other daughter will leave the niche to differentiate. Because of the intimate relationships between stem cells and their niches, mimicking the in vivo microenvironment could also help stem cell with in vitro expansion and functional integration into damaged tissues for future stem cell-based therapies. Thus, a comprehensive understanding of the molecular mechanism underlying the niche function not only contributes to our understanding of tissue homeostasis control and diseases, but also helps to put a step forward for the clinical application of stem cells.

Owing to advantages in simple tissue structure and availability of sophisticated genetic tools, studies in simple model organisms such as *Drosophila melanogaster* have pioneered our understanding of the niche, with clear demonstration of cellular composition and molecular basis of physical interaction and signaling regulation in the stem niches. Although adult stem cells in mammals are usually difficult to identify due to tissue complexity, with the identification of more reliable stem cell markers and endeavors of many researchers, tremendous progresses have also been made for adult stem cell niches in mammals. In the following parts, some examples of the best studied stem cell niches from invertebrates to mammals are introduced, with emphases on the structural composition and molecular functions. Subsequently we summarize the general features of the stem cell niche and discuss future challenges and clinical perspective on the stem cell niche.

2.2 *C.elegans* Germline Stem Cell Niche

The principle of cell-cell interaction in controlling stem cell behavior was first described in the worm gonad in early 1980s. In the *C. elegans* hermaphrodite gonad, there is one somatic cell at the distal end known as the distal tip cell (DTC). Germline stem cells (GSCs) are localized within the mitotic germ cell region close to the DTC tip (Fig. 2.1a). Moving along the distal-proximal axis, germ cells gradually switch from mitosis to meiosis and subsequently develop into functional gametes (Kimble and Crittenden 2007). DTC is crucial for maintaining GSCs, because laser ablation of DTC causes GSC elimination, as GSCs are switched from mitosis to meiosis and

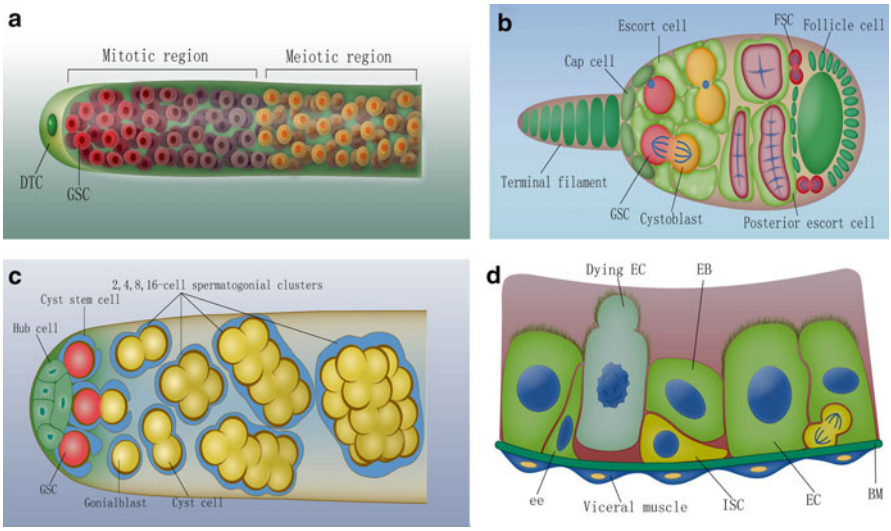

Fig. 2.1 The anatomy of *C. elegans* and *Drosophila* stem cell niches
(**a**) *C. elegans* germline stem cell (*GSC*) niche. GSCs are located in the mitotic region (*red*). The distal tip cell (*DTC*) (*green*) provides both physical support and signaling instructions to maintain GSCs.
(**b**) *Drosophila* ovarian GSC and follicle stem cell (*FSC*) niches. Cap cells together with terminal filament and escort cells constitute the ovarian GSC niche. Cap cells anchor the GSCs by forming adherens junctions, and produce instructive signals to maintain GSCs. Daughter cells of GSCs positioned outside the GSC niche are differentiating cystoblasts. Two FSCs at the mid region of the germarium are responsible for the generation of the follicle cells that encapsulating the developing germline cysts. FSCs are in contact with the neighboring posterior escort cells and underlying basal lamina.
(**c**) *Drosophila* male GSC niche. The male GSC niche is composed of hub cells and cyst stem cells. Similar with the ovarian counterparts, male GSC daughter cells positioned outside the niche become differentiating gonialblasts, which subsequently undergo four rounds of transit amplifying divisions with incomplete cytokinesis, generating 16-cell spermatogonial clusters.
(**d**) *Drosophila* intestinal stem cell (*ISC*) niche. ISCs in the midgut are directly associated with a thin layer of basement membrane. The underlying visceral muscle secretes multiple signaling molecules to regulate ISC maintenance. The dying ECs may produce mitogens to stimulate ISC proliferation in response to various damage. *EB* enteroblast, *EC* enterocyte, *ee* enteroendocrine cell.
Art works in this and subsequent figures are provided by Ning Yang

subsequently differentiate. Also, when the location of male DTC was genetically manipulated, the axis of the gonad was disrupted and ectopic mitotic germ cells were formed around the mislocalized DTC (Kimble and White 1981). These data demonstrate that DTC is both necessary and sufficient for the maintenance of GSCs. Interestingly, the DTC sends short processes to encapsulate distal-most germ cells and long processes extending as many as 30 germ cells (Crittenden et al. 2006), which might provide a unique physical environment to support a pool of stem cells by a single niche cell.

The DTC controls GSC self-renewal via GLP-1/Notch signaling pathway (Crittenden et al. 1994; Henderson et al. 1994). The two DSL ligands LAG-2 and

APX-1 are expressed in the DTC (Nadarajan et al. 2009), while the Notch-like receptor GLP-1 is expressed in germ cells in the mitotic region. Disruption of GLP-1/Notch signaling results in stem cell loss, whereas GLP-1 gain-of-function mutation leads to GSC overproliferation (Austin and Kimble 1987; Berry et al. 1997; Lambie and Kimble 1991). Activation of GLP-1/Notch signaling in GSC leads to the expression of downstream target fbf-2, which in turn represses expression of differentiation-promoting genes including GLD-1,2 and 3 (Byrd and Kimble 2009; Crittenden et al. 2002; Eckmann et al. 2004; Kimble and Crittenden 2007; Suh et al. 2009).

A body of knowledge has been acquired regarding the mechanisms regulating the DTC formation and maintenance. Briefly, the DTC is descended from somatic gonadal progenitor cell (SGP) through asymmetric division. The Wnt/β-catenin asymmetric (WβA) pathway plays central role in DTC specification. Activation of WβA pathway promotes the DTC fate through upregulating the expression of its direct target *ceh-22* (Lam et al. 2006). By contrast, NHR-25 represses the DTC fate by antagonizing WβA pathway (Asahina et al. 2006). In addition, the HLH-2/daughterless transcription factor is implicated in the DTC specification as well as maintenance (Chesney et al. 2009; Karp and Greenwald 2004). Of note, both WβA pathway and *ceh-22* are required and sufficient to specify the DTC fate. Loss of WβA pathway or *ceh-22* results in loss of the DTC, while over-activation of WβA pathway or *ceh-22* produces extra DTCs (Kidd et al. 2005; Lam et al. 2006; Siegfried et al. 2004; Siegfried and Kimble 2002).

2.3 Stem Cell Niches in *Drosophila*

2.3.1 Germline Stem Cell Niche in the Drosophila *Ovary*

The anatomic structure of the *Drosophila* gonad is well defined. The female and male GSCs can be reliably identified in vivo by their localization and by specific cellular markers, and remain accessible to sophisticated genetic manipulations. Consequently, they serve as excellent model systems to study niche regulation of stem cells. In fact, the molecular mechanisms of *Drosophila* GSC-niche regulation are among the best studied and have provided a conceptual framework for the niche study in mammalian systems.

In the Drosophila ovary, GSCs can be identified by their anterior-most location in the germarium and the presence of a unique organelle named spectrosome. In each germarium, five to ten terminal filament (TF) cells, four to six cap cells and GSC-contacting escort cells constitute the female GSC niche that houses two to three GSCs (Fig. 2.1b). Normally, GSCs undergo asymmetric divisions. Upon each division, one daughter remains within the niche and takes the GSC fate, while the other daughter is positioned outside the niche and invariably differentiates into a cystoblast (CB), which will commit four rounds of incomplete mitosis to generate a 16-cell cyst and ultimately a new oocyte.

The cap cells are the principal component of the GSC niche (Xie and Spradling 2000), which anchor GSCs by forming DE-cadherin-mediated adherens junctions between the GSCs and the cap cells (Song et al. 2002). Loss of this adhesion would cause GSCs to leave their niche and differentiate. In addition to the role in physical support, the cap cells also provide signals that are essential for GSC maintenance. They secrete BMP family ligands Dpp and Gbb, which locally activate receptors on GSCs and suppress the expression of a differentiation-promoting gene, *bag of marbles* (*bam*). In cystoblasts, the BMP signaling activity diminishes, which results in the release of *bam* repression and the initiation of differentiation. BMP signaling is required for GSC maintenance, as compromised BMP signaling pathway transduction in GSCs causes their precocious differentiation. Dpp overexpression is also sufficient to stimulate GSC self-renewal and block GSC differentiation, leading to the accumulation of GSC-like cells in the ovariole (Chen and McKearin 2003; Song et al. 2004; Xie and Spradling 1998). GSC-contacting escort cells are also an important component of GSC niche, as blockade of JAK/STAT signaling in escort cells results in loss of GSCs (Decotto and Spradling 2005). In addition, unpaired (Upd) produced from TF cells activates JAK/STAT signaling in cap cells and escort cells, leading to augmented expression of Dpp (Lopez-Onieva et al. 2008; Wang et al. 2008). Therefore, TF cells also contribute to the niche.

Much progress has been made in understanding how niche controlled BMP signaling activity is restricted to GSCs. That has been reviewed somewhere else (Chen et al. 2011; Losick et al. 2011). Briefly, JAK/STAT signaling seems to be necessary and sufficient for dpp expression in cap cells, while Lsd1 inhibits dpp expression in escort cells, as knockdown of Lsd1 in escort cells augments dpp transcription (Eliazer et al. 2011). In addition, the heparin sulfate glycoprotein Dally, and the type IV collagen Viking are required to restrict diffusion of Dpp outside the niche (Guo and Wang 2009; Hayashi et al. 2009; Wang et al. 2008). Moreover, the serine/threonine kinase Fused, together with the E3 ligase Smurf direct the degradation of BMP receptor Thickvein (Tkv) in CBs, allowing for CB differentiation (Xia et al. 2010). Interestingly, escort cells are also essential for restricting BMP signaling to GSCs, as defective function of escort cells causes elevated BMP signaling which in turn leads to defects in germ cell differentiation (Kirilly et al. 2011; Liu et al. 2010a; Wang et al. 2012).

The niche function also requires Yb and Piwi, which are required in the somatic niche cells to maintain GSCs (Cox et al. 1998; King and Lin 1999; Ma et al. 2014). GSCs also send signals to the niche to regulate niche function. Delta, the ligand for the Notch pathway, is specifically expressed in the germ cells, and activates Notch in the niche cells for their specification during the development for their maintenance during adulthood (Song et al. 2007; Ward et al. 2006).

2.3.2 *Follicle Stem Cell Niche in the* Drosophila *Ovary*

In each germarium, two follicle stem cells (FSCs), which generate follicle cells to envelop the developing germ cells, are located near the boundary between the 2A and 2B regions (Nystul and Spradling 2007) (Fig. 2.1b). So far there is no reliable

cellular marker to identify FSCs. It has been suggested multiple signal molecules produced from the TF/cap cells, including Hedgehog(Hh), Wingless(Wg) and Dpp, are all required for the long-term maintenance of FSCs, indicating that these signaling pathways function cooperatively to regulate FSC behavior (Forbes et al. 1996; Kirilly et al. 2005; Song and Xie 2003; Zhang and Kalderon 2001). Therefore, the GSC niche also functions as a part of the niche for FSCs.

Apart from that, FSC-contacting posterior escort cells located near the region 2A/2B border could be an essential component of the FSC niche as well. Escort cells do not turn over regularly and do not move along with cysts at the junction of 2A and 2B region (Morris and Spradling 2011). In addition, E-cad and Armadillo/β-catenin enriched at the junctions between FSCs and its adjacent cells are required for the maintenance of FSCs (Song and Xie 2002), suggesting adherens junctions anchor FSCs to the escort niche cell. Besides, integrin-mediated FSC anchoring to the basal lamina is also required for the long-term maintenance of FSCs (O'Reilly et al. 2008), suggesting that extracellular matrix is a critical component of the FSC niche.

Although it is poorly understood how these extrinsic niche signals act on FSCs to regulate their self-renewal, some intrinsic factors have been identified to be involved in this process. The ATP-dependent remodeling factor Domino(DOM) is required for FSC self-renewal (Xi and Xie 2005), while two polycomb genes Psc and Su(z)2 function redundantly and necessarily in FSCs for their differentiation. Loss of Psc and Su(z)2 ultimately leads to neoplastic tumor (Li et al. 2010). Further studies would provide more profound insights into the fundamental yet intricate mechanisms by which the niche signals link to intrinsic factors for the control of FSC self-renewal.

2.3.3 Germline Stem Cell Niche in the Drosophila *Testis*

The male GSC niche is also well-studied in Drosophila. A cluster of somatic cells (which form a hub) are located at the anterior tip of the testis and serve as the niche for both GSCs and the cyst stem cells (CySCs, or cyst progenitor cells) (Fig. 2.1c). About 8–10 GSCs reside around each hub, and each GSC is encapsulated by two CySCs. After each asymmetric division, a GSC produces one new GSC that remains in contact with the hub and a differentiating daughter namely gonialblast, which is positioned outside the niche and subsequently undergoes four rounds of transit amplifying divisions with incomplete cytokinesis, generating a 16-cell spermatogonial cluster. Spermatogonia further differentiate into spermatocytes which undergo meiosis and ultimately produce sperms. GSCs and gonialblasts contain a spectrosome as their counterparts in the ovary, while differentiated germ cell clusters have a branched fusome. The CySC divides coordinately with GSC division to produce a pair of cyst cells which enclose the differentiating gonialblast.

The activation of JAK/STAT signaling by the hub cells secreted ligand Upd was initially suggested to be necessary and sufficient for both GSCs and CySCs self-renewal (Kiger et al. 2001; Tulina and Matunis 2001). However, intrinsic activation

of JAK/STAT signaling pathway in GSC alone stimulates the expression of DE-cadherin, which mediates GSC adhesion to hub cells, but is not sufficient to promote GSC self-renewal (Leatherman and Dinardo 2010). It turns out that activation of JAK/STAT signaling in CySCs induces the expression of zfh-1, which stimulates the expression of BMP ligands Dpp and Gbb. BMP signaling activation in GSCs represses the transcription of differentiation-promoting factor *bam* and ultimately leads to GSCs self-renewal away from the hub cells (Kawase et al. 2004; Leatherman and Dinardo 2008). Therefore, in addition to the hub, CySCs may also be important components of the male GSC niche.

Like the ovarian counterpart cap cells, hub cells also express BMP ligands Gbb and Dpp. In addition, the male GSC niche also ultilizes ECM to restrict ligand diffusion. Dally-like but not Dally is involved in this process (Hayashi et al. 2009).

The hub is derived from somatic gonadal precursors (SGPs) in the embryonic gonad. Notch and EGFR signaling have been implicated in hub cell specification. Notch signaling promotes hub specification, while EGFR signaling acts antagonistically with Notch to suppress hub differentiation (Kitadate and Kobayashi 2010). Interestingly, CySCs shares a common precursor with hub cells and can contribute to hub replenishment under certain circumstances (Dinardo et al. 2011; Voog et al. 2008). Conversely, the hub cells can be induced to give rise to functional CySCs following genetic ablation of CySCs or depletion of a single transcription factor Escargo, highlighting the dynamic nature of stem cell-niche identities in certain tissues (Hetie et al. 2014; Voog et al. 2014).

Studies in the male GSC niche also provide insights into the mechanisms of spindle orientation for asymmetric division of stem cells. The centrosome is replicated during interphase, and during mitosis, the mitotic spindle is mostly perpendicular to the hub-GSC interface. DE-cadherin could act through membrane-bound β-catenin and adenomatous polyposis coli (APC) to anchor the spindle pole (Yamashita et al. 2003). Interestingly, the mother and daughter centrosomes are asymmetrically inherited after mitosis by the two daughters of a stem cell, as the mother centrosome is always inherited by the daughter retaining stem cell fate (Yamashita et al. 2007).

2.3.4 Intestinal Stem Cell Niche in the Drosophila *Midgut*

The *Drosophila* gastrointestinal tract shows a high similarity to the mammalian intestine in development, cell composition and physiological function. In addition, the *Drosophila* intestinal epithelium is also maintained by multipotent intestinal stem cells (ISCs) (Micchelli and Perrimon 2006; Ohlstein and Spradling 2006). The epithelium is composed of a layer of cells projecting to the gut lumen, with highly organized apical-basal polarity. The ISCs, the only epithelial cells that are competent to undergo mitosis, reside at the basal surface of the epithelium and directly contact with the basement membrane (BM) composed of ECM, which separates the gut epithelium with the surrounding visceral muscles. An ISC undergoes asymmetric

division to produce two daughters with one retaining ISC fate and the other undergoing differentiation. The differentiated daughter, named enteroblast (EB) will differentiate into either an absorptive enterocyte (EC) or a secretory enteroendocrine (ee) cell (Fig. 2.1d). Notch signaling plays a critical role in the cell fate determination of intestinal cell linage (Micchelli and Perrimon 2006; Ohlstein and Spradling 2006). ISCs specifically express a Notch ligand Delta (Dl), which actives Notch in the EBs and promotes them to differentiate into ECs or ee cells. The expression level of Dl in the ISCs is variable from one ISC to another. It is believed that the high Dl level activates Notch at a high level in EB to promote its differentiation towards EC fate, whereas the low Dl level activates Notch at a low level to allow EB to differentiate toward ee fate (Ohlstein and Spradling 2007).

ISCs do not directly contact with any fixed stromal cells. The underlying visceral muscle is proposed to be a major component of the ISC niche. Wingless is the first identified molecule produced by the niche, which is able to traverse through the BM and activates the canonical Wnt signaling pathway in ISCs to regulate their long-term maintenance and proliferation (Lin et al. 2008). The visceral muscle also expresses Unpaired (Lin et al. 2009), the ligand of JAK/STAT pathway, and Vein (Biteau and Jasper 2011; Buchon et al. 2010; Jiang et al. 2010; Xu et al. 2011), the ligand for EGFR, which respectively activate JAK/STAT and EGFR/Ras signaling in ISCs to regulate ISC maintenance and proliferation. Recently, the Drosophila insulin-like peptides, dILP3, was found to be produced by the visceral muscle cells as well, which activates ISCs and expands ISC population to promote adaptive growth of intestine in response to nutrition availability (O'Brien et al. 2011). It is noteworthy that activation of any one of Wingless, JAK/STAT or EGFR signaling pathway alone in ISCs is not sufficient to completely block ISC differentiation (Lee et al. 2009; Lin et al. 2009; Xu et al. 2011). Therefore, the self-renewal of ISCs is likely controlled by a cooperative action of multiple signaling pathways. Several JAK/STAT and EGFR ligands, such as Upd3, Spitz and Karen, could also be detected in epithelial cells, including ISCs, progenitor cells and ECs (Beebe et al. 2009; Biteau and Jasper 2011; Jiang et al. 2010; Jiang et al. 2009; Lin et al. 2009; Liu et al. 2010b; Xu et al. 2011), especially under stress conditions (Buchon et al. 2009; Buchon et al. 2010; Jiang et al. 2010), suggesting that non-stem cells in the intestinal epithelium could also contribute to niche function. The diverse and dynamic expression of those maintenance signals suggest that the niche function can be dynamically regulated in coordinance with environmental changes.

2.4 Stem Cell Niches in Mammals

Increasing evidence suggests that adult stem cells in mammals are also housed and maintained by the niches, although most of the tissue-specific stem cell niches have not been rigorously verified largely due to their associated tissue complexity. In addition to the common scenarios regarding the functional relationships between the stem cells and the stem cell niches, there could be distinct mechanisms uniquely

exploited in mammalian stem cells but not stem cells in invertebrate. For example, the invertebrate stem cells are usually mitotically active. In contrast, the mammalian adult stem cells are often in a relatively quiescent state. In many cases, there seems to be two populations of stem cells with distinct niche locations: quiescent and active stem cells. In the following parts, some examples of the best studied mammalian stem cells and their associated niches are described and discussed, focusing on the physical composition and signaling interactions within the stem cell niches.

2.4.1 The Hematopoietic Stem Cell Niche

As mentioned before, the niche hypothesis was first proposed based on studies on the rodent hematopoietic stem cell (HSC) system several decades ago. Although it is known that the bone marrow (BM) is the primary site of adult HSC maintenance and haematopoiesis, the exact location of the HSCs in the bone marrow (BM) was a mystery and had been the subject of controversies. Until recent years, considerable progress has been made to understand the HSC niche in the BM due to advances in mouse genetics and imaging technologies (Joseph et al. 2013). The current view is that HSCs reside within the perivascular niche partly consisted of mesenchymal stromal cells and endothelial cells (Morrison and Scadden 2014) (Fig. 2.2a).

Before the in vivo HSC niche was characterized, a series of in vitro studies showed that osteoblastic cell lines were capable of supporting primitive hematopoietic cells for a long term in ex vivo culture systems (Taichman et al. 1996). These observations provided an important hint for finding the HSCs niche in the BM. Osteoblastic cells were first demonstrated to participate in HSC regulation in vivo by two simultaneous studies working with different engineered mouse models (Calvi 2003; Zhang 2003). Both cases of genetic manipulation of the mouse models induced an increase in the number of osteoblasts and trabecular bone, and the number of primitive haematopoietic cells (scored as HSCs at that time) increased accompanyingly. Consistently, ablation of osteoblasts by expression thymidine kinase specific in the osteoblasts leads to a decrease of primitive hematopoietic cells in the BM and an increase of extramedullary haematopoiesis (Visnjic et al. 2004). It was suggested that only N-cadherin + osteoblasts are associated with HSCs (Zhang 2003). However, N-cadherin is not required for HSC maintenance as loss of N-cadherin does not lead to HSC depletion or defective hematopoiesis (Kiel et al. 2009). In addition, few HSCs could be observed in contact with osteoblastic cells in live imaging studies using validated stem cell (Kiel 2005; Kiel et al. 2009; Nombela-Arrieta et al. 2013).

There are additional molecules produced by the osteoblasts that have been implicated in the regulation of HSCs, such as Angiopoietin-1, Thrombopoietin, Osteopontin (Opn), and CXCL12 (also called SDF-1). Angiopoietin-1 and Thrombopoietina interact with their receptors (Tie-2 and MP1 respectively) expressed on the HSCs to maintain HSC quiescence (Arai et al. 2004; Yoshihara et al. 2007). Opn, a glycoprotein, negatively regulate HSC proliferation and the size of the HSC pool, perhaps via interaction with integrins and CD44 (Nilsson et al.

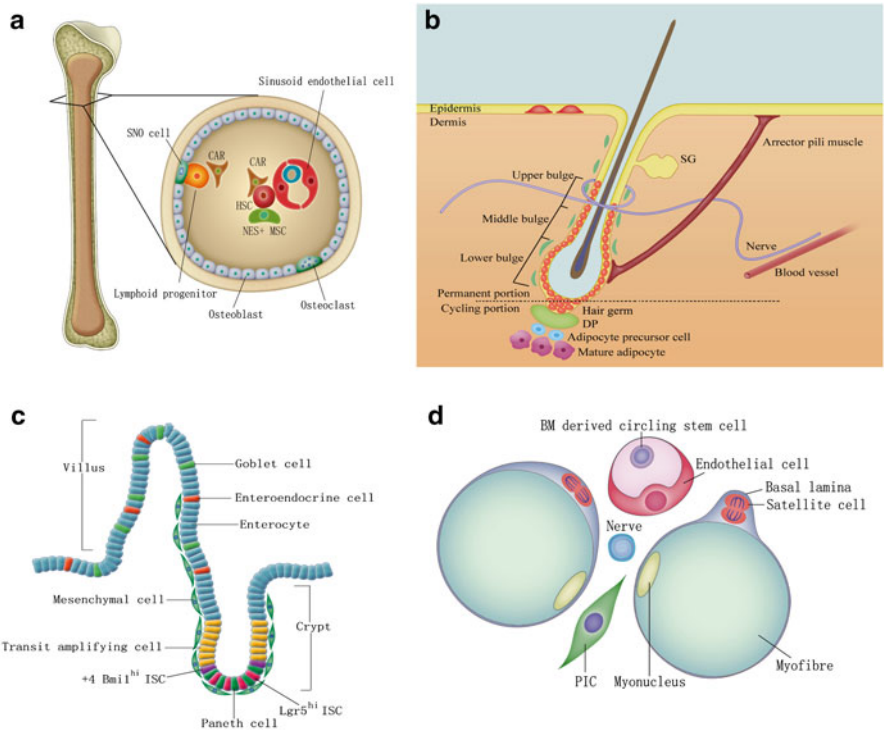

Fig. 2.2 The anatomy of mammalian stem cell niches
(**a**) Hematopoietic stem cell (*HSC*) niche. HSCs in the bone marrow primarily reside adjacent to sinusoids throughout the bone marrow. Sinusoid endothelial cells and mesenchymal stromal cells (*MSCs*) as well as CXCL12-abundent reticular (*CAR*) cells are the major components of HSC niche. Osteoblasts do not directly contribute to HSC maintenance but are crucial for the maintenance of certain lymphoid progenitors.
(**b**) Stem cell niches in skin. A diagram of hair follicle (*HF*) in telogen. In the epidermis, stem/progenitor cells are located in the basal layer and differentiate into suprabasal cells. The basement membrane separates basal layer from the underlying dermis. The HFSCs reside in the bulge region below the sebaceous gland (*SG*). The mesenchymal dermal papilla (*DP*) and adipocyte lineages are crucial for follicle stem cells maintenance and activation. The upper bulge is wrapped by sensory nerve fibers, which release Sonic hedgehog (*Shh*) to induce Gli1 expression in adjacent upper stem cells. The activation of Hh pathway is essential for the upper stem cells to gain the potential to become epidermal stem cells during wound healing.
(**c**) Intestinal stem cell (*ISC*) niche in the small intestine. Bmi1^{hi} ISCs are located at the +4 position from the crypt bottom and contact with paneth cells and transit amplifying cells. Lgr5^{hi} ISCs are located at the crypt bottom and surrounded by paneth cells which form the niche for Lgr5^{hi} ISCs. A hierarchy between Bmi1^{hi} ISCs and Lgr5^{hi} ISCs has been suggested recently.
(**d**) Muscle stem cell niche. Two types of muscle-resident stem cells have been described. Satellite cells are located beneath the basal lamina and are in contact with myofibers. They could undergo planar symmetric divisions and apical-basal asymmetric divisions. The recently identified muscle stem cells – PW1+Pax7- interstial cells (*PICs*) are located between myofibres. Both PICs and bone marrow-derived cells are able to generate functional satellite cells during regeneration

2005; Stier et al. 2005). CXCL12, a chemokine that activates the receptor CXCR4 in HSCs, is also important for HSC quiescence and maintenance in the BM (Nie et al. 2008; Sugiyama et al. 2006). CXCL12 is also expressed in other non-osteoblast cells, including endothelial cells, and a subset of reticular cells scattered in the BM (Sugiyama et al. 2006). Systemic analysis of the role of CXCL12 produced from each individual cell types in regulating HSCs shows that deletion of Cxcl12 from osteoblasts does not deplete HSCs nor mobilizes these cells into circulation. Instead, only certain early lymphoid progenitors are depleted (Ding and Morrison 2013). Therefore osteoblastic cells may contribute to the maintenance of lymphoid progenitors. The Wnt signaling may also regulate HSC quiescence, as osteoblast-specific overexpression of the canonical Wnt inhibitor Dikkopf1 (Dkk1) results in HSC activation (Fleming et al. 2008), although the requirement of Wnt signaling has not been directly demonstrated.

Increasing evidence indicates that the vasculature in the BM may also serve as the HSC niche. Multiple cell types have been reported to make up the HSC vascular niche. A simple combination of three SLAM family receptors is found to be able to specifically distinguish the stem and progenitor cells and thus make it possible to detect the HSC niche in tissue section (Kiel et al. 2005). With the help of these new markers, many of the hematopoietic stem/progenitor cells (HSPCs) were found to be mainly located in the perivascular region. Consistently, an in vivo imaging study revealed that after transplantation, the labeled primitive hematopoietic cells could home to SDF-1–rich subdomains of microvessels in the bone marrow, where they persisted and increased in number over time (Sipkins et al. 2005). These studies suggest the perivascular region could serve as the HSC niche. VEGFR2 and VEGFR3 are expressed in sinusoidal endothelial cells (SECs), but not smooth-muscle-invested arterioles or osteoblasts. VEGFR2 is not required for normal HSC homeostasis. However, upon severe myelosuppressive damage, VEGFR2-mediated SEC regeneration is critical for HSC engraftment and reconstitution (Hooper et al. 2009). Besides, conditional deletion of Cxcl12 from endothelial cells could lead to depletion of HSCs, suggesting endothelial cells are an important component of HSC niche (Ding and Morrison 2013).

Recently, a population of nestin-expressing (NES+) mesenchymal stem cells (MSCs), which are exclusively distributed in perivascular region, has been identified to act as an unique niche of bone marrow HSC. NES+ cells are physically associated with HSCs and express multiple HSC maintenance genes including CXCL12 and Angiopoietin-1. In vivo ablation of NES+ MSC cells leads to significant reduction of long term HSCs (LT-HSCs) number (Mendez-Ferrer et al. 2010).

Additionally, CXCL12-abundant reticular (CAR) cells are the major source of CXCL12. And most HSCs near endosteum or the sinusoidal endothelium, if not all, are in contact with CAR cells (Sugiyama et al. 2006). Selective ablation of CAR cells cause reduction of HSCs number by approximately 50 % and HSCs become more quiescent, suggestive of CAR cells as an essential HSC niche component (Omatsu et al. 2010). Both CAR cells and NES+ MSCs are competent to differentiate into adipocytes and osteoblases, suggesting that there may be some overlap between these two cell types.

2.4.2 Skin Stem Cell Niche

The mammalian skin, which is under constant turnover, serves as a physical barrier to protect the body from many environmental stresses such as bacteria infection, water loss and UV-irradiation. The epidermis appendages such as hair follicles, nail, oil and sweat glands endow additional sophisticated functions to the body. The epidermis is comprised of stratified layers of progenitors and differentiated cells, and the stem cells or progenitors are believed to reside in the basal layer above the dermis (Fuchs 2009; Watt 1998) (Fig. 2.2b). Attached to the BM that separates epidermis from dermis, the basal cells can undergo asymmetric division to generate suprabasal spinous cells, which subsequently move upward and became enucleated and finally shed from the body. Notch signaling, p63 and microRNAs are important for the basal-to-suprabasal switch of the progenitor cell (Blanpain and Fuchs 2006; Moriyama et al. 2008; Yi et al. 2008).

The skin with hair can be divided into the following structural units: each with a hair follicle (HF), sebaceous gland (SG) and interfollicular epidermis (IFE). Sequentially down from the SG is the bulge where stem cells reside, outer root sheath, inner root sheath, hair shaft, transit amplifying matrix cells that envelop a group of mesenchymal cells, and dermal papilla (DP) (Fig. 2.2b). The adult HF constantly undergoes rounds of degeneration (catagen), rest (telogen) and growth (anagen), known as hair cycle. HF stem cells (HFSCs) provide the source of proliferation during anagen. In the destructive catagen phase, the matrix cells undergo programmed cell death and bring the DP upward underneath the (secondary) hair germ, the early progenies of bulge stem cells. The DP plays an inductive role in maintaining HFSCs in quiescent state and competent for the next cycle of growth (Blanpain and Fuchs 2006) (Fig. 2.2b). Normally, HFSCs do not contribute to the maintenance of SG and IFE. However, during the repairing process after wounding, they can regenerate the damaged epidermis and SG. HFSCs can be divided into two populations based on their location with the basal lamina: basal and suprabasal populations. These cells differ in their expression signatures, but both populations are able to self-renew in vitro and share the same differentiation potential (Blanpain et al. 2004).

The epithelial-mesenchymal interactions are important to regulate HFSCs (Blanpain and Fuchs 2009). Among the signaling pathways, Wnt and BMP are the most intensively studied. From embryonic HF initiation to adult stem cell self-renewal and differentiation, Wnt signaling plays multiple important roles during these processes. Loss of β-catenin, which complexes with TCF/LEF transcription factors to activate Wnt-response genes, completely blocks HF formation, while overexpression of an activated form leads to de novo HF morphogenesis (Gat et al. 1998; Huelsken et al. 2001). Elegant genetic and mathematical modeling show that Wnt ligands and the inhibitor Dkks pattern the HF spacing by a reaction-diffusion mechanism (Sick et al. 2006). In adult HF, β-catenin nuclear accumulation correlates with the transition from telogen to anagen, indicating the important roles of Wnt signaling in regulating stem cell self-renewal (Lowry et al. 2005). Wnt/beta-catenin

signaling activities are also detected during matrix cell differentiation towards hair shaft (DasGupta and Fuchs 1999), and LEF1 rather than TCF3 in the bulge are required for matrix cell differentiation. Despite these prominent roles, the source of Wnt ligands is difficult to probe, as there are dozens of Wnts in mammals with some expressed in the epithelium, yet others in the mesenchyme (Reddy et al. 2001). The BMP pathway has long been known for its inhibitory effects on HF morphogenesis and adult HFSC proliferation (Blessing et al. 1993; Botchkarev et al. 1999). The mesenchyme produces a balanced level of BMP ligands and the antagonist noggin (Blanpain and Fuchs 2009). Inactivating the BMP receptor BMPR1a in HF epithelium leads to enhanced cycling of HFSCs and impaired differentiation (Kobielak et al. 2007). Other signaling pathways such as hedgehog and Notch are also involved in either regulating HF proliferation or differentiation (Blanpain and Fuchs 2009).

Recently, it has been found that sensory nerves regulate stem cell function in the upper bulge by producing Sonic hedgehog (Shh), which induces expression of Gli1 expression in adjacent stem cells. Gli1+ cells have the potential to become epidermal stem cells during wound healing. And the activity of these cells depends on Shh released from the perineural niche (Brownell et al. 2011). It is also worth additional attention that adipocyte precursor cells positively regulate follicle stem cell activity by producing platelet derived growth factos (PDGFs). Lack of adipocyte precursor cells due to the inhibition of adipogenesis at early developmental phase in Efb1 knockout mice leads to defects in stem cell activation. And injection of WT adipocyte precursor cells into Efb1$^{-/-}$ skin at P21 is able to activate stem cell and rescue hair cycling defects. In addition, adipocyte precursor cells are sufficient to activate follicle stem cells (Festa et al. 2011). Therefore, adipocyte lineage cells are both necessary and sufficient for follicle stem cell activation.

2.4.3 Intestinal Stem Cell Niche

The mammalian intestinal epithelium turns over in every 3–5 days, making it one of the most rapid self-renewing tissues in the adult. In the mouse small intestine, the gut epithelium is organized into numerous crypt/villi units, with the invaginations known as crypts and protrusions termed villi, surrounded by pericryptal fibroblasts and mesenchyme. The intestinal stem cells (ISCs) reside in the crypt and give rise to transit amplifying cells, which move upward and differentiate into absorptive enterocytes, mucos-secreting goblet cells and hormone-secretive enteroendocrine cells in the villi. Upon reaching the tip of villi these cells undergo programmed cell death and are shed into the lumen. The ISCs also generate bactericidal Paneth cells, which are located in the bottom of the crypt (van der Flier and Clevers 2009) (Fig. 2.2c).

Two populations of stem cells have been identified with compelling evidence. Conventional long-term BrdU label retaining assay based on the "immortal strand" hypothesis suggests that ISCs are located just above the paneth cells at the +4 position from the crypt bottom. The polycomb group gene *Bmi1* is found to be specifically expressed in the cells located at the +4 position. Genetic lineage tracing

mediated by Bmi1-CreER demonstrates that the *Bmi*-1 expressing cells can populate the whole epithelium 12 months after tamoxifen induction, further supporting that the *Bmi1*$^+$ cells at the +4 position behave as intestinal stem cells (Sangiorgi and Capecchi 2008). +4 position ISCs can be marked by mouse telomerase reverse transciptase (*mTert*)-GFP as well. Similar lineage tracing mediated by *mTert* -CreER further confirms that cells at +4 position give rise to all differentiated intestinal cell types (Breault 2008; Montgomery 2011).

Similar genetic tracing studies done by Clevers group identify the crypt base columnar (CBC) cells which express a Wnt target gene Lgr5 and are interspersed among the paneth cells as bona fide ISCs. The Lgr5-expressing cells can regenerate the vili-crypt unit within 2 months after induction (Barker 2007). Interestingly, a single isolated Lgr5$^+$ stem cell could regenerate the intact crypt-villus organoid in vitro without the long postulated mesenchymal niche, suggesting that ISCs have an innate and robust self-organizing ability to direct the formation of a functional epithelium (Sato 2009). The identification of CBC as intestinal stem cells is further sustained by lineage tracing studies conducted with *Prominin 1* (Zhu 2009). Most recently, Clevers and colleges have shown that paneth cells constitute the niche for Lgr5$^+$ stem cells. Paneth cells produce multiple niche factors including epidermal growth factor (EGF), WNT3A and Notch ligand Dll4. Co-culture of sorted Lgr5$^+$ cells with paneth cells significantly promote the crypt-villus organoid formation (Sato et al. 2011). Interestingly, neither genetic ablation of paneth cells nor disruption of WNT3A expression from epithelial cells could block intestinal epithelial self-renewal, suggesting the existence of other important niche components apart from paneth cells (Durand et al. 2012; Farin et al. 2012; Kim et al. 2012). The paneth cells are able to regulate the size of the ISC pool by sensing calorie availability via mTORC1 signaling activity (Yilmaz et al. 2012). Notably, Lgr5$^+$ stem cells divide symmetrically in their niche. They undergo "neutral competition" for niche occupation and the loser is expelled from the niche to undergo differentiation (Lopez-Garcia et al. 2010; Snippert et al. 2010).

Until most recently, the relationship between +4 position ISCs and Lgr5$^+$ ISCs was unclear. Interestingly, mTert-expressing ISCs have been reported to be able to give rise to Lgr5$^+$ ISCs, suggestive of a hierarchy between the slow-cycling and fast-cycling ISCs (Montgomery 2011). However, the Lgr5$^+$ ISCs also display significant telomerase activity (Schepers et al. 2011). Therefore it requires reconsideration whether mTert-expressing ISCs overlap with Lgr5$^+$ ISCs. Interestingly, a recent study shows that complete loss of Lgr5$^+$ ISCs by genetic ablation does not perturb the architecture and homeostasis of the intestinal epithelium, suggesting other stem cell pools can compensate for the loss of Lgr5$^+$ ISCs. Lineage tracing studies suggest that *Bmi1*$^+$ ISCs can replenish the fast-cycling Lgr5$^+$ ISCs both under normal condition and after injury (Tian et al. 2011), further supporting the existence of slow-cycling and fast-cycling ISCs, which can be marked by Bmi1 and Lgr5, respectively. The current view is that active Lgr5$^+$ ISCs are responsible for routine epithelial homeostasis, whereas the relatively quiescent +4 cells serve as 'reserve' stem cells for tissue repair after injury (Barker 2014).

Multiple signaling pathways participate in the regulation of the gut homeostasis, including Wnt, BMP, Notch, Hedgehog, EphB and Ras pathways, and each of them have different roles in regulating cell proliferation, differentiation and migration. The Wnt/β-catenin pathway is the major pathway controlling ISC maintenance and self-renewal. High levels of nuclear β-catenin are found in the epithelial cells at the crypt bottom, but not in the epithelial cells in the villus. Disrupting Wnt pathway activity causes crypt loss, indicating that Wnt signaling is essential for ISC maintenance (Korinek et al. 1998). On the other hand, Wnt pathway activation by the loss of APC, a negative regulator of Wnt signaling, produces giant crypts because of hyperproliferation of intestinal progenitor cells (Andreu et al. 2005; Sansom et al. 2004). The source of the active Wnt ligand remains elusive. In situ results show that several Wnts are expressed in the crypt bottom, while several other Wnts are expressed in the mesenchymal cells (Girgenrath et al. 2006). BMP signaling activated by the BMP ligands produced from the mesenchymal cells functions to restrict ISC proliferation and facilitate differentiation, as loss of Bmpr1a or expression of noggin inhibitor in intestine epithelium leads to intestinal polyposis (Haramis et al. 2004; He et al. 2004). Hedgehog signaling inhibits ISC proliferation and promotes their differentiation by inducing the expression of BMP ligands in the mesenchymal cells (Madison et al. 2005; van den Brink et al. 2004). These observations also indicate that the mesenchyme beneath the crypt bottom has important role in regulating ISC behavior and could be an important constitute of the ISC niche.

2.4.4 Muscle Stem Cell Niche

Satellite cells, the best understood muscle-resident stem cells, are believed to be crucial for postnatal skeletal muscle growth and regeneration after injury. They are located between the plasma membrane of muscle fibre and basement membrane surrounding the muscle fibre (Fig. 2.1d). After injury, satellite cells are activated to generate myogenic precursor cells, which undergo transit amplification and differentiation before finally fuse to form multinucleated myofibers. Recent studies demonstrate that satellite cells are heterogeneous populations consisting of slow-cycling stem cells and fast-cycling progenitor cells. Both stem cells and progenitor cells express Pax7, but only progenitor cells express Myf5. $Pax7^{+}$ $Myf5^{-}$ satellite cells can undergo planar divison (usually symmetric) and apical-basal division (usually asymmetric). There is a strong correlation between the fate and location of their daughter cells upon division. The daughter cell attached to basement membrane remains a self-renewing stem cell, and the other daughter positioned away from basement membrane becomes a committed myogenic cell (Kuang et al. 2007).

The host muscle fibre, extracellular matrix, microvasculature and interstitial cells constitute the niche for satellite cells (Kuang et al. 2008). Mice lacking the ECM component Laminin-α2 show defects in muscle growth and regeneration (Miyagoe et al. 1997), indicating a critical role of ECM in satellite cell function. Injured muscles could release HGF to activate the quiescent satellite cells, and the macrophage

could release the TNF ligand TWEAK to promote muscle progenitors regeneration (Girgenrath et al. 2006; Tatsumi et al. 1998). Other growth factors and cytokines such as bFGF, IGF, BNDF, VEGF, PDGF, IL-6 and LIF could also regulate satellite cell proliferation and differentiation (Kuang et al. 2008). The Delta/Notch signaling pathway plays an important role for maintaining muscle stem cells (Conboy and Rando 2002). The ligand Delta-1 enriched in $Pax7^{+}Myf5^{+}$ progenitor cell is assumed to activate Notch signaling to promote self-renewal of the adjoining $Pax7^{+}$ $Myf5^{-}$ stem cell. Blockade of Notch signaling leads to reduced stem cell self-renewal and regeneration ability (Conboy et al. 2003; Kuang et al. 2007). Intriguingly, crosstalk between wnt and Notch signaling via GSK3β has been shown to be involved in the cell fate choices of activated satellite cells. Overactivation of Wnt signaling pathway leads to premature muscle differentiation while its inactivation prevents muscle differentiation. The defects in muscle differentiation caused by enhancement of Notch signaling can be rescued by enhancement of Wnt signaling (Brack et al. 2008).

Emerging evidence suggest that non-satellite cells may contribute to myogenesis in response to injury. Transplanted adult bone marrow-derived cells (BMDC) can be converted to functional satellite cells following irradiation-induced damage (LaBarge and Blau 2002). Recently, a population of $PW1^{+}Pax7^{-}$ interstitial cells (PICs) have been identified to be able to generate satellite cells during regeneration, suggesting a hierarchy between these two muscle stem cell populations (Mitchell et al. 2010). The potential niche for PICs remains to be defined.

2.5 Key Components of the Stem Cell Niche

As described above, niche structure varies greatly from tissues to tissues and in different organisms. In terms of physical composition, some niches are relatively simple, composed of a single type of stromal cell, but some are rather complex, composed of multiple types of stromal cells and also non-cellular components. In terms of the stem cell types they host, some niches specifically host a single type of stem cells, and some rather simultaneously control more than one type of stem cells. However, all of these relatively well-characterized niches share certain common components, which are summarized as the following.

1. Physical support. The residence of stem cells within specific anatomic locations requires particular physical support including association with supportive stromal cells or basement membrane or both. The physical support keeps stem cells from being exposed to detrimental environment and prevents them from undergoing precocious differentiation. On the basis of physical association between stem cells and niches, two general types of niche -stromal niches and epithelial niches have been proposed (see below) (Morrison and Spradling 2008).
2. Secreted signals. The stromal cells in the niche commonly produce secreted signal molecules to directly regulate stem cell maintenance and self-renewal. Some niches require one principal signal for this function, whereas some niches

require the cooperative function of multiple signals. These signaling activities often function to prevent the initiation of differentiation programs, thereby keeping stem cells in the undifferentiated states. The niche signaling also frequently regulates stem cell activity by promoting or inhibiting their division, therefore controls stem cell quiescence and activation.

3. Cell adhesion molecules. Stem cells commonly produce cell adhesion molecules for their anchorage in the niche. Cadherin-mediated cell-to-cell adhesion between the stem cells and the niche cells and integrin-mediated cell-to-ECM adhesion between the stem cells and the basement membrane are two general types of cell adhesion utilized in the stem cell niches. In addition to the role of adhesion molecules in anchoring stem cells, they also participate in regulating stem cell division by anchoring and orientating mitotic spindles and regulating signaling cascades (Marthiens et al. 2010; Xi 2009).

2.6 Classification of Stem Cell Niches: Stromal Versus Epidermal

Based on the comparison of physical structures among these well-characterized stem cell niches in simple organisms, the niche can be categorized into two general types, stromal niche and epidermal niche (Morrison and Spradling 2008), which may also be applicable to the stem cell niches in mammals.

The stromal niche is best exemplified by the GSC niches in *Drosophila*. The stromal niche is constituted of fixed stromal cells. For example, cap cells or hub cells constitute the female and male GSC niches, respectively. In the stromal niche, the stem cells are usually anchored to the niche cells by forming cadherin-mediated adherens junctions. The junctional structure at the stem cell-niche interface may be utilized for spindle pole anchorage for asymmetric stem cell division. In the stromal niche, short range self-renewal signals from the niche cells are critical for stem cell self-renewal, such that stem cells that are out of the niche could not receive self-renewal signals and will commit differentiation. On the other hand, stem cells could also send signals back to the niche cells to maintain their fate and function (Fig. 2.3a).

In the epidermal niche, exemplified by the FSC niche in the *Drosophila* ovary and the ISC niche in the *Drosophila* midgut, stem cells do not directly contact any fixed stromal cells but are constantly associated with the basement membrane composed of ECM. In addition, both stem cells and their differentiating daughter cells are exposed to seemingly similar surrounding environments without apparently distinctive compartalization. Stem cell anchorage and self-renewal mechanisms are different from that utilized in the stromal niche, and may be diverse from one system to another (Fig. 2.3b). In the FSC niche, stem cells are anchored in a fixed location by integrin-mediated cell adhesion between the stem cell and the ECM. Stem cells receive multiple signals produced from a relative distant source at the anterior tip for their self-renewal. There is no evidence for a specific composition of ECM at the

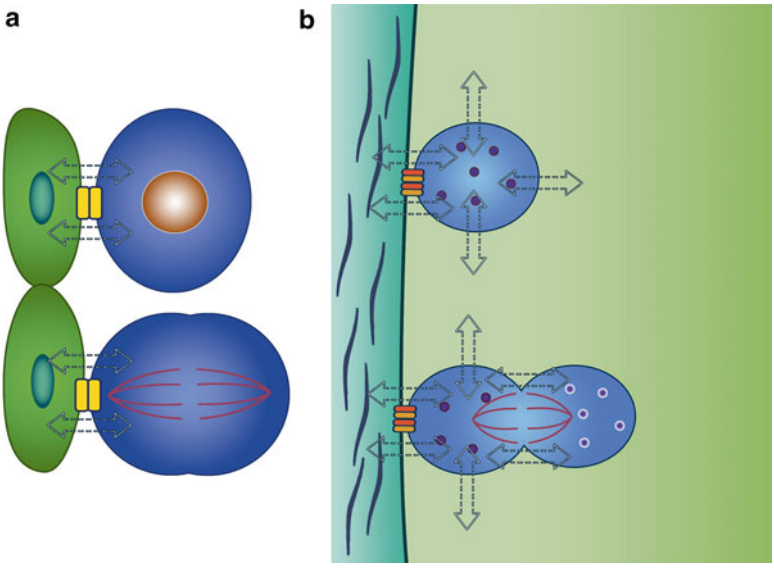

Fig. 2.3 Classification of stem cell niches based on cellular and structural composition. (**a**) A stromal niche. In the stromal niche, stem cells are anchored in the niche cells by forming cadherin-mediated cell-to-cell adhesion between the stem cells and the niche cells. Signaling between the niche cells and the stem cells is critical for stem cell maintenance and self-renewal. (**b**) An epidermal niche. In the epidermal niche, stem cells are anchored in the niche by forming integrin-mediated cell-to-ECM adhesion between the stem cells and the basement membrane. Signaling interactions between the stem cells and the niche environment, including the ECM, the neighboring cells and the immediate daughters may cooperatively regulate stem cell fate or symmetric or asymmetric segregation of cell fate determinants

stem cell location and the location of the FSC is probably controlled by both the levels of self-renewal signaling activity and communications between the stem cells and nearby non-stem cells and ECM. In the single-layered *Drosophila* midgut epithelium, ISCs are lining along the basement membrane that separates the epithelial layer with the muscular niche. The non-stem epithelial cells including enterocytes and enteroendocrine cells are also in direct contact with the basement membrane, and Wingless and Unpaired self-renewal signals are expressed in the muscle cells along the length of the midgut. Thus, it seems that in addition to ISCs, non-stem epithelial cells are also exposed to the niche microenvironment. It is therefore possible that stem cell self-renewal could be controlled by additional mechanisms in addition to the instructive signals from the muscular niche. Delta expressing ISC could direct daughter cell fate by activation of Notch in the differentiating daughter cells, and Delta-Notch mediated lateral inhibition may further reinforce each other's cell fate. Thus, stem cell self-renewal in the epidermal niche is possibly controlled by both the instructive communications between the stem cells and the niche, and the instructive communications between the stem cells and neighboring differentiated cells, including the differentiating daughter cells (Fig. 2.3b).

2.7 Stem Cell Self-Renewal in the Niche: Division Asymmetry Versus Population Asymmetry

As the ultimate defense for tissue homeostasis, stem cells have to accomplish two tasks throughout adult life: one is to generate more stem cells (self-renewal), the other is to produce committed cells (differentiation). And these two tasks must be tightly coordinated. Accumulating data from studies in invertebrates together with vertebrates point out two plausible strategies used by stem cells to interpret how the balance between self-renewal and differentiation is achieved. Stem cells can adopt either division asymmetry or population asymmetry strategy to maintain tissue homeostasis (Morrison and Kimble 2006; Simons and Clevers 2011; Watt and Hogan 2000).

Division asymmetry refers to that each individual stem cell divides to produce two daughters with distinct fates: one remains as a new stem cell and the other commits differentiation. Asymmetric division can be achieved either through asymmetric segregation of cell fate determinants, such as for Drosophila neuroblasts (Knoblich 2008), or through cues from the niche. The well-characterized *Drosophila* GSCs in the ovary and testis use the latter strategy. In this scenario, the highly asymmetric niche architecture directs and facilitates the outcome of stem cell division: the daughter cell remained in the niche will self-renewal, while the daughter cell positioned away from the niche will differentiate.

In population asymmetry, each stem cell gives rise to two daughter cells upon division, the fate of which is unpredictable and depends on the extrinsic input. Some stem cells may be lost through differentiation and some stem cells can expand to replace the lost stem cells. And the replacement rate is comparable to the loss rate. Therefore, the net effect of population asymmetry is the same as division asymmetry. The total number of stem cells remains constant at the level of stem cell population. Stem cells in many mammalian tissues adopt this strategy to achieve homeostasis. For instance, the $Lgr5^{hi}$ ISCs in mouse intestine divide symmetrically to generate two daughter cells, which subsequently undergo "neutral competition" for contact with Paneth cells with the neighboring stem cells. And the loser cells in the competition are squeezed out of the niche to initiate the differentiation program (Lopez-Garcia et al. 2010; Snippert et al. 2010). Besides, the GSCs in mammalian testis and epidermal stem cells in mouse interfollicle epithelium might fall into this category as well.

2.8 Stem Cell Behavior Within the Niche

Studies on the *Drosophila* GSC niche have also revealed several interesting stem cell behaviors that may be important for stem cell long-term maintenance and function, and those phenomena have enriched our understanding of the stem cell niche concept. Here are some examples.

1. Stem cell replacement. It is evident that adult stem cells have limited half-life. They turn over regularly, but the stem cell number within each niche could remain relatively constant. This is probably due to a phenomenon named stem cell replacement. One example is the GSC in the *Drosophila* ovary. When one GSC is depleted from the niche, the other GSCs could undergo symmetric division to supplement the lost GSC (Xie and Spradling 2000). This indicates that the niche has the capability to sustain a stable number of GSCs by controlling symmetric and asymmetric division of GSCs.
2. Stem cell dedifferentiation in the niche. This represents another potentially important mechanism for maintaining constant stem cell number in the niche. When GSCs in the *Drosophila* ovary and testis are forced to differentiate, the early differentiating germ cells could be dedifferentiated into functional GSCs and reoccupy the niche, if they again receive the niche signaling. This reveals the plasticity of progenitor cells and a dominant role of niche in determining stem cell fate (Brawley and Matunis 2004; Kai and Spradling 2004).
3. Stem cell competition. The regular turn-over of stem cells and replacement by the neighboring stem cells may also indicate that these stem cells within the same niche may constantly compete with each other for niche occupation. Studies of GSCs with different genetic background in the same niche have shown that cell adhesion molecules are involved in stem cell competition (Jin et al. 2008). Stem cell competition may be important for the quality control of stem cells, and for coordinating the functions of different types of stem cells that share a single niche (Rhiner et al. 2009). It is also possible that cancer stem cells could potentially make more devastating damages by utilizing this mechanism to hijack the niche and get rid of the normal stem cells.

2.9 Future Perspective

The study of the stem cells and their niches has provided important implications on the relationships between dysregulation of the stem cell niche and human diseases and aging, and may provide useful strategies for clinical applications. Increasing evidence suggests that many cancers are stem cell diseases, in which a rare population of cancer stem cells is responsible for the initiation and recurrence of cancers (Clarke and Fuller 2006). Understanding stem cell self-renewal mechanisms could help to provide novel therapeutic strategy to treat cancers. For example, the CD44 adhesion receptor, which is known to mediate Osteopontin signaling from the niche, could be a therapeutic target of acute myeloid leukemia (AML) cancer stem cells, as administration of CD44 antibody efficiently eliminates leukemia stem cells in the mouse model of human AML (Jin et al. 2006). In addition, abnormalities in the niche, rather than stem cells themselves, may also lead to the development of cancers. For example, increasing evidence suggests that leukemia could be contributed by both cell autonomous abnormalities and dysfunction of the microenvironment in the bone marrow (Lane et al. 2009). Microenvironmental deletion of retinoic acid

gamma receptor (RARγ) or retinoblastoma leads to a phenotype reminiscent of myeloproliferative disease in mouse, which raises the possibility that some leukemia may result from disorder of the microenvironment (Walkley et al. 2007). Therefore, targeting abnormal niche function could be another therapeutic strategy to treat cancers.

Understanding of the stem cell and niche regulation may also lead to improved methods for stem cell manipulation in vivo and in vitro to facilitate replacement therapies in the future. For example, osteoblastic cells, the niche cells for HSCs, can be manipulated by PTH in mouse models of clinical use of HSCs. PTH administration can increase stem cell harvest, protect HSC from chemotherapy and promote HSC function in transplant recipients (Adams et al. 2007).

The ability of adult stem cells to regenerate tissue declines with age and this phenomenon, regarded as stem cell aging, is contributed by the changes in the niche microenvironment, systemic environment and intrinsically within the stem cells, although the contribution of each factor could vary greatly in different tissues and organisms. For example, in the *Drosophila* testis and ovary, the GSC activity declines greatly with age, largely due to the functional decay of niche signaling (Boyle et al. 2007; Pan et al. 2007; Zhao et al. 2008). In mouse satellite stem cell niche, systemic change-induced Wnt signaling activation has been linked to the decline of regeneration potential in aged satellite stem cells (Brack et al. 2007; Carlson et al. 2008). Therefore, modulating stem cell niche function could also be a useful strategy to delay the development of aging and promote tissue regeneration and damage repair.

Aside from these promising clinical prospective, there are still a lot of mysteries about the stem cells and their associated niches. The identification and characterization of these less understood mammalian stem cell niches would be an urgent task. How the extrinsic signals integrate with intrinsic circuitries to maintain the stemness and how stem cell self-renewal and differentiation are precisely balanced only begins to be understood. Again, studies on simpler genetic model systems would certainly continue to pioneer our understanding of stem cells and their niches.

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Chapter 3
Stem Cells and Asymmetric Cell Division

Rita Sousa-Nunes and Frank Hirth

Abstract Asymmetric stem cell division is a widespread process used to generate cellular diversity in developing and adult organisms whilst retaining a steady stem cell pool. When dividing asymmetrically, stem cells self-renew and generate a second cell type, which can be either a differentiating progenitor or a postmitotic cell. Studies in model organisms, most notably the nematode worm *Caenorhabditis elegans*, the fruitfly *Drosophila melanogaster*, and the mouse *Mus musculus*, have identified interrelated mechanisms that regulate asymmetric cell division, from polarity formation and mitotic spindle orientation, to asymmetric segregation of fate determinants and organelles, that impact growth and proliferation. Mechanisms linking extrinsic signals to cellular asymmetry are also beginning to emerge. These cellular processes are mediated by evolutionary conserved molecules, and together equilibrate numbers of progenitor and differentiated cells. Insights into asymmetric division have enhanced our understanding of stem cell biology and of hypo- or hyper-proliferation as a consequence of its disruption, including cancer formation. These insights are of major interest for regenerative medicine, since asymmetrically dividing stem cells provide a powerful source for targeted cell replacement and tissue regeneration.

Keywords Stem cell • Progenitor cell • Neural stem cell • Neuroblast • Cell polarity • Apicobasal polarity • Asymmetric cell division • Self-renewal • Differentiation • Cell fate determinant • Growth • Proliferation • Mitotic spindle orientation • Centrosome • Primary cilium • Midbody • *Drosophila* • *C. elegans* • Mouse • Cell replacement • Tissue regeneration • Cancer

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List of Abbreviations

Ago1	Argonaute protein 1
AurA	Aurora-A
Baz	Bazooka
Brat	Brain tumor
Cdc42	Cell division cycle 42
C. elegans	*Caenorhabditis elegans*
Cnn	Centrosomin
CNS	Central Nervous System
c-Myc	cellular Myelocytomatosis oncogene
aPKC	atypical Protein Kinase C
Dlg	Discs large
EpiSC	epiblast stem cells
ES	embryonic stem (cell)
ECT-2	Epithelial cell transforming gene 2
Flfl	Falafel
Gαi	G-protein alpha, subunit i
Gβ13F	G-protein beta at 13 F (cytological location in *Drosophila* genome)
Gγ1	G-protein gamma 1
GAP	GTPase activating protein
GDI	Guanine dissociation inhibitor
GDP	Guanosine diphosphate
GDPase	Guanosine diphosphatase
GEF	Guanine exchange factor
GMC	ganglion mother cell
GTP	Guanosine-5'-triphosphate
GTPase	Guanosine triphosphatase
Hh	Hedgehog
INP	intermediate neural precursor
Insc	Inscuteable
iPS	induced pluripotent stem (cells)
Jar	Jaguar
Khc-73	Kinesin heavy chain 73
KIF13B	Kinesin Family Member 13B
KLP23	Kinesin-Like Protein 23
Lgl	Lethal (2) giant larvae
LGN	Leucine-Glycine-Asparagine repeats-containing protein (also known as G-protein-signaling modulator 2, GPSM2)
Mira	Miranda
mRNA	messenger Ribonucleic Acid
Mts	Microtubule star
Mud	Mushroom body defect
NHL	NCL-1, HT2A, and LIN-41 (protein domain)

Nin	Ninein
NudE	Nuclear distribution E
NuMA	Nuclear Mitotic Apparatus
PAR	Partitioning defective
Par-3	Partitioning defective 3
Par-6	Partitioning defective 6
Pav	Pavarotti
PCM	pericentriolar material
PCP	planar cell polarity
PDZ	Post synaptic density 95, Discs large, and Zonula occludens-1 domain
Pins	Partner of Inscuteable
Pon	Partner of Numb
PP2A	Protein Phosphatase 2A
Pros	Prospero
Prox1	Prospero homeobox protein 1
RNA	Ribonucleic Acid
SOP	sensory organ precursor (of *Drosophila*)
Sqh	Spaghetti squash
TRIM 3	Tripartite motif protein 3
TRIM 32	Tripartite motif protein 32
Zip	Zipper

3.1 Introduction

Stem cells are characterised by their ability to both self-renew and give rise to distinct cell types. They are present in developing and adult tissues, and the greater the variety of cell types they can generate the greater their potential or "potency" is said to be, ranging from pluripotent (which can form cell types belonging to any of the three germ layers: endoderm, mesoderm and ectoderm), to multipotent, to oligopotent (Smith 2006). Pluripotency of early embryonic cells (eg., mammalian inner cell mass and epiblast cells or fish blastomeres) is transient, after which they generate progressively more lineage-restricted progenitors (Murry and Keller 2008; Slack 2008; Martello and Smith 2014; Morgani and Brickman 2014; Loh et al. 2015; Paranjpe and Veenstra 2015). Notwithstanding, prolonged maintenance of the pluripotent state can be achieved by in vitro culture, as is the case for mammalian embryonic stem (ES) cells or epiblast stem cells (EpiSC), derived from the inner cell mass of the blastocyst or the egg cylinder, respectively; or for fish blastomere-derived cell lines (Solter 2006; Hong et al. 2011; Nichols and Smith 2012). Furthermore, cellular reprogramming can revert even fully differentiated cells back to pluripotent stem cells, seen in regenerating organisms and cancer, or induced experimentally, as is the case for mammalian induced pluripotent stem (iPS) cells (Takahashi and Yamanaka 2006; Stadtfeld and Hochedlinger 2010; Inoue et al. 2014).

Cultures of pluripotent cells are invariably heterogeneous, containing a mixture of pluripotent and non-pluripotent progenitors, which re-emerge even after clonal

selection. This raises the intriguing possibility that regulated or stochastic community effects may be at play in stem cell lineages that promote collective decision making, perhaps via processes analogous to bacterial quorum sensing (Halley et al. 2012; Cahan and Daley 2013; Casadesús and Low 2013; Fujimoto and Sawai 2013; Morgani and Brickman 2014; Torres-Padilla and Chambers 2014). Whether this phenomenon is exclusive to *in vitro* culture or whether it can occur *in vivo* is currently unknown. It may be that, depending on the tissue, examples of either deterministic or homeostatic stem cell behaviours will be found (Clevers et al. 2014).

Most, if not all, adult tissues contain stem cells. The origin of adult stem cells is largely unclear but recent experimental evidence suggests that some originate from cells selected already at embryonic stages (Furutachi et al. 2015; Shyer et al. 2015; Campos-Sánchez and Cobaleda 2015). Adult stem cells are thought to be tissue-specific, therefore, multipotent at best. Nonetheless, this constraint might only apply in vivo due to the environment they normally encounter, with larger potency revealed – or else induced – by exposure to an appropriate milieu (Clarke et al. 2000; Krause et al. 2001).

Altogether, the above illustrates the importance of taking context into account when considering stem cell behaviour, with implications for development, disease and therapeutic intervention. Stem cells are of major clinical interest. In regenerative medicine, they offer the promise of cell, tissue and organ regeneration or replacement; they also provide a valuable source for developmental and disease modelling as well as drug testing. iPS cells in particular have contributed to the vision of personalised medicine via autologous cell transplants and clinical trials (Onder and Daley 2012; Ader and Tanaka 2014; Inoue et al. 2014; Lancaster and Knoblich 2014; Sterneckert et al. 2014). Nevertheless, cellular reprogramming is a double-edged sword as it is also implicated in tumourigenesis (Meacham and Morrison 2013; Suvà et al. 2013; Kemper et al. 2014; Campos-Sánchez and Cobaleda 2015; Vlashi and Pajonk 2015). Therefore, therapeutic safety requires understanding how to keep stem cells under control, able to generate the cell types and numbers desired whilst precluding them from generating unwanted ones, namely tumours.

The fundamental challenge to moving stem cell-based therapies into the clinic is the incompleteness of our knowledge about the molecular machineries governing stable differentiation programs and limiting the proliferation potential of stem cells. These are two sides of the same coin as the path to differentiation generally restricts proliferation, and failure in differentiation can lead to tumourigenesis (Harris 2005; Miller et al. 2007; Hindley and Philpott 2013; Hardwick and Philpott 2014). Therefore, safe therapies require that stem cell proliferation be restricted to a certain number of mitotic divisions in vivo until a defined and limited amount of differentiating progeny is generated. Then, each pool of differentiating progenitors too needs to be regulated in the context of its neighbours so that a cell cluster, tissue or organ reaches a proportionate composition of cell types with an appropriate and functional final size. Finally, successful cell replacement and tissue regeneration is only achieved once new cells integrate into existing cell clusters, tissues and organs. This is particularly challenging for the central nervous system (CNS), since it is the organ with the largest variety of cell types, in which the majority of cells are post-

mitotic and part of elaborate neural circuits underlying complex behaviour (Lindvall and Kokaia 2006; Li et al. 2008; Ormerod et al. 2008; Kim and de Vellis 2009; Vishwakarma et al. 2014).

One mechanism by which cell lineages or cell populations equilibrate proliferation and differentiation is asymmetric cell division. Most knowledge concerning mechanisms of asymmetric cell division derives from studies of the nematode worm *Caenorhabditis elegans* (*C. elegans*) and the fruitfly *Drosophila melanogaster*, although studies with the laboratory mouse, *Mus musculus*, and more recently the zebrafish, *Danio rerio*, have also made significant contributions (Gönczy 2008; Sousa-Nunes and Somers 2013; Alexandre et al. 2014; Gómez-López et al. 2014). These animals are seemingly very different to humans and the ancestors of nematodes and flies already separated from the vertebrate lineage more than 600 Ma ago during the course of evolution (Adoutte et al. 2000; Peterson et al. 2004). Still, they all share key features relevant to human stem cell biology and tissue regeneration. Whole genome sequencing has revealed striking similarities in the structural composition of individual genes, providing compelling evidence for structural conservation due to common origin (homology). Moreover, molecules, molecular networks and/or mechanisms underlying core elements of cell biology are conserved as well: homologous genes mediate homologous mechanisms such as Cyclin/Cyclin Dependent Kinase modules regulating the eukaryotic cell cycle (Edgar and Lehner 1996; Bähler 2005; Sánchez and Dynlacht 2005; Cross et al. 2011; Harashima et al. 2013), the Target of Rapamycin and Insulin signalling pathways regulating metazoan cell growth (Hietakangas and Cohen 2009; Dibble and Manning 2013; Jewell and Guan 2013; Ochocki and Simon 2013), and their downstream effectors, such as the Forkhead box transcription factor FoxO, mediating neural stem cell reactivation from quiescence (Paik et al. 2009; Renault et al. 2009; Chell and Brand 2010; Sousa-Nunes et al. 2011). These are but a few examples of evidence for deep homology underlying cell biological mechanisms pertinent to stem cell regulation. At a tissue level, evolutionary conservation is further supported by experiments demonstrating that *Drosophila* and human genes can substitute each other even in the development of the brain (Leuzinger et al. 1998; Nagao et al. 1998; Hanks et al. 1998). Principles of homology appear applicable to the numerous processes underlying asymmetric stem cell division. Therefore, knowledge gained in model organisms is invaluable to enhance our understanding of this process.

In summary, a solid and comprehensive understanding of the molecular mechanisms underlying stem cell proliferation and differentiation, in particular of asymmetric cell division, are fundamental prerequisites for curbing cancer as well as for the successful application of stem cells in regenerative medicine.

3.2 Classifications and Definitions

An asymmetric cell division is one that generates two daughters that differ at birth. Upon an asymmetric cell division, the two sisters may be born with distinct size, shape, content or exposure to signals, which translates into different fates and

behaviours. This mode of division is prevalent and ancestral, seen across all kingdoms of life (Bernander 2000; Neumüller and Knoblich 2009; Thanbichler 2009; Dettmer and Friml 2011; Pereira and Yamashita 2011; Rasmussen et al. 2011; Bi and Park 2012; Li 2013; Williams and Fuchs 2013; Kieser and Rubin 2014; Roubinet and Cabernard 2014; Tan and Ramamurthi 2014; Vevea et al. 2014).

In metazoans, asymmetric stem cell divisions are employed to generate cellular diversity whilst retaining a steady stem cell pool. Asymmetric divisions also provide a flexible means by which to vary the size and cell type proportions of a lineage, by a method that is highly amenable to evolutionary tuning. Upon an asymmetric mitosis, stem cells self-renew and generate a non-stem cell, which can be either a committed progenitor (also called simply "progenitor", "intermediate progenitor" or "transit-amplifying cell") or a postmitotic cell. The term "commitment" describes exit from self-renewal, on the path to differentiation and here we reserve the word "differentiated" for what some call "terminally differentiated" or postmitotic. Stem cells do not all divide asymmetrically and not all cells that divide asymmetrically are stem cells (Fig. 3.1). Indeed, stem cell number can expand by self-renewing symmetric divisions; and can decrease by symmetric

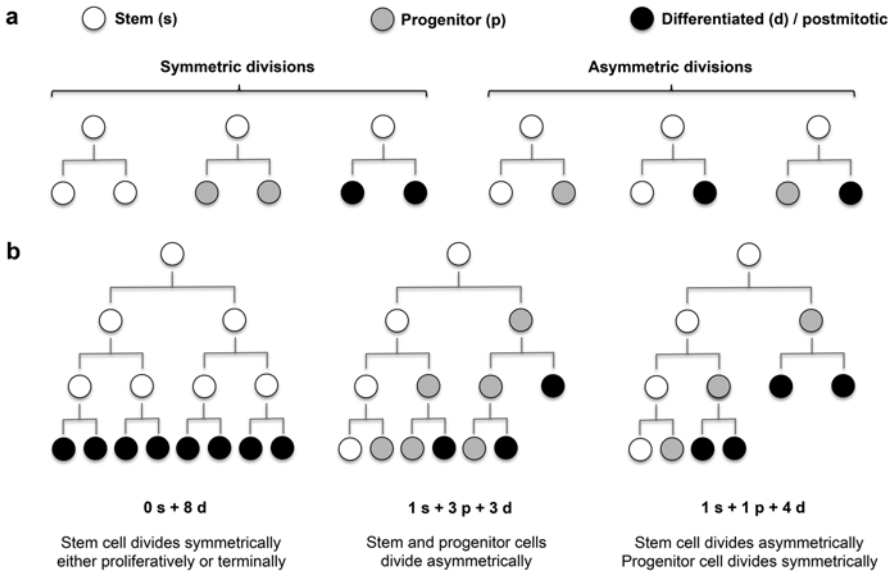

Fig. 3.1 Modes of stem cell division. (**a**) Stem cells (s, *white circles*) can divide symmetrically or asymmetrically in developing and adult organisms. Symmetric, proliferative stem cell division expands the stem cell pool (*left-most* scenario), whereas symmetric, differentiative stem cell division depletes the stem cell pool by generating differentiating progenitors (p, *grey circles*) and/or fully differentiated, i.e., postmitotic cells (d, *black circles*). Asymmetric stem cell division can include a mixture of both proliferative and differentiative stem cell division depending, respectively, if it results in another self-renewing stem cell and/or a differentiating progenitor and/or postmitotic cells. (**b**) Examples of proliferation patterns in lineages where stem and/or progenitor cells divide symmetrically or asymmetrically. Note the different proportions (numbers) between stem, progenitor and differentiated cells in these examples

divisions that generate two progenitors or two postmitotic cells (the latter called a terminal division). Asymmetric divisions commonly maintain stem cell number but they can reduce it if divisions produce two distinct progenitors or a progenitor and a postmitotic cell (Fig. 3.1a). Concerning cell-type proportions, there are infinite permutations, the more possibilities the more types of intermediate progenitors and depending on whether these, in turn, divide symmetrically or asymmetrically (Fig. 3.1b).

For an asymmetric division to take place, the parental cell must be polarized, either as a result of position with respect to extrinsic cues or intrinsically (Fig. 3.2a). What starts as an extrinsic cue may be inherited or otherwise transmitted to and stabilised in the stem cell (Fig. 3.2a dashed arrow). If a cell divides asymmetrically when in isolation, the mechanism is said to be intrinsic. Such is the case for *Drosophila* CNS stem cells, called neuroblasts (Broadus and Doe 1997). Cell polarisation does not imply an asymmetric division, however (eg., epithelial cells exhibit apicobasal polarity and usually divide symmetrically, Bergstralh et al. 2013) (Fig. 3.2b cell on left). What determines whether a division is asymmetric or not is whether the plane of division creates asymmetry (Fig. 3.2b, dotted lines). The best understood means by which this is achieved is by regulation of spindle orientation with respect to cortical polarity cues such that the two daughter cells inherit different cellular constituents, which impart distinct fates on the siblings and so are called "fate determinants". Yet, other mechanisms have been found to promote division

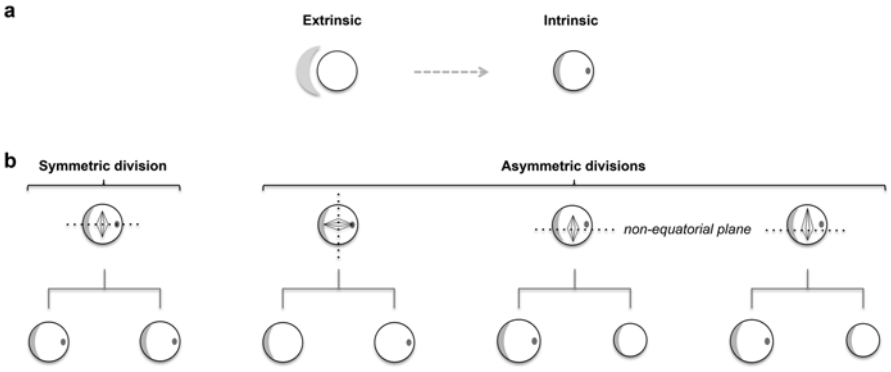

Fig. 3.2 Conditions underlying asymmetric stem cell division. (**a**) For an asymmetric stem cell division to take place, symmetry must be broken in the parental cell (*circle*), which can be achieved either by extrinsic signals coming from the niche (*grey* crescent adjacent to cell on the *left*) or by inheritance of intrinsic polarity from them parental cell (*grey* crescent and spot inside cell on the *right*). An originally external cue can become converted into intrinsic polarity (*dashed arrow*). (**b**) Cell polarity does not necessary lead to an asymmetric stem cell division. The cell on the *left*, though polarised, is undergoing a symmetric cell division as the cleavage plane (*doted line*) partitions polarised components equally. For an asymmetric stem cell division to take place, the cleavage plane must segregate cell components unequally, which can be achieved either by an equatorial cleavage plane that is asymmetrically positioned with respect to polarised components; or by non-equatorial cleavages due to spindle displacement off-centre or asymmetric spindle geometry. The three methods of asymmetric cleavage are not mutually exclusive

asymmetry, namely asymmetric spindle positioning and asymmetric spindle geometry, which lead to unequal sibling cell size in addition to differential distribution of fate determinants (Fig. 3.2b) (Neumüller and Knoblich 2009; Begasse and Hyman 2011; Roubinet and Cabernard 2014). Some cells use more than one of these mechanisms; for example, *Drosophila* neuroblasts orient their mitotic spindle such that self-renewing and differentiation factors are segregated exclusively into one of the two daughters and, in addition, their spindle becomes asymmetric and displaced at anaphase, all of which cooperate in generating daughters of different content and size (Roubinet and Cabernard 2014).

Extrinsic cues are provided by the microenvironment, called "niche" in the case of stem cells in their natural *in vivo* context. The cellular, mechanical and topological composition of the niche provides the stimuli and support necessary for stem cell self-renewal and it may not only be a source but also encouraged by asymmetric divisions since some stem cells create their own niche (Neumüller and Knoblich 2009; Clevers et al. 2014; Januschke and Näthke 2014; Lane et al. 2014; Scadden 2014; Wabik and Jones 2015).

3.3 Mechanisms of Asymmetric Stem Cell Division

From the establishment of cell polarity to completing an unequal cytokinesis, asymmetric division is a complex task yet one that some cells achieve in minutes. Numerous molecular events are concomitant and inter-regulated as opposed to necessarily successive. In the interest of clarity, the following sections break up the intricate choreography of asymmetric stem cell divisions into themes. Molecular machineries and processes underlying asymmetric stem cell divisions are outlined, with emphasis on lessons from *C. elegans* and *Drosophila* from which most understanding originated.

3.3.1 Initial Polarity

Stem cell polarity can be induced by signals from the niche or inherited from the tissue of origin, and thereafter modified and/or maintained. For example, localised Wnt signalling can induce asymmetric stem cell divisions in systems as different as the *C. elegans* embryo and mammalian ES cells (Goldstein et al. 2006; Habib et al. 2013). Cases of polarity inheritance include *Drosophila* neuroblasts (Fig. 3.3) and vertebrate neural stem cells, which derive initial apicobasal polarity from parental neuroepithelial cells. The cytoskeleton is well suited to transmit, establish and maintain cell polarity given its inherent molecular polarisation, dynamics, as well as structural and transport roles (Li and Gundersen 2008). Generation or maintenance of polarity in the *C. elegans* zygote or *Drosophila* neuroblasts requires the actomyosin cytoskeleton, shown by the use of drugs and mutant analyses (Hill and Strome 1990; Knoblich et al. 1995, 1997; Kraut et al. 1996; Broadus and Doe 1997; Shen

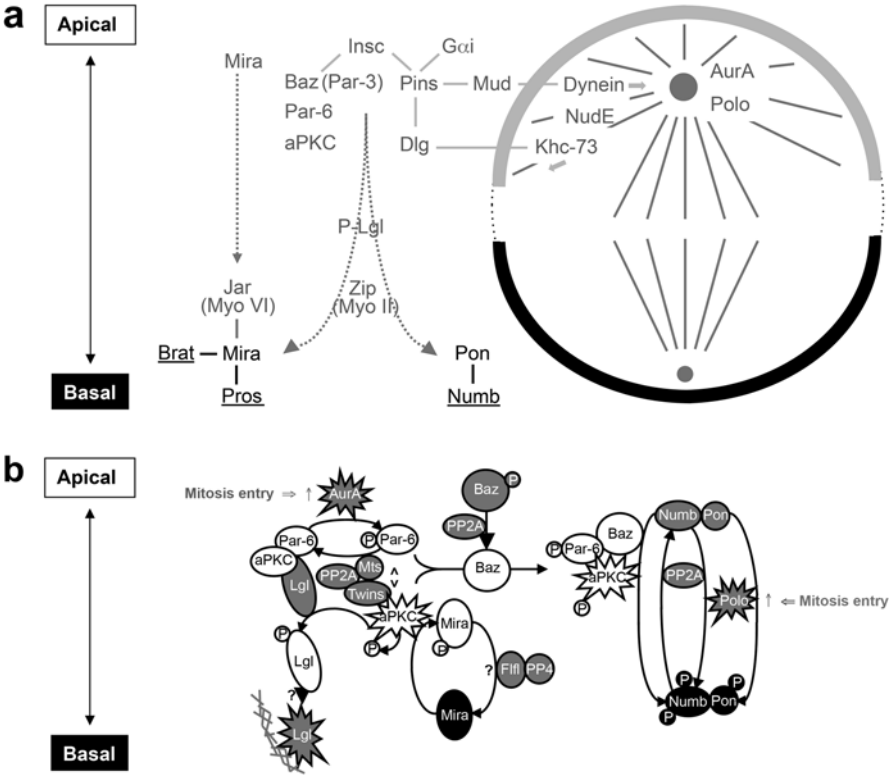

Fig. 3.3 Asymmetric stem cell division in the *Drosophila* CNS. (**a**) Neuroblast asymmetry during mitosis – protein interaction schematic (see main text for details). *Grey*: apical cortex complex members, uniformly cortical or centrosomal proteins; *black*: basal cortex complex members, with "differentiation factors" underlined; *straight lines* between words represent direct protein-protein interactions; hatched lines represent possibly indirect protein interactions. On the schematic on the *right*, *small circles* represent centrosomes; *straight lines* represent spindle and astral microtubules; *arrows* represent direction of movement of the microtubule-based motors. (**b**) Neuroblast asymmetry during mitosis – mechanistic schematic of (de)phosphorylation events. *White shapes*: apical complex members; *Black shapes*: basal complex members; *grey shapes*: uniformly cytoplasmic/cortical proteins; *grey mesh*: actin microfilaments; star-like shapes: activated forms of proteins; *black arrows*: (de)phosphorylation events; *grey upward arrows*: upregulation of protein activity upon mitosis entry; encircled *P* phosphorylation event(s), *Mts* (Microtubule star) and Twins are PP2 subunits, *Flfl* (Falafel) is a PP4 subunit (see main text for other acronyms) (Modified from Sousa-Nunes and Somers 2013)

et al. 1998; Lu et al. 1999; Ohshiro et al. 2000; Barros et al. 2003; Petritsch et al. 2003; Severson and Bowerman 2003).

Asymmetric actomyosin activity is best understood in the *C. elegans* zygote, facilitated by its relatively large size (~50 μm length and 30 μm diameter) and transparency (Begasse and Hyman 2011). Here, the first polarity cue is sperm entry. Following completion of meiosis, the cell cortex starts "ruffling" throughout due to

actomyosin contractions in the context of cortical tension and a viscous cytoplasm. However, once the paternal centrosome nears the cortex and matures (recruiting various proteins), both membrane and the actomyosin cytoskeleton become polarised: contractions stop on the side of the paternal pronucleus (future posterior) and the membrane smoothens due to a cortical posterior-to-anterior actomyosin flow (Hird and White 1993; Cowan and Hyman 2004, 2006; Munro et al. 2004; Motegi and Sugimoto 2006; Mayer et al. 2010). The mechanism remains unclear but, interestingly, this centrosome regulation of asymmetric actomyosin flow is independent of its microtubule nucleating activity and involves downregulation of the activity of the small GTPases Rho and Cdc42 (Cowan and Hyman 2004; Jenkins et al. 2006; Motegi and Sugimoto 2006; Schonegg and Hyman 2006). The evolutionarily conserved family of Rho GTPases are regulators of cytoskeleton remodelling that act like molecular switches as they cycle between an active GTP-bound state, predominantly associated with membranes, and an inactive GDP-bound state, that is cytoplasmic. They are activated by GTPase activating proteins (GAPs) and inactivated by guanine exchange factors (GEFs) or guanine dissociation inhibitors (GDIs) (Mack and Georgiou 2014). The activity of Rho GTPases plays a key role in the initial steps of polarity formation in various cell types and tissues, from yeast to T lymphocytes and epithelial cells of the lung, gut and skin (Iden and Collard 2008; Li 2013).

Redistribution of cortical actomyosin underlies polarisation of the PAR proteins (named for "Partitioning-defective" *C. elegans* embryos when mutated) (eg., Aceto et al. 2006; Hao et al. 2006). Two of these, the PDZ domain proteins Par-6 and Par-3 (Bazooka, Baz, in flies) form a complex with atypical Protein Kinase C (aPKC) that is deployed again and again to establish complementary domains along the polarity axis of various cell types including mammalian epithelial cells and various stem cells (Suzuki and Ohno 2006; Goldstein and Macara 2007). It will be referred to henceforth as the aPKC/Par complex. In the *C. elegans* zygote this complex defines the anterior and in epithelial cells it marks the apical cortex. Epithelial-derived stem cells inherit the aPKC/Par complex, which thus provide a first polarity cue. This can be the case even for stem cells that delaminate from the epithelium, as seen in *Drosophila* neuroblasts (Schober et al. 1999; Wodarz et al. 1999; Betschinger et al. 2003; Rolls et al. 2003; Izumi et al. 2004) (Fig. 3.3). Conversely, Par proteins are themselves necessary for the actomyosin flow (Cheeks et al. 2004; Munro et al. 2004).

Apical complex proteins play several roles: orientation of the mitotic spindle, by interaction with microtubule binding proteins; generation of asymmetric spindle geometry and positioning of differentiation-promoting factors to the opposite (basal) side of the neuroblast, by employment mainly of the actomyosin cytoskeleton to which we shall return.

3.3.2 Mitotic Spindle Orientation and Geometry

Following their specification, *Drosophila* neuroblasts initiate expression of an adaptor protein called Inscuteable (Insc), which associates with the pre-existing aPKC/Par complex (Li et al. 1997; Schober et al. 1999). In turn, Insc recruits another adaptor,

Partner of Inscuteable (Pins) that binds the heterotrimeric G protein α subunit i (Gαi) (Yu et al. 2005; Nipper et al. 2007) (Fig. 3.3a). Expression of Insc is necessary and sufficient to reorient the neuroblast mitotic spindle from parallel to the epithelial surface (as in the overlying neuroepithelium), to perpendicular, thus switching symmetric divisions to asymmetric ones (Kraut et al. 1996). Mouse Insc too plays a critical role in spindle reorientation in cortical progenitors of the mouse neocortex: both loss and gain of function mutations affect correct mitotic spindle positioning and the number of neurons in all cortical layers (Postiglione et al. 2011).

The current model places Pins at the heart of the mechanism orienting the mitotic spindle in *Drosophila* neuroblasts. Two distinct structural domains in Pins coordinate interactions with plus- and minus-end directed microtubule motors: Kinesin heavy chain 73 (Khc-73; homologous to mammalian Kinesin Family member 13B, KIF13B) and Dynein (Fig. 3.3a). These are respectively engaged in capture of astral microtubules by the apical cortex, which orients the mitotic spindle; and force generation, to keep astral microtubules taut (Lu and Prehoda 2013). Astral microtubules are those nucleated by the centrosome during mitosis and which do not connect to a kinetochore. On the one hand, Pins binds to a membrane-associated guanylyl kinase protein called Discs large (Dlg), which in turn binds Khc-73. Recruitment of Dlg requires Pins phosphorylation by the mitotic kinase Aurora-A (AurA) (Johnston et al. 2009). On the other hand, binding of Insc to Gαi enables Pins to recruit a microtubule and Dynein-binding protein called Mushroom body defect (Mud) (Izumi et al. 2006; Siller et al. 2006; Bowman et al. 2006; Nipper et al. 2007; Wang et al. 2011). Asymmetric localisation of Mud is also stimulated by AurA phosphorylation and its association with Dynein is proposed to mediate force generation (Wang et al. 2006; Lu and Prehoda 2013). Mud mutations do not alter cortical polarity, whereas AurA mutations can, but both proteins are required for correct spindle orientation (Berdnik and Knoblich 2002; Giet et al. 2002; Bowman et al. 2006; Izumi et al. 2006; Lee et al. 2006b; Wang et al. 2006; Siller et al. 2006; Wirtz-Peitz et al. 2008; see other AurA substrates below). The two microtubule-interacting pathways (Khc-73 and Dynein-dependent) are thought to be linked not only physically by Pins but also enzymatically by Khc-73 delivery of the Dynein co-factor Nuclear distribution E (NudE), essential for Dynein activation (Siegrist and Doe 2007; Lu and Prehoda 2013) (Fig. 3.3a).

These data from *Drosophila* provide a direct link between asymmetric protein localisation and mitotic spindle orientation. Comparable machineries and processes have been found in *C. elegans* and mammals (Gönczy 2008; Siller and Doe 2009; Knoblich 2010). The mammalian cerebral cortex and retina contain multipotent neuroepithelial progenitor cells with pronounced apicobasal polarity. Their apical domain or "apical endfoot" contains a complex of Cdc42/aPKC/Par, as well as the transmembrane protein Prominin/CD133 (subsequently found to control the apical compartment of *Drosophila* photoreceptor cells) (Götz and Huttner 2005; Farkas and Huttner 2008; Gurudev et al. 2014; see also chapter by Huttner and colleagues). The mammalian homologue of Pins, termed LGN, can bind the Mud homologue NuMA, linking the latter to heterotrimeric G-proteins, thereby regulating mitotic spindle orientation (Zheng 2000; Du et al. 2001; Du and Macara 2004; Sun and

Schatten 2006). In mouse skin progenitor cells, the switch from symmetric to asymmetric divisions concomitant with stratification relies on LGN, NuMA, Gαi3 and Dynactin activity (Williams et al. 2011, 2014). In human cells, the conserved NuMA/LGN/Gαi triad also orients the mitotic spindle via cortical Dynein (Kotak et al. 2012). These data suggest that at least some of the mechanisms underlying apical polarity formation and mitotic spindle alignment, namely interactions between the aPKC/Par complex, heterotrimeric G-proteins and mitotic spindle orientation, represent a highly conserved mechanism underlying asymmetric stem cell division.

One striking feature of *Drosophila* neuroblast divisions is the generation of unequal sized daughter cells. The self-renewing daughter cell is larger than its sibling, primarily the result of asymmetric spindle geometry but also uneven displacement from the two cortices (Fig. 3.2b). Prior to anaphase the mitotic spindle is symmetric and the metaphase plate assembles in the centre of the cell (Kaltschmidt et al. 2000; Cai et al. 2003). During anaphase, apical astral microtubules enlarge while basal astral microtubules shrink, resulting in displacement of the cleavage plane closer to the basal cortex pole. Mitotic spindle asymmetry is controlled by two parallel pathways involving the apical aPKC/Par and Pins/Gαi apical complexes, downstream of two other G proteins, Gβ13F and Gγ1 (Cai et al. 2003; Fuse et al. 2003; Izumi et al. 2004). Basal displacement of the cleavage furrow involves Pins-mediated localisation of proteins normally found associated with cleavage-furrows, including the homologue of Kinesin-like protein 23 (Pavarotti, in flies), the actin-binding protein Anillin (Scraps, in flies), and Myosin II heavy chain (Zipper, Zip, in flies) (Cabernard et al. 2010). This so-called "basal furrow domain" has recently been shown to function in the asymmetric positioning of the cleavage furrow by inhibiting cortical extension specifically on the basal side (Connell et al. 2011). Analogy has been drawn with the *C. elegans* zygote, whereby Myosin II polarisation could drive asymmetric cortical behaviour. During neuroblast mitosis, Myosin II is enriched apically during prometaphase and on the basal cortex during anaphase (Barros et al. 2003; Connell et al. 2011). Here, it presumably exerts strongest force, which, along with lack of resistance from the opposite (apical) cortex, has been proposed to promote asymmetric cortical extension at anaphase, contributing to the size difference of resulting daughter cells (Connell et al. 2011).

In short, the actomyosin cytoskeleton coordinates cortical asymmetry and mitotic spindle position and geometry, whereas the microtubule cytoskeleton orients the spindle with respect to that asymmetry, all of which directly contribute to the regulation of asymmetric stem cell division.

3.3.3 Basal Fate Determinant Localisation

Asymmetric stem cell division secures differential segregation of fate determinants into the resulting daughter cells to regulate their distinct behaviours: self-renewal versus commitment. In dividing *Drosophila* neuroblasts this results in apically

localised proteins being maintained in the self-renewing cell, whereas basally localised proteins are segregated into differentiating progenitors. So-called type I neural lineages contain a single type of differentiating progenitor, termed ganglion mother cell (GMC); type II neural lineages contain an additional one, termed intermediate neural progenitor (INPs) that in turn divides asymmetrically to generate another INP and a GMC (Kim and Hirth 2009; Sousa-Nunes et al. 2010). Cortical aPKC kinase activity is necessary and sufficient to induce neuroblast self-renewal (Lee et al. 2006a,b,c) and three key factors that promote progenitor commitment to differentiation are the homeodomain transcription factor Prospero (Pros) (Vaessin et al. 1991; Doe et al. 1991; Matsuzaki et al. 1992), the NHL-domain protein Brain tumour (Brat) (Arama et al. 2000; Reymond et al. 2001; Sardiello et al. 2008) and the negative regulator of Notch signalling, Numb (Uemura et al. 1989) (Fig. 3.3a).

Basal targeting of fate determinants in neuroblasts is achieved via adaptor proteins: Miranda (Mira), which carries Pros and Brat (Shen et al. 1997; Ikeshima-Kataoka et al. 1997; Schuldt et al. 1998; Broadus et al. 1998; Matsuzaki et al. 1998; Bello et al. 2006; Betschinger et al. 2006; Lee et al. 2006c); and Partner of Numb (Pon), which carries Numb (Lu et al. 1998) (Fig. 3.3a). Disruption of one complex does not affect the other, nor does it interfere with localisation of the apical complexes or mitotic spindle orientation. Mira is essential for the asymmetric localisation of the cell fate determinants Pros and Brat. Pros can bind to a central Pros-binding domain of Mira and Brat binds to the coiled-coil cargo binding domain of Mira (Fuerstenberg et al. 1998; Betschinger et al. 2006). Moreover, the interaction between the NHL domain of Brat and the C-terminal domain of Mira appears to be essential for promoting asymmetric localisation of Pros to the GMC, where it is required for cell cycle exit and neuronal fate determination (Lee et al. 2006c). Thus, it is conceivable that Mira and its cargo proteins Brat and Pros maybe transported across the dividing neuroblast as a complex.

Since basal determinants are excluded from the self-renewing daughter upon each cytokinesis, they must be transcribed and localised de novo in each cell cycle. Intriguingly, newly synthesised Mira localises apically at late interphase before rapidly relocalising to the basal cortex from prometaphase (Li et al. 1997; Shen et al. 1997, 1998; Matsuzaki et al. 1998; Schuldt et al. 1998). It is unclear whether there is a function for this dynamic displacement of Mira, since its apical localisation has been demonstrated to be dispensable for normal basal localisation in neuroblasts (Broadus and Doe 1997).

What drives basal protein targeting of adaptor proteins and their respective cell fate determinants? An intact actomyosin cytoskeleton is required (Broadus and Doe 1997; Ohshiro et al. 2000; Barros et al. 2003; Petritsch et al. 2003) and an important mediator of the process is the membrane and actin cytoskeleton interacting protein Lethal (2) Giant Larvae (L(2)gl or simply Lgl) which localises throughout the neuroblast cortex (Ohshiro et al. 2000; Peng et al. 2000; Albertson and Doe 2003; Betschinger et al. 2003) (Fig. 3.3a).

Upon mitosis entry, AurA phosphorylates various substrates in addition to Mud and Pins, towards coordinating cell-cycle entry and neuroblast polarity. AurA and aPKC lead to a phosphorylation cascade that determines a series of protein-protein

interactions and exchanges that propagate polarity from initially apical signals to stabilise differentiation-promoting factors on the basal cortex (Fig. 3.3b). AurA phosphorylates aPKC, which is necessary for its asymmetric localisation (Lee et al. 2006b; Wang et al. 2006; Wirtz-Peitz et al. 2008). AurA also phosphorylates Par-6, preventing its interaction with aPKC (Wirtz-Peitz et al. 2008). Phosphorylated aPKC can act independently of Par-6 to phosphorylate Lgl, leading to Lgl inactivation on the apical cortex and its detachment from the Par-6/aPKC complex. Within the aPKC/Par complex, this sequence of events leads to the exchange of Lgl for Par-3, which recruits the cell fate determinant Numb. Phosphorylation of Numb by aPKC, dissociates it from the cortex. Another mitotic kinase, Polo (homologous to mammalian Polo-like kinases), directly phosphorylates Pon, which is required for its basal targeting (Wang et al. 2007). Together, the overall effect of this cascade is the stabilisation of phosphorylated Numb on the basal cortex (Betschinger et al. 2003; Lee et al. 2006a,b,c; Smith et al. 2007; Wirtz-Peitz et al. 2008) (Fig. 3.3b).

Other studies indicated an interaction between Lgl with the plus-end directed actin motor Myosin II (Ohshiro et al. 2000). The regulatory light chain of Myosin II (Spaghetti Squash, in flies) is required in neuroblasts for cortical localisation of determinants (Barros et al. 2003; Erben et al. 2008). Apical enrichment of Myosin II at prometaphase has been suggested to depend upon Lgl inactivation at the apical cortex (Betschinger et al. 2003). In line with Mira having been found to physically interact with Zip (Ohshiro et al. 2000), one model proposes that, as it is itself displaced from apical to basal, Myosin II would push Mira and other differentiation-promoting determinants along the cell cortex to the basal side (Barros et al. 2003). Still, how Myosin II regulates basal determinant localisation remains controversial. It has been argued that Mira can diffuse throughout the cell either passively or aided by the unconventional Myosin VI (called Jaguar, Jar, in flies) (Petritsch et al. 2003; Erben et al. 2008) (Fig. 3.3b). Another model, compatible with the previous, proposes that direct phosphorylation by aPKC leads to Mira exclusion from the apical cortex and that de-phosphorylation (possibly by Protein Phosphatase 4) is required for its basal attachment (Atwood and Prehoda 2009; Sousa-Nunes et al. 2009; Sousa-Nunes and Somers 2010). Indeed, for phosphorylation events to promote apicobasal displacement of differentiation factors molecule by molecule, dephosphorylation is required to re-set key residues. Protein Phosphatase 2A (PP2A) regulates apicobasal neuroblast polarity by dephosphorylating Par-6 (in AurA-targeted residues), Par-3 and Numb (in Polo-targeted residues) (Krahn et al. 2009; Ogawa et al. 2009; Ouyang et al. 2011a,b) (Fig. 3.3b). Loss of PP2A can lead to neuroblast polarity defects of varying severity, up to complete polarity reversal (Krahn et al. 2009).

The mammalian homologues of Pros, Brat and Numb (called Prox1, TRIM3 and TRIM32, and m-Numb/Numb-like) have also been implicated in mammalian neural differentiation. Several studies suggest that Prox1 regulates neurogenesis in various regions of the CNS (Stergiopoulos et al. 2015). The human Brat homologue TRIM3 acts as a tumor suppressor (Boulay et al. 2010) regulating asymmetric cell division in glioblastoma (Chen et al. 2014), while another Brat homologue TRIM32 has been shown to suppress self-renewal in dividing cortical progenitor cells, and to induce neuronal differentiation in mouse (Schwamborn et al. 2009). The two

mammalian Numb homologues are functionally redundant and m-Numb protein has been shown to localise asymmetrically in neural progenitor cells found in the ventricular and subventricular zones, segregating into one of the daughter cells (Zhong et al. 1996; Wodarz and Huttner 2003). Whether and how AurA or Polo homologues participate in asymmetric apportioning of fate determinants during mammalian neural progenitor asymmetric division remains to be seen. Unlike *Drosophila* neuroblasts, mammalian neural stem cells are clearly polarised even during interphase. Nonetheless, non-mitotic roles of AurA have been described to regulate polarity of a few mammalian cell types (eg. Pugacheva et al. 2007; Mori et al. 2009; Mahankali et al. 2015).

In summary, in epithelial-derived stem cells, apical complex members regulate localisation of fate determinants on the opposite (basal) side of the cell cortex. Fate determinants to be inherited by committed progeny induce differentiation by a number of mechanisms, transcriptional and post-transcriptional, that contribute to the regulation of asymmetric stem cell division.

3.3.4 Centrosomes and Other Microtubule-Based Structures

During mitosis, in addition to being apically enriched, Mud associates with mitotic centrosomes and other microtubule-based structures; AurA and Polo also associate with centrosomes (Giet et al. 2002; Izumi et al. 2006; Nipper et al. 2007; Archambault et al. 2008) (Fig. 3.3a). In recent years, more and more proteins and mRNAs are being found to localise to this organelle in a cell-type and context-dependent manner and a few studies have identified a role for the centrosome and centrioles in the regulation of stemness complementary to that of spindle pole organising centres.

Centrosomes are composed of two orthogonal centrioles and a matrix of dozens of proteins called pericentriolar material (PCM). In each cell cycle, prior to entry into mitosis, centrosomes duplicate. This involves centriole disengagement from each other, recruitment of proteins to duplicate each of the centrioles and of new PCM (a process referred to as centrosome maturation), regulated by largely unknown mechanisms. During mitosis there are thus two mother and two daughter centrioles, each centrosome containing a mother and a daughter. Still, among the two mother centrioles one is older, having been the mother of the other; the mitotic centrosome that carries the older centriole is called the mother centrosome (Pelletier and Yamashita 2012). Upon cytokinesis, each centrosome is segregated into each of the daughter cells and, at least for some asymmetrically-dividing stem cells, this segregation is non-random. Stereotypical inheritance of centrosomes was first reported for *Drosophila* male germline stem cells, which divide asymmetrically. Upon each division, these stem cells preferentially retain the mother centrosome (Yamashita et al. 2007). Interestingly, this was subsequently also observed in asymmetrically dividing mouse neural stem cells (Wang et al. 2009). The opposite, however, takes place during *Drosophila* neuroblast and female germline stem cell asymmetric divisions, which preferentially retain the daughter centrosome (Conduit

and Raff 2010; Januschke et al. 2011; Salzmann et al. 2014). These data indicate that it is not mother versus daughter centrosome that inherently transmit information conferring "stemness" but does suggest that centrosomes can be different and that the difference may matter.

Consistent asymmetric inheritance of centrosomes in *Drosophila* neural and germline lineages requires the PCM component Centrosomin (Cnn) (Conduit and Raff 2010) and in mouse neural stem cells requires the PCM protein Ninein (Nin) (Wang et al. 2009). Only a minority of PCM proteins is conserved at the primary sequence level (Dammermann et al. 2012; Mennella et al. 2014) rendering it difficult to establish whether Cnn and Nin might be acting in an analogous way to specify asymmetric centrosome inheritance. Notwithstanding, removal of Cnn in either fly stem cell system or attenuation of Nin in neural stem cells leads to randomization of centrosome inheritance (Yamashita et al. 2007; Wang et al. 2009; Conduit and Raff 2010). Importantly, Cnn removal led to fly spermatogenesis decline with age and Nin downregulation led to premature depletion of mouse neural progenitors (Cheng et al. 2008; Wang et al. 2009). These findings make it all the more mysterious that flies can develop into seemingly healthy adults, albeit sterile, in the absence of centrioles (Basto et al. 2006). Clearly, stem cell asymmetric divisions are regulated by numerous mechanisms, which likely present some degree of redundancy.

Stereotypical inheritance could be due to centrosomes carrying distinct fate determinants or to differential microtubule nucleation capacity promoting differential association with the niche (Pelletier and Yamashita 2012). There is suggestive data in support of both ideas. Differential presence of mRNAs in the two mitotic centrosomes has been found in cleavage-stage mollusc embryos. These mRNAs move in an actomyosin cytoskeleton dependent way to opposite sides of the cell cortex and impact daughter cell fates (Lambert and Nagy 2002). Cortically asymmetric mRNAs have also been found in *Drosophila* neuroblasts although they seem dispensible for cell fate asymmetry (Broadus et al. 1998; Schuldt et al. 1998; Knoblich et al. 1999; Erben et al. 2008); their selective advantage may lie in differentiation speed rather than cell fate. More recently, components of the canonical Wnt signaling pathway have been found in centrosomes in various cell types, including *C. elegans* early blastomeres and mammalian neural progenitors, which divide asymmetrically (Kaplan et al. 2004; Louie et al. 2004; Bahmanyar et al. 2008; Fumoto et al. 2009; Chilov et al. 2011; Steere et al. 2012; Vora and Phillips 2015). In the case of early *C. elegans*, both mitotic centrosomes carry β-Catenin but asymmetry in β-Catenin levels was observed following release from the PCM in daughter cells, due to differential proteasome processing (Vora and Phillips 2015). In mammalian neural stem cells, attenuation of β-Catenin led to acentrosomal cells that lacked a microtubule network and, consequently, presented spindle orientation defects, increased asymmetric divisions and premature neuronal differentiation (Chilov et al. 2011).

Could centrosomal asymmetry transduce niche-mediated Wnt signaling? As mentioned above, niche-provided Wnt signalling can induce asymmetric stem cell divisions (Goldstein et al. 2006; Habib et al. 2013) and nuclear β-Catenin is an effector of the canonical Wnt pathway. In cultured ES cells, beads coated with a ligand for canonical Wnt signalling not only induced asymmetric distribution of

components of the canonical pathway, they were sufficient to orient the mitotic spindle and lead to stereotypical asymmetric centrosome inheritance. The Wnt-proximal daughter ES cell preferentially inherited the mother centrosome (judged by presence of Nin) as well as pluripotency markers, in contrast to the Wnt-distal daughter, thus fated to differentiate (ten Berge et al. 2011; Habib et al. 2013). None of this was observed when beads were coated with a non-canonical pathway Wnt ligand (Habib et al. 2013). In this study, centrosomal β-catenin was not detected but, nonetheless, it provided a link between localised extracellular canonical Wnt, asymmetric stem cell division and asymmetric centrosomal inheritance. There might be various paths to the same end.

A nuclear β-Catenin antagonist called Chibby localizes to the distal end of the mother centriole in Human cells, where it is required for primary cilium assembly (Steere et al. 2012). The primary cilium (also known as immotile cilium) is an evolutionarily conserved organelle, commonly found in vertebrate cells, usually one per cell, that serves sensory and signaling functions (Goetz and Anderson 2010; Sasai and Briscoe 2012). The primary cilium is a cylindrical microtubule-based structure that protrudes the cell, with its microtubule cytoskeleton (called axoneme) anchored at the so-called basal body, a microtubule organising centre derived from mother centrioles (Goetz and Anderson 2010). Recent years have seen great excitement in following up the initial discovery that primary cilia are required for transduction of the Hedgehog (Hh) signalling pathway in vertebrates (Huangfu et al. 2003). Much attention is also being given to the possibility of a role for the primary cilium in transducing other signalling pathways, though this appears subtler. In particular, a role for primary cilia in switching between canonical and the non-canonical planar cell polarity (PCP) Wnt signalling pathway has been proposed, yet remains controversial (Goetz and Anderson 2010). *Drosophila* sensory organ precursors divide asymmetrically under the control of the PCP Wnt pathway yet cilia are not required for this or other pathways in *Drosophila*, the only cells that contain this structure being sensory neurons (Goetz and Anderson 2010; Satir et al. 2010; Furman and Bukharina 2011).

Unlike flies, whole mouse mutants without centrioles are lethal by mid-gestation due to elevated levels of p53 and cell death, as well as lack of primary cilia and consequent disruption to Hh signalling (Goetz and Anderson 2010; Bazzi and Anderson 2014). However, analyses of conditional mouse mutants lacking centrioles specifically in neural progenitors, with cell death rescued by combination with a p53 null mutation, showed very surprising results. Neural stem cell spindle orientation became randomised, yet neuron number and corticogenesis appeared grossly normal, arguing against the plane of division being critical (Insolera et al. 2014). Nonetheless, upon asymmetric division of mouse neural stem cells, mother versus daughter centriole inheritance impacts kinetics of cilium reformation and therefore, potentially, the kinetics of signal(s) reception. During mouse neural stem cells mitoses, a ciliary membrane remnant stays attached to one centriole and is inherited by only one of the daughter cells, which then reforms a cilium faster than its sibling (Paridaen et al. 2013). Altogether, the purpose of stereotypical centrosome and primary cilium inheritance is unclear; it could be another case where the mechanism

has been selected for speed but further analyses are required to determine whether it is or not inconsequential.

The primary cilium offers a clear opportunity for the niche to communicate with stem cells. Might the niche and centrosomes communicate in other ways, namely applicable to cells devoid of cilia such as *Drosophila* stem cells? In addition to effector of canonical Wnt signalling, β-Catenin is also a major component of Cadherin-mediated adherens junctions, which mediate cell-cell adhesion in various contexts, namely in epithelia and endothelial cells, as well as between some stem cells and their niche (Nelson and Nusse 2004). In the *Drosophila* male germline, adherens junctions appears to interact with astral microtubules, orienting the mitotic spindle for an asymmetric division and perhaps contributing to selection of the mother centrosome as the one to be retained by the stem cell (Yamashita et al. 2007). The adhesive role of adherens junctions can also promote asymmetric stem cell divisions by maintaining the stem cell in contact with the niche or by maintaining tissue integrity whilst an oblique plane of division takes place. In the *Drosophila* female germline, β-catenin and E-cadherin are required to maintain stem cells associated with their niche, preventing them from differentiating (Song et al. 2002). In epithelial cells of any species, adherens junctions are located just below the apical aPKC/Par domain (Tepass et al. 1990; Wodarz et al. 2000; Kuchinke et al. 1998; Bachmann et al. 2001; Hong et al. 2001; Lu et al. 2001; Petronczki and Knoblich 2001) and, depending on the plane of division, asymmetrically dividing mouse neural stem cells can segregate the most apical domain into one of the daughters whilst maintaining tissue integrity by partitioning adhesive proteins to both daughters (Marthiens and ffrench-Constant 2009). In vitro, E-Cadherin, is required for maintenance of ES cell "stemness" as well as for their differentiation, and for iPS cell induction (Pieters and van Roy 2014). In short, although they inhibit asymmetric divisions in an epithelial plane (Lu et al. 2001), adherens junctions can actually promote asymmetric stem cell divisions.

β-Catenin can also localise to another microtubule-rich organelle transiently formed at the end of mitosis: the midbody (Kaplan et al. 2004; Schiel et al. 2013). The midbody is located within the intercellular bridge of cells undergoing cytokinesis and upon physical separation of daughter cells (called abcission), it is either inherited by one of the daughters (even in symmetric cell divisions leading to equal fates) or shed into the extracellular space (Chen et al. 2013a,b; Schiel et al. 2013). Asymmetric inheritance of the midbody could provide a mechanism for alternate fates of daughter cells. Like for centrosomes, the evidence suggests that it is not that the midbody inherently transmits information conferring "stemness" but that its asymmetric inheritance may be regulated. Some stem cells preferentially exclude and others preferentially retain the midbody (Pohl and Jentsch 2009; Kuo et al. 2011; Salzmann et al. 2014). In fact, in mitosis, the two microtubule-based organelles (centrosome and midbody) probably communicate as preferential inheritance of the midbody is correlated with preferential centrosome inheritance, be it the mother or daughter centrosome which, as described above, can vary between cell type (Pohl and Jentsch 2009; Kuo et al. 2011; Salzmann et al. 2014).

In summary, these data suggest a role for the centrosome, the primary cilium and the midbody in asymmetric stem cell division. However, it remains to be shown whether these roles apply to all stem cells, which is currently an area of intensive research.

3.3.5 Growth and Proliferation

Perturbation of asymmetric segregation of fate determinants or of structures supporting stem cell interactions with their niche can result in the differentiation of both daughter cells or, conversely, in excessive proliferation and even metastatic tumours. *Drosophila* has played a major part in establishing the link between the disruption of asymmetric stem cell division and cancer. Numerous regulators of asymmetric stem cell division are tumour suppressors first identified in flies (eg., Gateff 1978; Caussinus and Gonzalez 2005). Current interpretation is that the common denominator lies in failure of one of the daughter cells to proceed along a committed path towards differentiation (Homem and Knoblich 2012). The severe consequences of disruption have likely led to evolutionary pressure towards selecting for the numerous and highly redundant mechanisms described here that strive to achieve the remarkable fidelity of asymmetric stem cell divisions.

The *Drosophila* larval brain is now an established model for understanding the mechanisms of stem cell self-renewal and tumourigenesis. Here, asymmetric divisions are not only regulated in proliferating neuroblasts but also in committed progenitors. Transplantation studies have revealed that ablation of certain apical components (Pins), basal fate determinants (Mira, Pros, Brat, Numb), cell cycle and spindle orientation regulators (AurA, Polo, Mud, Dlg), and others that control these (Lgl, PP2), result in metastatic tumour formation (Caussinus and Gonzalez 2005; Castellanos and Dominguez 2008; see also references below). Neuroblast-derived tumours possess a mixture of cell-types, including Pros-positive differentiated cells and, for some lineages, glial cells and, significantly, numerous supernumerary self-renewing Mira-positive neuroblasts (and/or INPs in type II lineages) (Caussinus and Gonzalez 2005; Beaucher et al. 2007). The tumourous brain tissue can be maintained indefinitely through serial-transplantation into the abdomen of host flies. Within the abdomen, the brain-derived tissue rapidly proliferates and has the potential to invade other tissues and eventually kill the host (Caussinus and Gonzalez 2005; Castellanos and Dominguez 2008). The serially transplanted tumour tissue develops characteristic signs of metastatic tumours including centrosome amplification and genome instability. Centrosome amplification was shown to be sufficient to initiate tumourigenesis in flies even without genome instability (Basto et al. 2008; Castellanos and Dominguez 2008).

In wild-type GMCs, Mira is degraded, thereby releasing Prospero from the cortex, which then translocates into the nucleus where it exerts a dual role, transcriptionally repressing cell cycle progression and neuroblast identity genes, as well as

activating a neural differentiation program (Hirata et al. 1995; Spana and Doe 1995; Li and Vaessin 2000; Liu et al. 2002; Choksi et al. 2006). Mutant analyses provide *in vivo* evidence that loss of *pros* results in enlarged neuroblast lineages essentially devoid of differentiating, post-mitotic neurons (Bello et al. 2006; Lee et al. 2006c; Betschinger et al. 2006). Instead, the vast majority of cells within these mutant clones show sustained expression of stem cell markers and increased mitotic activity, eventually leading to neoplastic tumour formation (Bello et al. 2006). These data indicate that loss of *pros* causes a transformation of GMCs into stem-like cells that are unable to exit the cell cycle and continue to proliferate. Based on these experimental observations, it is reasonable to consider Prospero a gate keeper in regulating self-renewal and differentiation in GMCs.

Similar to *pros*, *brat* mutation results in over-proliferating neuroblast lineages at the expense of differentiating neurons (Bowman et al. 2008; Bello et al. 2006; Lee et al. 2006c; Betschinger et al. 2006). The role of Brat in type I lineages in unknown but its mutation causes a dramatic phenotype in type II lineages. Studies in *Drosophila* have shown that Brat is a translational repressor (Sonoda and Wharton 2001), which also functions in the regulation of cell growth and ribosomal RNA synthesis (Frank et al. 2002). Growth and proliferation of *brat* mutant cells might be perpetuated by dis-inhibited dMyc activity (Betschinger et al. 2006), a transcription factor regulating cell growth and proliferation (Eilers and Eisenman 2008). The available data however suggest that Brat activity regulates a large number of direct and indirect targets involved in cell cycle progression and growth control. This notion is supported by genome-wide expression studies using adult wildtype and *brat* mutant brain tissue as a template (Loop et al. 2004). These studies identified several potential target genes of Brat, most prominent among them genes involved in cell cycle regulation and translation control, as well as RNA binding/processing, all being up-regulated in *brat* mutant tissue (Loop et al. 2004). In addition, *brat* gain of function can inhibit cell growth and ribosomal RNA accumulation, and slow-down cell division cycles (Frank et al. 2002). Considering its mutant phenotype, these data suggest that *brat* may inhibit cell growth by limiting the rate of ribosome biogenesis and protein synthesis. Pros overexpression can rescue the brain tumour formation seen in Brat mutants (Bello et al. 2006).

Homologues of Brat have been identified in mammals where they are also involved in progenitor cell proliferation control. Recent genetic evidence in mice suggests that the Brat homolog TRIM32 can bind Ago1, a protein involved in microRNA processing. TRIM32 functions both by degrading c-Myc as well as by activating certain microRNAs, among them the stem cell regulator Let-7a (Gangaraju and Lin 2009). TRIM32 activity thereby suppresses self-renewal in dividing cortical progenitor cells, and induces neuronal differentiation (Schwamborn et al. 2009). These findings indicate that Brat/TRIM-NHL proteins regulate self-renewal and differentiation of stem/progenitor cells by modulating microRNA activity as well as ribosome biogenesis and protein synthesis. Based on these observations, it is conceivable that similar to the situation in *Drosophila*, the machinery promoting asymmetric cell division may play an evolutionary conserved role in cell cycle control and tumour suppression. Indeed, mammalian homologues of Baz, Par-6, aPKC,

Lgl, Numb and Brat have been shown to regulate asymmetric cell fate determination and tumour suppression. Thus, mammalian aPKC, Par3, and LGN are involved in asymmetric division of basal epidermal progenitor cells of the skin and their dysregulation can lead to skin cancer (Lechler and Fuchs 2005). The human Brat homologue TRIM3 has been identified as a brain tumor suppressor gene (Boulay et al. 2009) that regulates asymmetric cell division in glioblastoma (Chen et al. 2014), suggesting that Brat/TRIM-NHL proteins act in a conserved genetic pathway regulating stem/progenitor cell self-renewal and differentiation.

The dual role of AurA linking asymmetric protein localisation and mitotic spindle orientation may explain to some extend why in AurA and Mud, but also in aPKC and Lgl mutants, the net result is the same: supernumerary neural stem cells at the expense of differentiating neurons. These observations have been substantiated by mutant studies, showing that neural lineages mutant for Lgl lead to supernumerary postembryonic neuroblasts due to occasional ectopic self-renewal (Lee et al. 2006a,b,c). Furthermore, overexpression of a membrane-targeted aPKC, but not a kinase-dead mutant isoform leads to a similar phenotype, whereas a decrease in aPKC expression reduces neuroblast numbers. Genetic interaction experiments showed that Lgl, aPKC double mutants have normal numbers of neuroblasts and that aPKC is fully epistatic to Lgl, suggesting that aPKC directly promotes neuroblast self-renewal (Lee et al. 2006a,b,c).

In addition, loss of Lgl1/Mlgl/Hugl, one of the two Lgl homologues in mice, results in failure to asymmetrically localize the fate determinant Numb and leads to severe brain dysplasia as neural progenitor cells fail to exit the cell cycle (Klezovitch et al. 2004). which in its cleaved, intracellular form is able to promote self-renewal and to suppress differentiation of neural stem cells in the larval central brain of *Drosophila*. In Polo mutants supernumerary neuroblast-like cells are also produced at the expense of neurons. Over-expression of Numb in polo mutant lineages is able to suppress over-proliferation, indicating that Polo inhibits progenitor cell self-renewal by regulating the localization and function of Numb. However, the mechanism by which Numb directly or indirectly regulates cell cycle activity and proliferation is poorly understood. Mammalian Numb homologues appear to play multiple context-dependent roles with regards to regulating neural cell-fate decisions as their disruption has been reported to result in an overproduction of neurons in the forebrain and a loss of proliferating progenitors (Petersen et al. 2002) while another study has reported a reduction in the number of differentiated motoneurons (Zilian et al. 2001). Double knockouts of Numb and Numb-like in the mouse dorsal forebrain have been found to lead to impaired neuronal differentiation, hyperproliferation of neural progenitors, and delayed cell-cycle exit (Petersen et al. 2002, 2004; Li et al. 2003). In addition, recent data provide compelling evidence that also mammalian homologues of Notch, NuMa and dynactin as well as Inscuteable contribute to maintain a proper balance between neuronal proliferation and differentiation in the developing mouse neocortex (Postiglione et al. 2011).

Other well-characterized human tumour suppressors, such as the kinase Lkb1, whose loss-of-function phenotype results in Peutz-Jeghers syndrome, and p53, regulate cell polarity in worms, flies and humans and might be involved in asymmetric

cell division as well (Marignani 2005; Cicalese et al. 2009; Ouyang et al. 2011b). Thus, similar to the situation in *Drosophila*, asymmetric cell division in mammals appears to be involved in the regulation of stem and progenitor cell self-renewal, and the regulation of cell cycle progression and growth control. Midbody inheritance has recently been shown to be inherited by less differentiated, stem cell-like cell populations both in vitro and in vivo and to correlate with cancer cell proliferation and disease aggressiveness (Pohl and Jentsch 2009; Ettinger et al. 2011; Kuo et al. 2011).

Altogether, data originating from studies of the developing CNS of *Drosophila* provide compelling evidence that one strategy to regulate stem cell self-renewal and differentiation is asymmetric segregation of cell fate determinants in a dividing cell. This is achieved, in part, by asymmetric protein localisation and related mitotic spindle orientation, thereby providing a template for unequal distribution of key regulators. Interestingly, however, such a cascade of events does not explain why mutant stem cells continue to proliferate, thereby self-renewing for an extended period of time. Furthermore, despite size heterogeneity in *pros* mutant neuroblast clones, some supernumerary cells clearly increase in volume following ectopic divisions, for example, in *pros* mutant neuroblast clones (Bello et al. 2006). Thus, in *pros* mutant clones, cell growth appears to be maintained over many rounds of self-renewing divisions, indicating that Pros may also act as a transcriptional repressor on genes involved in growth control. However, genome-wide expression profiling did not identify growth control genes as potential targets of Pros, maybe because embryos had been used as a template (Choksi et al. 2006). A possible link between asymmetric protein localisation, cell cycle progression and growth control might be provided by Brat.

These data also suggest that deregulated stem/progenitor cell division can lead to uncontrolled cell growth and tumor formation (Caussinus and Hirth 2007). Indeed, recent experimental evidence suggests that so-called cancer stem cells drive the growth and metastasis of human tumors and cancer stem cells have already been identified in leukemia, and in solid tumors of the breast and brain (Reya et al. 2001; Pardal et al. 2003; Al-Hajj and Clarke 2004; Fomchenko and Holland 2005; Stiles and Rowitch 2008; Visvader and Lindeman 2008; Schatton et al. 2009). Moreover, inappropriate activation of the WNT, sonic hedgehog (SHH), Notch, PTEN, and BMI1 pathways have all been shown to promote the self-renewal of somatic stem cells, and their dysregulation can lead to neoplastic tissue formation (Pardal et al. 2003; Jiang and Hui 2008).

3.4 Conclusions and Perspectives

Studies using model organisms, from invertebrates to mammals, have revealed insights into the molecular mechanisms underlying asymmetric stem cell division. These studies identified essential steps of asymmetric cell division that are characterised by symmetry break / polarity formation, mitotic spindle orientation and

segregation of cell fate determinants, unequal partitioning of microtubule-based organelles and selective interaction with or response to niche signals. Not only are these processes conserved, they are mostly mediated by evolutionarily conserved molecules and organelles. Asymmetric segregation of other molecules and organelles, namely of those with different age and in invertebrate and vertebrate stem cell systems continue to be added to the vast list of possible mechanisms regulating stemness (eg., Tran et al. 2012; Bufalino et al. 2013; Liu et al. 2013; Yadlapalli and Yamashita 2013; Yennek et al. 2014; Katajisto et al. 2015).

Asymmetric stem cell division lies at the interface of stem cell self-renewal and differentiation and thus regulates the number and identity of differentiating progeny. Therefore, this ancestral mode of cell division is of major therapeutic interest in regenerative medicine as asymmetrically dividing stem cells provide a powerful source for targeted cell replacement and tissue regeneration. For therapeutic applications, it will be essential to determine further details of the machinery involved, in order to be able to manipulate asymmetric stem cell division *in vitro* for the unlimited generation of differentiated cells at will, yet with sufficient understanding to then prevent uncontrolled proliferation *in vivo*. Several key questions need to be addressed and answered in order to achieve these goals. These include determining the molecules, organelles and mechanisms that define and maintain stemness; to identify molecules that regulate the binary switch between self-renewal and differentiation; to determine the mechanisms that direct cell type specific differentiation; and to determine ways how an *in vitro* generated cell can integrate into an existing cellular context while remaining differentiated. There is no doubt that elucidating the molecular mechanisms regulating asymmetric stem cell division will carry on making significant contributions to our understanding of cancer as well as to the successful application of stem cells in regenerative medicine.

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Chapter 4
Stem Cells in the Developing and Adult Nervous System

Fumitaka Osakada and Masayo Takahashi

Abstract The fertilized egg is a totipotent stem cell that can produce all cell types of the organism, including the embryonic and the extraembryonic tissues. As development proceeds, cells lose their capacity to proliferate and differentiate into different cell types, and gain specialization. However, advances in stem cell biology have provided new insights into development and regenerative medicine. For example, neural stem/progenitor cells have been found to exist not only during embryonic development, but also in the adult nervous system of mammals. Moreover, although development of an organism proceeds irreversibly from embryo to adult with cells differentiating progressively toward specialized cell types, somatic cells can be artificially reprogrammed to adopt a different cell fate, as exemplified by induced pluripotent stem cells (iPS cells) and induced neuronal cells (iN cells). Complex 3D tissue including the retina, cortex and adenohypophysis can be generated from pluripotent stem cells *in vitro*. Notably, autologous transplantation of an iPS cell-derived retinal pigment epithelium sheet was carried out for patients with aged macular degeneration in Japan in 2014. iPS cell technology is opening a new era in regenerative medicine and drug discovery. Here, we summarize the current views of stem cell biology during embryogenesis and adult neurogenesis, and then discuss therapeutic potential of stem cells, focusing on retinal development and regeneration.

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4.1 Stem Cell Hierarchy

Stem cells and progenitor cells possess the remarkable ability to give rise to multiple cell types while maintaining their capacity to self-renew, or produce more stem or progenitor cells (Gotz and Huttner 2005; Morrison and Kimble 2006). This is accomplished in part through different types of cell division. Symmetric divisions of stem cells increase the stem cell population, whereas asymmetric divisions produce another stem cell and a progenitor cell that is more restricted in its differentiation capacity (Fig. 4.1a, b). In contrast, symmetric divisions of progenitor cells, which are generally thought to be in a slightly more differentiated state than stem cells, generate two daughter cells that are identical to each other but different from the mother cell (Fig. 4.1c). Asymmetric divisions of progenitor cells generate two types of differentiated daughter cells that are different from the mother cell (Fig. 4.1d).

During development, cells differ in their ability to differentiate into other cell types. The fertilized egg is totipotent, meaning that it can develop into every cell type in an organism (Fig. 4.2). However, with successive divisions, cells in the embryo lose their potential and progressively become more and more specialized. For example, the pluripotent inner cell mass (ICM) of the mammalian blastocyst stage embryo gives rise to cells in all three embryonic germ layers, but not to the extraembryonic trophoblast lineage (Niwa 2007). Embryonic stem (ES) cells, which are cell lines derived from the ICM that can grow indefinitely *in vitro* (Evans and Kaufman 1981), are also pluripotent. Within each germ layer, multipotent stem cells or progenitor cells are able to develop into several different cell types, but are more restricted in potential than ICM or ES cells. For example, a cell in the ectoderm gives rise to neural stem cells or neural progenitor cells, which divide to produce three types of terminally differentiated cells: neurons, astrocytes, and oligodendrocytes. Within tissues and organs, bipotent cells are able to develop into two cell types. Finally, when a cell's potential has been completely restricted, it is committed to undergo terminal differentiation into only a single cell type.

Although the development of an embryo into an adult normally entails progressive and irreversible differentiation of cells into their final, specialized fates, adult somatic cells can be artificially reprogrammed and returned to the naive state of pluripotency found in the early embryo (Fig. 4.2). Over 50 years ago, Dr. J. Gurdon and his colleagues showed that frog somatic cells can be reprogrammed after fusion with an enucleated oocyte, and that they can develop into a tadpole (Gurdon 1962). Reprogramming in vertebrates was also demonstrated by the creation of cloned animals from sheep (Campbell et al. 1996) and mouse (Wakayama et al. 1998) somatic cells fused with enucleated oocytes. Human and mouse ES cells can also

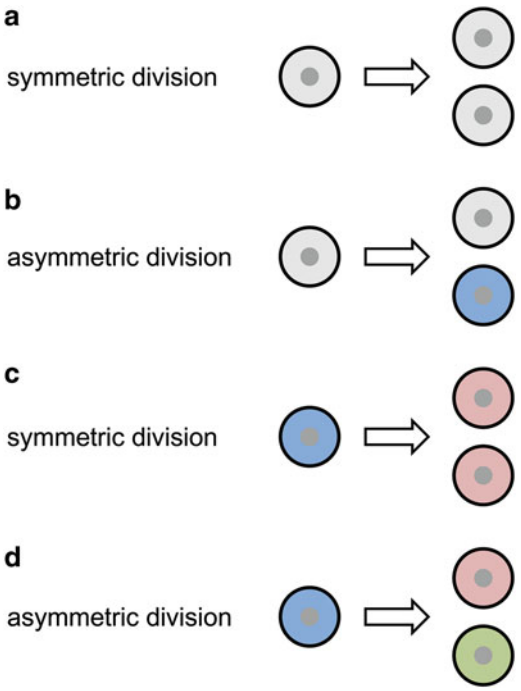

Fig. 4.1 Symmetric and asymmetric divisions of stem cells. (**a**) Symmetric division of stem cells generates two identical copies of the mother cell. (**b**) Asymmetric division of stem cells produces one new stem cell that is identical to the mother cell and another cell that is destined to differentiate along a particular lineage. (**c**) Symmetric division of progenitor cells generates two daughter cells that are identical to each other but different from the mother cell. (**d**) Asymmetric division of progenitor cells generates two types of differentiated daughter cells. *Gray*: stem cell, *Blue*: progenitor cell, *Red or green*: differentiated cell

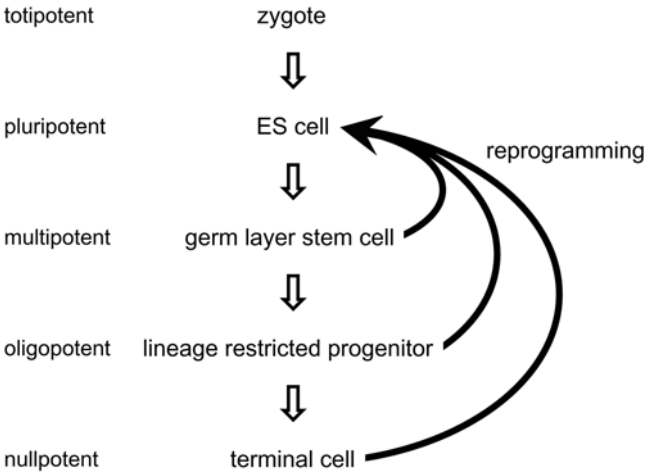

Fig. 4.2 Stem cell hierarchy during development and reprogramming. As development proceeds, cells lose their capacity to proliferate and differentiate into different cell types, and gain specialization. Normally, cells undergo progressive and irreversible differentiation into specialized cell types (*white arrow*). However, differentiated cells can be artificially reprogrammed and returned to the naive state of pluripotency found in the early embryo, as exemplified by iPS cells (*black arrow*)

reprogram somatic cells by cell fusion or treatment with cell extracts (Tada et al. 2001). These results indicate that somatic cells can become pluripotent following exposure to certain reprogramming factors present within oocytes and ES cells.

In 2006, Dr. S. Yamanaka and his colleagues identified these reprogramming factors (Takahashi and Yamanaka 2006). Forced expression of four transcription factors, Oct3/4, Sox2, Klf4, and cMyc, into mouse embryonic and adult fibroblasts was able to return somatic cells to a pluripotent state. They named these cells induced pluripotent stem (iPS) cells. The first generation of iPS cells resembled ES cells in terms of morphology, proliferation, expression of some ES cell marker genes, and formation of teratomas. However, the global gene expression pattern of iPS cells differed from that of ES cells, and these iPS cells failed to produce adult chimeric mice. In 2007, germline transmission was achieved with mouse iPS cells (Maherali et al. 2007; Okita et al. 2007; Wernig et al. 2007). The current generation of iPS cells has been shown to be functionally equivalent to ES cells; they express ES cell markers, have similar gene expression profiles, form teratomas, and contribute to all cell types in chimeric animals, including the germline. The molecular mechanisms underlying the reprogramming process are poorly understood (Hochedlinger and Plath 2009; Yamanaka 2009).

iPS cell technology has opened up the possibility of directly reprogramming somatic cells to adopt a different cell fate. In 2010, nuclear reprogramming allowed direct conversion of somatic cells into neurons, cardiomyocytes, and blood cell progenitors without first passing through a pluripotent state (Ieda et al. 2010; Szabo et al. 2010; Vierbuchen et al. 2010). This concept, however, was not new. A pioneering study performed in 1898 by Weintraub et al. demonstrated that the expression of a single transcription factor, MyoD, is sufficient to convert fibroblasts and numerous other cell types into skeletal muscle cells (Weintraub et al. 1989). Based on these findings, it is likely that the key reprogramming factors are developmental regulators of the target cell lineage. For example, a combination of neural lineage-specific transcription factors, Ascl1, Brn2, and Myt1l, was used to convert mouse fibroblasts directly into functional neurons, known as induced neuronal (iN) cells (Vierbuchen et al. 2010). Moreover, the combination of Ascl1, Brn2, Myt1l, Lmx1a, and FoxA2 or of Ascl1, Nurr1, and Lmx1a can induce midbrain dopaminergic neurons (Caiazzo et al. 2011; Pfisterer et al. 2011). Interestingly, Yamanaka 4 factors (Oct4, Sox2, Klf4 and c-Myc) induced $Pax6^{+}$ neural progenitors (Kim et al. 2011). iN cells have advantages in obtaining neural progenitors and neurons for a shorter time of period over iPS cells since it takes time to induce neural progenitors and neurons from iPS cells, for example several months from human iPS cells. On the other hand, several lines of evidence indicate that characteristics of most iN cells are different from those of endogenous neural progenitors during development, suggesting that iN cells do not completely reconstitute a transcriptional network of endogenous neural cells. Future studies will focus on revealing a transcriptional network charactering a cell type as well as identifying the minimal set of factors sufficient for reprogramming for each cell type, particularly therapeutically significant cell types (Osakada 2011).

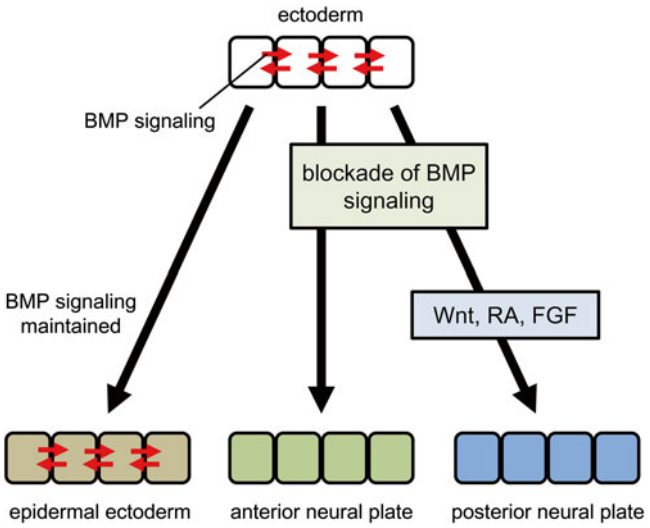

Fig. 4.3 BMP signaling and the specification of ectodermal cell fates. Ectodermal cells exposed to BMP4 differentiate into epidermal ectoderm cells. Blockade of BMP4 signaling by Chordin, Noggin, or Follistatin induces the formation of anterior neural plate tissue. Exposure of this tissue to Wnt, retinoic acid (*RA*), or FGF leads to the generation of posterior neural plate tissue

4.2 Neural Development

The construction of the central nervous system (CNS) is an integrated series of developmental steps, beginning with the decision of a few early embryonic cells to adopt a neural fate. Following fertilization, multiple cell divisions generate a large number of cells from the fertilized oocyte. The three germ layers, the ectoderm, endoderm, and mesoderm, arise through complex movements during gastrulation. Ectodermal cells give rise to different tissue derivatives, depending on the axial position. The dorsal-most ectoderm thickens to form the neural plate, which through a morphogenetic process gives rise first to the neural tube and subsequently to the CNS. Ectodermal cells at the ventral edges of the neural plate, the neural folds, come to lie at the dorsal surface of the neural tube during neurulation. Neural crest cells delaminate from this population of cells and migrate out to give rise to most of the peripheral nervous system. The ectodermal cells lying more ventral to the cranial neural plate form the placodes from which the sensory ganglia will arise. Finally, ectodermal cells on the extreme ventral side of the embryo give rise to the epidermis.

The ventral ectoderm undergoes epidermal differentiation in response to bone morphogenetic protein (BMP) ligands (Fig. 4.3). BMPs activate intracellular proteins, such as Smads, that regulate the transcription of *Gata* and *Msx* genes, which encode transcription factors. Gata and Msx proteins then inhibit *Sox* transcription to promote epidermal fate. The dorsal mesoderm, known as Spemann's organizer,

expresses multiple BMP antagonists, such as Chordin, Noggin, and Follistatin, that induce neural tissue by inactivating Smad signaling (Hemmati-Brivanlou et al. 1994; Hemmati-Brivanlou and Melton 1994; Lamb et al. 1993; Sasai et al. 1994, 1995; Sasai et al. 1994). This results in Sox protein expression, which directly activates proneural gene transcription.

After neural induction, the embryonic CNS is patterned along its anterior-posterior, dorsal-ventral, and left-right axes. The neural tube is regionalized along the anteroposterior axis, with most of the neural tube giving rise to the spinal cord and the rostral end enlarging to form the three primary brain vesicles: the prosencephalon, mesencephalon, and rhombenchephalon. These become further subdivided into five vesicles. The prosencephalon gives rise to both the telencephalon and diencephalon. The diencephalon eventually produces the thalamus, hypothalamus, and retina (the neural retina and retinal pigmented epithelium). The mesencephalon gives rise to the midbrain, and the rhombencephalon divides into the metencephalon and myelencephalon, which form the cerebellum and medulla, respectively.

Neural induction causes the early neural plate to adopt an anterior neural fate bias. The presumptive neural plate is then patterned by caudalizing signals to generate different brain regions (Fig. 4.3). Diffusible morphogens secreted from a localized source establish concentration and activity gradients that act as a positional code to generate distinct progenitor domains, and ultimately to specify subtype identity. These signaling molecules restrict the expression of specific transcriptional factors, which go on to regulate the expression of downstream target genes that define regional identity within the nervous system.

The Wnt, retinoic acid, and FGF signaling pathways play a major role in specifying the anterior-posterior axis (Fig. 4.4a). Concomitant with anteroposterior extension and patterning of the neural plate, the dorsoventral axis is also patterned. Cell fate determination along the dorsal-ventral axis involves the action of two opposing signaling molecules: Sonic hedgehog, which originates from the notochord and later from the floor plate, and TGF-β proteins, especially BMP4, BMP7, and Activin, which originate from the dorsal ectoderm and later from the roof plate (Fig. 4.4b). Patterning along the left-right axis also occurs during gastrulation, at the same time as anterior-posterior and dorsal-ventral axis patterning. A leading candidate for initiating asymmetry is Activin, which acts through Nodal and Lefty before any morphological differences are evident.

Stem cells are defined by their ability both to self-renew and to produce diverse cell types. During development, neural stem cells that arise from the neuroectoderm proliferate throughout the induction and patterning of the neural primordium. Stem cells in the early embryonic nervous system undergo many symmetric cell divisions to generate more stem cells, while those in the late embryo undergo asymmetric divisions to generate progenitor cells that are more restricted in their differentiation capacity (Fig. 4.1a, b). These progenitor cells eventually exit the cell cycle and differentiate into neurons, astrocytes, and oligodendrocytes (Fig. 4.1c, d).

The fates of neural stem/progenitor cells are restricted temporally, with early neural progenitors generating neurons but not glia, and later embryonic and adult

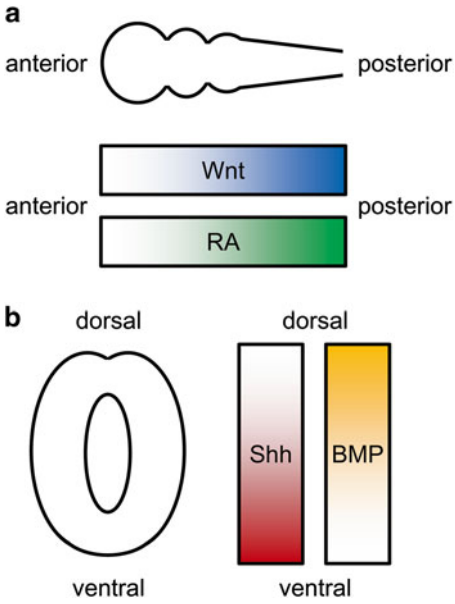

Fig. 4.4 Patterning of the nervous system. (**a**) Gradients of Wnt and retinoic acid (*RA*) specify the anterior-posterior axis of the neural tube. Wnt and RA posteriorize the neural tube. Suppression of Wnt and RA causes anteriorization of the neural tube. (**b**) Gradients of BMP and Shh specify the dorsal-ventral axis of the neural tube. Shh is expressed first in the notochord and later in the floor-plate, and induces ventral differentiation in the neural tube. BMP is expressed in the ectoderm overlying the neural tube and then in the dorsal neural tube cells, and induces dorsal differentiation of the neural tube

neural progenitors generating both neurons and glia. However, these late neural progenitors do not produce early-born neurons, such as forebrain cholinergic neurons, midbrain dopaminergic neurons, and spinal motor neurons. In addition, neural progenitor cells maintain the regional identity of their origin; for example, it is difficult to transform telencephalon-derived neural progenitors into retinal neurons and midbrain dopaminergic neurons.

4.3 Retinal Development

The eye primordium can be identified as early as the neural plate stage. As the neural plate rolls into a tube, the lateral aspects of the anterior neural tube evaginate to form paired optic vesicles, which then fold inward to form bilayered optic cups (Fig. 4.5a–d). The inner layer of the optic cup develops into the neural retina, while the outer layer develops into the retinal pigmented epithelium (RPE) (Fig. 4.5e–i).

Eye field specification in the neural plate is regulated by a set of transcription factors, Pax6, Rx/Rax, Six3, Six6/Optx2, and Lhx2. Functional inactivation of these

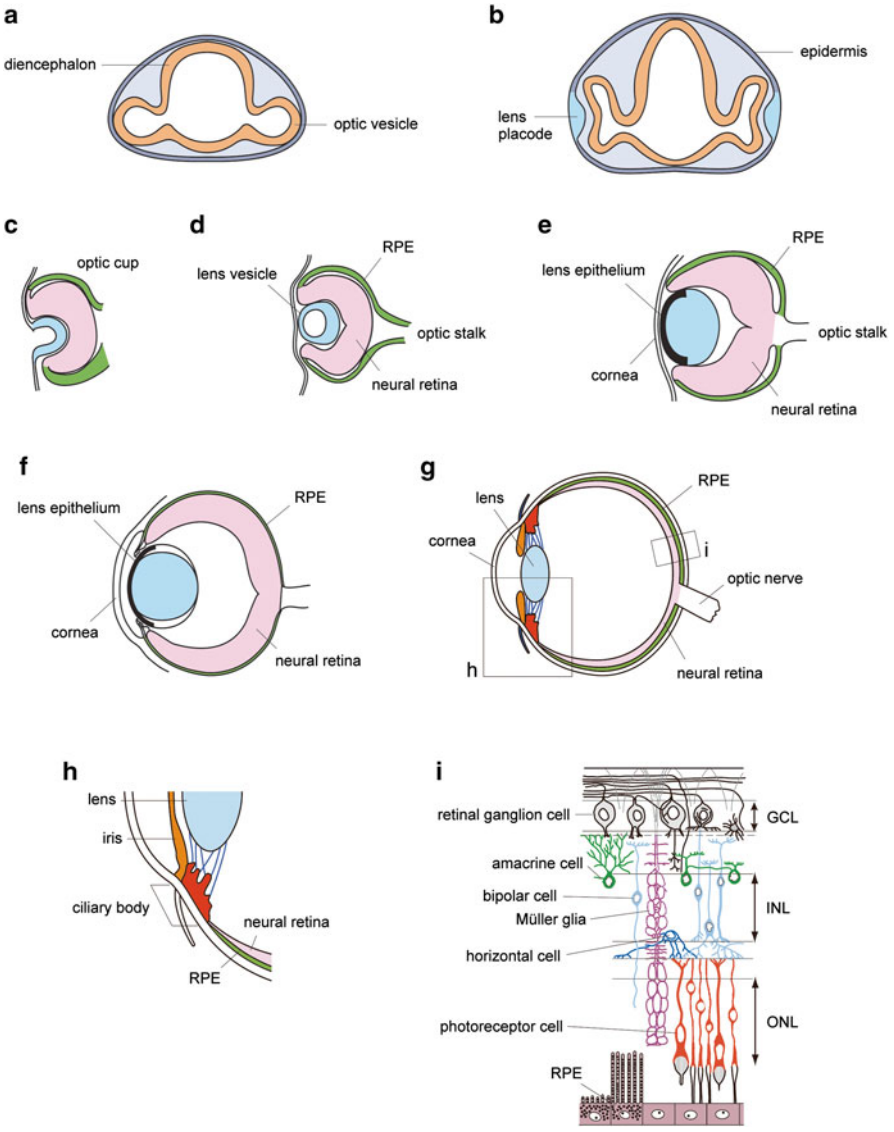

Fig. 4.5 Development of the eye. (**a-f**) Mouse embryos at embryonic days (E)9.5 (**a**), E10.0 (**b**), E10.5 (**c**), E11.5 (**d**), E13.5 (**e**) and E18.5 (**f**). (**g**) Adult eyes. (**h, i**) Magnified views of boxed region in panel (**g**). (**h**) Ciliary body and iris. (**i**) Cell types and layers in the adult retina. *RPE* retinal pigmented epithelium, *ONL* outer nuclear layer, *INL* inner nuclear layer, *GCL* ganglion cell layer (Figures from Experimental Medicine, 2006 by Osakada and Takahashi)

eye field transcription factors (EFTFs) in frogs, fish, rodents, and humans results in loss or abnormalities of the eye. Conversely, overexpression of *Pax6, Rx/Rax, Six3*, and *Six6/Optx2* expands or induces ectopic eye tissues in the vertebrate nervous system. For example, *Pax6* is expressed in the anterior neural plate at the end of gastrulation and is then restricted to the optic vesicle and lens ectoderm. Injection of *Pax6* mRNA into *Xenopus* embryos induces ectopic eyes, indicating that *Pax6* is sufficient for eye formation (Chow et al. 1999). In addition, mutations in *Pax6* result in eye malformations and reduced eye size. *Rx/Rax* is expressed in the presumptive eye field as well as the ventral diencephalon (Furukawa et al. 1997a). $Rx^{-/-}$ mice completely lack eyes, whereas overexpression of Rx in *Xenopus* and zebrafish embryos results in the formation of ectopic retinal tissue and hyperproliferation of the neural retina and the RPE (Andreazzoli et al. 1999; Chuang et al. 1999; Mathers et al. 1997). *Six3* is expressed in the anterior neural plate and in the presumptive eye field (Lagutin et al. 2001; Oliver et al. 1995). *Six3* plays a critical role in the formation of the forebrain, as mouse embryos lacking Six3 function lack most of the head structures anterior to the midbrain (Lagutin et al. 2003). The specific role of *Six3* in eye development, however, remains unknown due to the early head truncation phenotype of *Six3* mutants. In addition, *Six6/Optx2* plays a role in proliferation of retinal progenitors. These EFTFs interact directly with one another and form a self-regulating feedback network, though it remains unclear how this coordinated expression is established.

Little is known about the extracellular signaling molecules that regulate the EFTFs. Wnt1 and Wnt8b activate a Wnt/ß-catenin pathway, and cause reduction of the eye field by suppressing Rx and Six3 expression when overexpressed in *Xenopus* embryos. Wnt11 activates the non-canonical Wnt pathway and causes enlarged eyes when overexpressed in *Xenopus* (Cavodeassi et al. 2005). Overexpression of Frizzled-3, a Wnt receptor, also results in formation of multiple ectopic eyes in *Xenopus.* In mutants lacking the function of Dickkopf-1 (Dkk-1), an inhibitor of canonical Wnt signaling, cranial structures anterior to the midbrain are lost, including the eye (Mukhopadhyay et al. 2001). In *Xenopus*, the BMP inhibitor Noggin induces the expression of EFTFs, including Pax6, Rx, Six3, Six6, and Lhx2 (Zuber et al. 2003). In addition, overexpression of ectonucleoside triphosphate diphosphohydrolase 2 (E-NTPDase2), an ectoenzyme that converts ATP to ADP, causes ectopic eye-like structures in *Xenopus*, while downregulation of endogenous *E-NTPDase2* decreases *Rx1* and *Pax6* expression (Masse et al. 2007). Alterations to the *E-NTPDase2* locus on human chromosome 9 cause severe head and eye defects. Finally, Notch signaling also participates in retinal specification. Overexpression of a constitutively active Notch internal cytoplasmic domain (NICD) induces expression of *Pax6* and the formation of ectopic eyes in *Xenopus* (Onuma et al. 2002). Hes1, a component of the Notch signaling pathway, is expressed in the anterior neural plate and subsequently in the optic cup (Lee et al. 2005). Loss of *Hes1* alone results in reduced eye size, while combined loss of *Hes1* and *Pax6* or *Hes1* and *Hes5* prevents optic cup formation (Hatakeyama et al. 2004; Lee et al. 2005).

During retinal development, stem cells proliferate extensively to increase their cell number and give rise to distinct subtypes of cells over time by changing their

Table 4.1 Intrinsic factors regulating retinal cell differentiation

Cell type	Homeobox genes	bHLH genes
Photoreceptor cells	Crx/Otx2	NeuroD/Mash 1
Horizontal cells	Pax6 / Six3 / Prox1	Math3
Bipolar cells	Chx10	Mash1/Math3
Amacrine cells	Pax6 / Six3	NeuroD / Math3
Ganglion cells	Pax6	Math5
Müllerglia	Rx	Hes1/Hes5

competency. The seven cell types in the retina are born from retinal stem cells in the following temporal sequence: retinal ganglion cells, cone photoreceptors, amacrine cells, and horizontal cells, followed by rod photoreceptors, bipolar cells, and Müller glia. These cells are organized into three cell layers: rod and cone photoreceptors in the outer nuclear layer (ONL), Müller glia, horizontal, bipolar, and amacrine cells in the inner nuclear layer (INL), and ganglion and displaced amacrine cells in the ganglion cell layer (GCL).

The differentiation of each retinal cell type is a highly complex process requiring both extrinsic and intrinsic factors. Intrinsic factors include combinations of bHLH and homeodomain transcription factors (Table 4.1) that work together to specify retinal cell subtype. It is likely that homeodomain factors regulate layer specificity but not neuronal fate, while bHLH proteins determine neuronal fate within the homedomain factor-specified layers. For example, the generation of photoreceptors is regulated by Crx, Otx2 (homeobox gene products), and NeuroD (basic helix-loop-helix proteins). Mice lacking *Crx* function exhibit deficits in outer segment formation in their photoreceptors (Chen et al. 1997; Furukawa et al. 1997b). On the other hand, overexpression of Crx in P0 progenitors promotes the photoreceptor formation and inhibits amacrine fate *in vivo*. Conditional *Otx2* knockout mice lack photoreceptor differentiation (Nishida et al. 2003), while loss of NeuroD, which is expressed in photoreceptors and amacrine cells (Morrow et al. 1999), results in moderately decreased photoreceptor number.

Extrinsic factors regulating retinal differentiation have also been identified (Table 4.2). For example, retinoic acid promotes photoreceptor differentiation, and inhibition of endogenous retinoic acid synthesis results in a reduction in rod differentiation (Hyatt et al. 1996). The amino acid taurine promotes rod differentiation via the α2 glycine receptor and the $GABA_A$ receptor (Altshuler and Cepko 1992; Young and Cepko 2004).

Once cells are committed to a particular fate, they migrate to stereotyped positions throughout the laminated retina and establish synaptic connections to other neurons. Synapse formation proceeds centrifugally from the inner to the outer retina, first among the horizontal connections within the plexiform layers, followed by vertical connections between layers.

Table 4.2 Extrinsic factors regulating retinal cell differentiation

Cell type	Soluble factor
Photoreceptor cells	(+) Retinoic acid
	(+) Taurine
	(+) Thyroid hormone
	(+) Shh
	(+) FGF
	(−) CNTF
Horizontal cells	
Bipolar cells	(+) CNTF
Amacrine cells	
Ganglion cells	(−) Shh
Müller glia	(−) Retinoic acid
	(−) FGF

(+): promotes differentiation, (−): inhibits differentiation

4.4 Adult Neurogenesis

For many decades, it was believed that neurons in the adult mammalian CNS could not regenerate after injury, as postulated by Ramón y Cajal in 1913. However, recent evidence has overturned this long-held dogma. Neural stem cells are present not only during embryonic development, but also in the adult brains of mammals, including humans (Eriksson et al. 1998; Reynolds et al. 1992; Reynolds and Weiss 1992; Sanai et al. 2004). The production of neurons occurs primarily during nervous system development, but throughout adulthood, new neurons are generated in two locations of the brain under normal conditions: the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus. A recent study using radioactive carbon dating revealed a striking annual turnover rate of 1.75 % in the human dentate gyrus where approximately 700 new neurons are added every day (Spalding et al. 2013).

Adult neurogenesis can be divided into three major steps: proliferation, neuronal determination, and maturation. These different developmental stages are regulated by distinct processes. Adult neural stem cells can self-renew and are multipotent, differentiating into three types of neural cells: neurons, astrocytes, and oligodendrocytes. Neurons born in the adult SVZ migrate over a great distance through the rostral migratory stream and become granule neurons and periglomerular neurons in the olfactory bulb (Lois and Alvarez-Buylla 1994; Lois et al. 1996). Neurons born in the adult SGZ migrate into the granule cell layer of the dentate gyrus and become dentate granule cells. These newborn neurons in the adult brain integrate into the existing circuitry (Lepousez et al. 2015) (Frankland et al. 2013). Increasing evidence suggests that adult neural stem cells significantly contribute to specialized neural functions under physiological and pathological conditions, such as learning, memory, olfaction, depression, epilepsy, and stroke (Frankland et al. 2013; Kheirbek

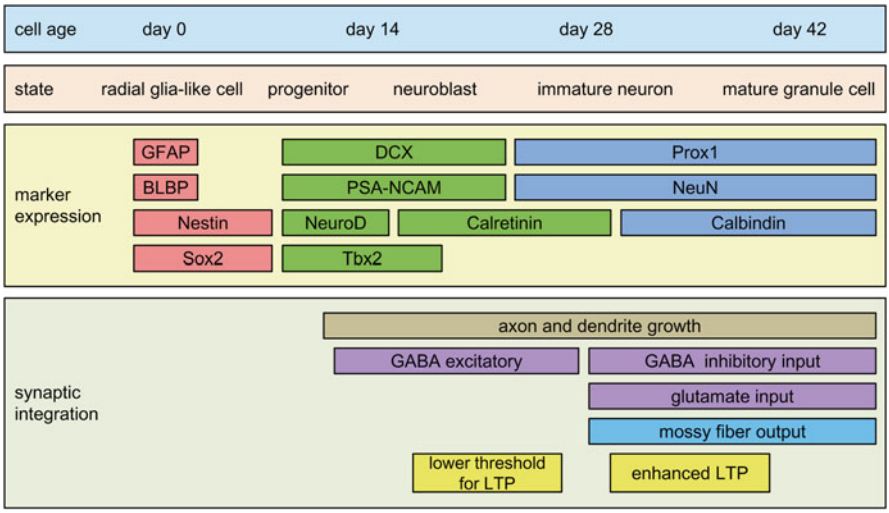

Fig. 4.6 Developmental stages in adult hippocampal neurogenesis. Development of newly generated neurons in the dentate gyrus proceeds through a series of stages characterized by expression of specific markers, morphogenesis, synapse formation, acquisition of electrophysiological properties, and functional integration into neural circuits. *LTP* long-term potentiation

et al. 2012). Moreover, alterations in granule neuron maturation in the dentate gyrus have been implicated in the etiology and pathogenesis of schizophrenia and bipolar disorder (Reif et al. 2006).

Though the existence of neural stem cells in the adult brain has been established, their precise identity remains controversial, because the SVZ and SGZ are heterogeneous in terms of cell morphology and marker expression (Chojnacki et al. 2009). In the adult SVZ, neural stem cells correspond to SVZ astrocytes (type B cells), which are derived from radial glia, the neural stem cells of the embryonic and early postnatal brain (Doetsch et al. 1997, 1999). Type B cells generate transit amplifying cells (type C cells) that give rise to young neurons or neuroblasts (type A cells). Type B cells express GFAP, and are quiescent and less susceptible to anti-mitotic treatment (Doetsch et al. 1999), while type C cells are most frequently labeled with BrdU. Type A cells express PSA-NCAM and doublecortin, both of which are associated with neuronal migration. On the other hand, in the adult SGZ, radial glia-like cells, whose cell bodies are located in the SGZ and whose long processes extend through the granule cell layer into the inner molecular layer, are neural stem cells (Type 1 cells) (Fig. 4.6). Type 1 cells are infrequently labeled by BrdU and generate Type 2 cells, which possess short processes and high proliferative activity. Type 1 cells express GFAP, while Type 2 cells express Nestin but not GFAP.

Neuroblasts originating from SVZ progenitors migrate tangentially toward the olfactory bulb along the rostral migratory stream (Lois and Alvarez-Buylla 1994; Lois et al. 1996). Once they arrive in the olfactory bulb, migrating neuroblasts detach from the chain and migrate radially into the granule and glomerular cell

layers of the olfactory bulb. Newborn neurons go through morphological and physiological development, and integrate as granule neurons in the granule cell layer and as periglomerular neurons in the glomerular layer. Interestingly, recent evidence has demonstrated that SVZ Type B cells are heterogeneous and predetermined to generate specific types of neurons in the olfactory bulb (Merkle et al. 2007). SVZ Type B cells in different locations within the germinal region generate different types of interneurons.

During the maturation process in adult neurogenesis, the first functional synaptic innervation of progenitor cells by hippocampal circuitry is GABAergic (Fig. 4.6). Similar to immature neurons in the developing brain, newborn granule cells initially become depolarized in response to GABA because of their higher intracellular concentration of chloride ions (Ben-Ari 2002; Ge et al. 2006). The response to GABA switches from depolarization to hyperpolarization at 2–4 weeks after neuronal birth, which coincides with the growth of dendritic spines and the onset of glutamatergic responses. Within this time window, new neurons have lower thresholds for long-term potentiation (Ge et al. 2007; Schmidt-Hieber et al. 2004). Newborn neurons in the dentate gyrus display typical features of mature granule cells at 4 weeks of age, but they continue to change both physiologically and morphologically. They have round cell bodies in the GCL, complex spiny dendrites reaching the hippocampal fissure, and an axon that projects through the hilus toward CA3. The amplitude of long-term potentiation is greater in new neurons 4–6 weeks after birth. This may be mediated by the NR2B subunit of the NMDA receptor. Once they mature, newborn granule cells receive glutamatergic (excitatory) and GABAergic (inhibitory) inputs, send functional synaptic projections to CA3 pyramidal cells and hilar interneurons by releasing the neurotransmitter glutamate, and become completely integrated into the hippocampal circuitry in the dentate gyrus.

Adult neural stem cells contribute to specialized neural functions under physiological and pathological conditions, such as learning, memory, olfaction, depression, epilepsy, and stroke (Aimone et al. 2010) (Lepousez et al. 2015). The new neurons are generated at the subgranular zone (SGZ) of the dendate gyrus and integrate into the existing hippocampal circuitry, where they play a fundamental role in learning and spatial memory formation by performing pattern separation on inputs from the entorhinal cortex, an area of the brain located in the medial temporal lobe (McHugh et al. 2007; Zhao et al. 2008; Aimone et al. 2010).

Adult neurogenesis contributes to both the plasticity and regenerative capacity of the adult brain, and opens the possibility for potential future therapeutic applications based on the manipulation of this regenerative capacity. In particular, a better understanding of the basic mechanisms regulating adult neurogenesis may provide the foundation for treating neurodegenerative diseases. Importantly, adult neurogenesis in both the SVZ and SGZ declines during aging. Many lines of evidence indicate that neurotransmitters (GABA, glutamate, dopamine, acetylcholine and serotonin) (Cameron et al. 1995; Hoglinger et al. 2004; Liu et al. 2005), hormones (corticosteroids and prolactin), growth factors (FGF, EGF, BDNF, CNTF, IGF, VEGF, Shh, and Wnt) (Lai et al. 2003; Lie et al. 2005), and physiological and pathological stimuli (environmental enrichment, electroconvulsive shock stimulation,

stress and seizures) (Kempermann et al. 1997; Mirescu and Gould 2006; van Praag et al. 1999; Warner-Schmidt and Duman 2006) affect adult neurogenesis.

4.5 Retinal Regeneration

Regeneration in the CNS necessitates the reacquisition of pre-existing neural structures and function following injury and disease. The strategies for regeneration can be classified into two approaches: (i) activation of endogenous neural stem cells and (ii) transplantation of lost cell types (Goldman 2005; Osakada et al. 2010; Osakada and Takahashi 2009).

Visual impairment is usually caused by specific loss of different cell populations within the retina (Osakada et al. 2010; Osakada and Takahashi 2009). For example, glaucoma is a retinal degenerative disease in which the retinal ganglion cells (RGCs) forming the optic nerve are selectively lost. In retinitis pigmentosa, photoreceptors are lost due to genetic mutation (Hartong et al. 2006; Wright et al. 2010). In age-related macular degeneration (AMD), degeneration of the retinal pigmented epithelium (RPE) is followed by loss of photoreceptors (Rattner and Nathans 2006). Since first order neurons are selectively affected in retinitis pigmentosa and AMD, the neural circuitry mediating higher order visual processing is maintained in the early phase of degeneration (Bi et al. 2006; Busskamp et al. 2010; Humayun et al. 2003; Lagali et al. 2008; Mazzoni et al. 2008). Thus, repair of photoreceptor or RPE cells may permit recovery of visual function. It should be noted that retinal regeneration differs from regeneration of the optic nerve. Retinal regeneration aims to replace photoreceptors and reconstruct their synapses with proximal secondary neurons (bipolar cells and horizontal cells) within the retina. In contrast, optic nerve regeneration to treat glaucoma and other diseases requires replacement of RGCs and reconstruction of distant synaptic connections to the brain.

The capacity for adult neurogenesis in the retina is greatest in fish and amphibians. The ciliary margin zone (CMZ) of fish and amphibians contributes to retinal growth throughout the animal's life. In response to damage, retinal progenitors in the CMZ generate new retinal neurons in amphibians, fish, and birds. Thus, the CMZ resembles other regions containing neural stem cells, like the SVZ and the SGZ. Interestingly, sphere culture methods have shown that retinal stem cells persist in the mammalian ciliary epithelium (Ahmad et al. 2000; Tropepe et al. 2000). Moreover, iris cells from birds and mammals can generate retinal neurons *in vitro* (Haruta et al. 2001; Sun 2006). In amphibians, the RPE is the primary source of new retinal progenitors (Reh et al. 1987). After removal of the retina, the RPE loses pigmentation and proliferates to generate two new epithelial layers, a pigmented layer and a non-pigmented layer. The non-pigmented layer begins to express genes typical of retinal progenitors and undergoes extensive cell division to produce neurons for the new retina (Reh and Nagy 1987). In fish, birds, and mammals, Müller glia act as endogenous progenitors and generate new neurons in response to damage (Fischer and Reh 2001; Ooto et al. 2004).

In adult mammals, Müller glia have the potential to generate retinal neurons after injury *in vivo* (Karl et al. 2008; Ooto et al. 2004). The neural stem cell properties of Müller glia have been also verified *in vitro*. Dissociated Müller glia derived from injured retinas form neurospheres *in vitro*, which can differentiate into neurons and glia (Das et al. 2006). In addition, Müller glia-derived progenitors can be identified and purified as a side population of cells by the Hoechst dye efflux, another characteristic of progenitor cells (Das et al. 2006). After transplantation into the retina, these Müller glia-derived neurosphere cells can differentiate into retinal neurons.

Several lines of evidence support a close relationship between Müller glia and retinal progenitors. Recent gene expression profiling studies have demonstrated a large degree of overlap in the genes expressed in the Müller glia and late retinal progenitors. Moreover, the proliferation and differentiation of Müller glia-derived progenitors can be regulated by both intrinsic (homeobox and basic helix-loop-helix genes) and extrinsic (Wnt, Notch, Shh, FGF, EGF and BDNF) factors, similar to what has been observed in retinal progenitors during eye development (Das et al. 2006; Harada et al. 2011; Osakada et al. 2007; Wan et al. 2007). However, how Müller glia in the mammal reacquire neurogenic potential is still unknown. Several lines of evidence have demonstrated that activation of Shh, Wnt and Notch is sufficient to stimulate Müller glia to enter a neurogenic mode in the absence of injury (Del Debbio et al. 2010; Wan et al. 2007). Epigenetic modifications in Müller glia might be also involved in reacquisition of neurogenic potential. Since Müller glia are a potential source of regenerating cells in the adult mammalian retina, developing drugs that target these cells is a promising approach that may lead to new retinal regeneration therapies (Osakada and Takahashi 2009).

For photoreceptor transplantation, cells from the developing retina can be used as a donor source for transplantation. Importantly, integration of donor rod photoreceptors in the host retina requires rod photoreceptors corresponding to postnatal days 3–6 (MacLaren et al. 2006). However, use of human fetal tissue presents ethical problems, and the quantity of available fetal retinal cells is limited. Thus, *in vitro* expansion of retinal cells derived from stem/progenitor cells, if possible, would be ideal. When adult stem cells from the SGZ are transplanted into the developing eye, they integrate into the retina and exhibit morphologies and positions characteristic of Müller, amacrine, bipolar, horizontal, and photoreceptor cells (Takahashi et al. 1998). However, none acquire end-stage markers unique to retinal neurons. Thus, adult brain-derived stem cells cannot adopt retinal fates even when exposed to the cues present during retinal development. Although the brain and the retina are both generated from the ectodermally-derived neural tube, neural progenitors in different CNS regions differ in their competence to generate specific types of mature neurons. Alternatively, retinal progenitors in the embryonic retina can be expanded *in vitro* and can differentiate into various types of retinal neurons; however, they lose their ability to differentiate into photoreceptors following massive expansion (Akagi et al. 2003).

The somatic progenitors in adult eye tissue are another potential source of donor cells. The ciliary marginal zone has been reported to contain stem cells even in adults (Ahmad et al. 2000; Tropepe et al. 2000). When cultured *in vitro*, these cells

give rise to retinal neurons, including photoreceptors. Iris-derived cells have also been reported to generate retinal neurons (Haruta et al. 2001). Adult tissues offer the advantage that they can be used as autografts, which do not cause immune rejection. Autologous iris tissue can be feasibly obtained by peripheral iridectomy. Unlike the hippocampus, both the ciliary margin and the iris derive from the optic vesicle and optic cup, suggesting that they may be more competent than brain stem cells to generate retinal neurons. However, cells differentiated from adult somatic progenitors in the eye express several photoreceptor marker proteins, but not all the genes responsible for photoreceptor function. Thus, it is likely that the generation of functional photoreceptors requires a recapitulation of the normal process of retinal development.

ES cells are another potential source of donor cells for retinal transplantation. Based on our knowledge of embryonic development, we have developed methods of inducing stepwise differentiation of ES cells into retinal progenitors (Rx^+, $Mitf^+$, $Pax6^+$, $Chx10^+$), photoreceptors (Crx^+, Nrl^+, $rhodopsin^+$, $recoverin^+$) and RPE ($Mitf^+$, $ZO1^+$, $RPE65^+$) (Ikeda et al. 2005; Osakada et al. 2008, 2009a, b) (Fig. 4.7a, b). Surprisingly, optic cup structure can be induced from three-dimension culture of mouse ES cells, indicating some self-organizing capacity that might be harnessed (Eiraku et al. 2011). Transplantation of ES cell-derived photoreceptor sheets might also be an effective approach (Aramant and Seiler 2004). While somatic progenitors derived from the ciliary body or iris are limited in both differentiation potential and proliferation capacity, human ES cells can generate a large number of retinal cells. Indeed, transplantation of photoreceptors or RPE derived from human ES cells has been reported to restore some visual function (Lamba et al. 2009). Regeneration of the RPE is also important because it is essential for photoreceptor function; indeed, RPE degeneration causes secondary photoreceptor degeneration. Several promising lines of evidence indicate that transplantation of ES cell-derived RPE can prevent photoreceptor degeneration in an RPE degeneration model, RCS rats (Haruta et al. 2004; Idelson et al. 2009; Lund et al. 2006).

iPS cell technology provided a paradigm shift not only in our understanding of cell biology, but also in regenerative medicine approaches (Takahashi et al. 2007; Takahashi and Yamanaka 2006; Yu et al. 2007). iPS cells are functionally equivalent to ES cells and therefore share the same advantages as ES cells: pluripotency and proliferation capacity. However, for clinical applications, they offer the additional benefits of avoiding problems faced by human ES cell technology: the ethical problems surrounding the use of human embryos and the biological problem of tissue rejection (Takahashi et al. 2007; Yu et al. 2007). Thus, patient-specific, customized cell therapy might be possible. The retinal differentiation methods for ES cells are applicable to iPS cells (Hirami et al. 2009; Osakada et al. 2009b) (Fig. 4.7c, d). Transplantation of human iPS cell-derived RPE can rescue photoreceptors in an animal model of RPE degeneration and has a therapeutic potential (Carr et al. 2009). We launched a clinical trial of RPE transplantation using human iPS cells in Japan in 2014. This trial aims to assess the safety and feasibility of autologous transplantation of iPS cell-derived RPE sheets in patients with wet AMD, which is characterized by progressive damage to the RPE due to leakage caused by neovascularization.

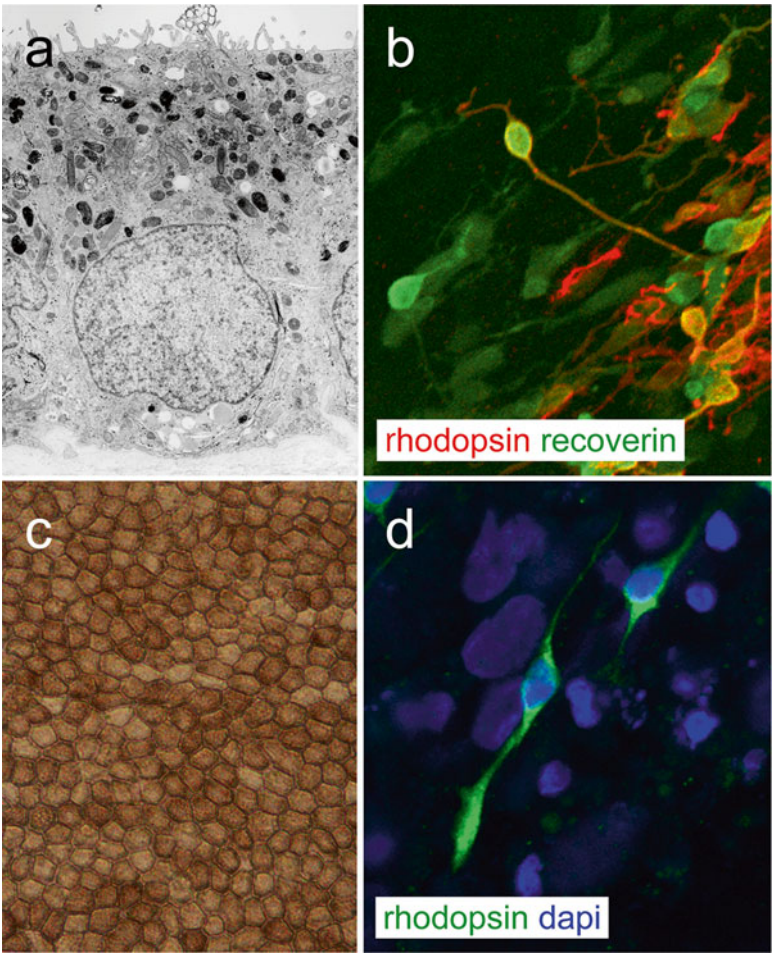

Fig. 4.7 Differentiation of retinal cells from human ES and iPS cells. (**a**) Electron micrograph of human ES cell-derived RPE cells. (**b**) Human ES cell-derived rod photoreceptor cells express both rhodopsin and recoverin. (**c, d**) Generation of RPE (**c**) and photoreceptors (**d**) from human iPS cells (Figures from Osakada et al. 2008)

By contrast, photoreceptor transplantation is still challenging. FACS-sorted photoreceptor cells from human iPS cells cannot integrate into the normal mouse retina and are inefficient for functional restoration, although unsorted iPS cell-derived cells can be transplanted and survive in the retina (Lamba et al. 2010). However, recent studies have shown that mouse and human ES cells can spontaneously form the 3D structure of the optic cup, the retinal primordia (Eiraku et al. 2011; Nakano et al. 2012; Zhong et al. 2014). The 3D retinal tissue generated from ES/iPS cells can overcome this situation. Gonzalez-Cordero et al. showed that dissociated photoreceptors derived from 3D retina of mouse ES culture can integrate within the

degenerating mouse retina and mature into outer segment-bearing photoreceptors (Gonzalez-Cordero et al. 2013) although photoreceptors did not survive and integrate in the degenerated retina that completely lost the photoreceptor layer. Remarkably, Assawachananont *et al.* showed transplantation of a photoreceptor sheet derived from ES/iPS cells allowed for making new synaptic connections to host bipolar cells even in the degenerated retina (Assawachananont et al. 2014). How these new photoreceptors functions in the degenerated retina and contribute to visual functions are still unknown (Osakada and Takahashi 2015). Many questions remain to be answered before photoreceptor regeneration can be applied in the clinic.

Nuclear reprogramming of somatic cells directly to retinal neurons and RPE could be also a promising approach to obtain retinal cells more quickly and more safely (Osakada 2011). One key issue in autologous transplantation for genetic disorders is that genetic defects due to mutations or deletion need to be repaired before transplantation. Gene correction by homologous recombination or zinc finger nuclease technology is feasible in mouse iPS cells and human iPS cells (Hanna et al. 2007; Liu et al. 2011; Yusa et al. 2011). For successful retinal regeneration, methods of making new synaptic connections between donor photoreceptors and host cells and optimizing host conditions, as well as use of animal models of human diseases to determine the efficacy and safety of treatments, will be crucial.

4.6 Conclusions and Perspectives

Over the past decade, significant progress has been made in stem cell biology. A better understanding of stem cells has shed light on the processes involved in embryonic development, adult neurogenesis, and regeneration. In particular, the discoveries of adult neural stem cells, ES cells, and iPS cells will stimulate both basic research and applied biomedical study.

It has been established that neurogenesis and neural regeneration take place even in the mammalian adult CNS, but many questions must still be resolved. For example, what are the physiological roles of neural stem cells in the adult brain? Why do only two regions generate new neurons in the intact adult brain? What is the difference between neurogenic and non-neurogenic regions? Can neurogenesis or neural regeneration be induced in non-neurogenic regions? How are new neurons integrated into preexisting neural circuits? How did the difference in regeneration capacity among species arise though evolution? A detailed understanding of stem/progenitor cells in the adult CNS will be important for therapeutic applications for CNS repair.

Despite tremendous progress in stem cell biology, there is still a large gap between the cellular and behavioral approaches towards understanding the pathophysiological roles of new neurons in the adult CNS (Aimone et al. 2010; Lepousez et al. 2015). A straightforward way to study their functional contribution is to eliminate new neurons in the adult CNS by irradiation or administration of anti-mitotic

drugs such as methylazoxymethanol acetate and temozolomide, which kill dividing cells in the adult brain (Madsen et al. 2003; Shors et al. 2001). However, these approaches cannot uncover the physiological properties of newly generated neuronal connections. Methods to analyze the integration of newly generated neurons or transplanted cells into existing neural networks are not well established at present. Addressing these questions at the circuit level will require visualizing newly formed connections, monitoring and manipulating the activity of these connections, and assessing the behavioral outcome (Osakada and Takahashi 2015). Recombinant viral vectors will be powerful tools for these purposes (Luo et al. 2008; Osakada and Callaway 2013; Osakada et al. 2011).

During vertebrate embryogenesis, the nervous system primordium arises from uncommitted ectoderm during gastrulation. While much is known about the mechanism of neural induction in amphibians, comparatively little is known about this process in mammals, in part because good experimental systems for *in vitro* neural differentiation comparable to the animal cap assay commonly used in *Xenopus* studies are still lacking in mice. Unlike the amphibian animal cap, which is large and easy to prepare in large quantities, the mammalian ICM and epiblast are tiny and technically demanding to handle. However, *in vitro* differentiation of ES cells recapitulates many aspects of embryonic development *in vivo* (Hansen et al. 2011; Osakada and Takahashi 2011). Indeed, the spatial and temporal aspects of neurogenesis can be recapitulated and manipulated in response to morphogens in ES cell culture (Gaspard et al. 2008; Mizuseki et al. 2003; Watanabe et al. 2005; Wichterle et al. 2002). Intriguingly, ES cells can generate the self-organized laminar structure of the cortex, including four distinct zones (ventricular, early and late cortical-plate, and Cajal-Retzius cell zones) along the apico-basal axis (Eiraku et al. 2008), and the optic cup structure in three dimension culture of ES cells (Eiraku et al. 2011). Differentiation culture of ES cells and iPS cells provides a versatile and powerful *in vitro* tool complementary to *in vivo* approaches. Such studies will provide an improved understanding of the mechanisms of mammalian development.

In addition to providing a promising approach towards cell transplantation therapy to treat disease or injury, stem cell technology has the potential to revolutionize drug discovery, making models available for primary screening, toxicity evaluation, and metabolic profiling. Mouse ES cells are already in use in drug discovery, and high-throughput screening is currently being developed. Since human models for disease are highly desirable, human ES cells and iPS cells will be powerful tools for drug discovery. In particular, the generation of patient-specific or disease-specific human iPS cells will be a strong tool for studying disease mechanisms, screening drugs, and developing new therapies (Brennand et al. 2011; Jin et al. 2011). For genetic diseases, iPS cells provide a new opportunity to analyze the molecular pathways that lead to disease pathogenesis at the cellular level (Jin et al. 2011). Moreover, drug effects during clinical treatment might be predicted and analyzed using iPS cells from patients, permitting personalized optimization of drug treatment.

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Chapter 5
Epigenetic Mechanisms Regulating the Transition from Embryonic Stem Cells Towards a Differentiated Neural Progeny

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Abstract Adult tissues preserve characteristic populations of self-renewing cells, which can give rise to various specialized cell types, and the brain is not an exception to this rule. The identification of neural stem cells (NSC) present in several areas of the adult brain has challenged conservative ideas regarding the applicability of regenerative medicine to the brain, creating a research field dedicated to unraveling the mechanisms of adult NSC self-renewal and differentiation, particularly within well defined tissue microenvironments termed neurogenic niches. Research over the past 50 years has revealed that NSC can give rise to different types of neural cells: neurons; astrocytes and oligodendrocytes; and recent observations have demonstrated that epigenetic mechanisms play a central role in the regulation of NSC self-renewal and differentiation under physiological and pathological conditions. In this chapter we review the literature describing these epigenetic mechanisms and discuss their possible implications for regenerative therapies for neurodegenerative disorders, which have been linked to alterations in the generation of new neurons from resident neural stem cells in the brain.

Keywords Neurodegenerative disease • DNA methylation • MicroRNA • Chromatin remodeling • Histone modification

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5.1 Introduction

The term "stem cell" was initially used in the late nineteenth century in the context of fundamental questions in embryology: the continuity of the germ-plasm and the origin of the blood system, where it was used to indicate that all blood cells develop from a common precursor cell and has evolved substantially ever since (Ramalho-Santos and Willenbring 2007). Nowadays we know several types of stem cells exist, and they are generally defined by a group of common properties, such as the capacity of self-renewing, the ability to generate multiple cell lineages and, in the case of adult stem cells, the ability to persist for the lifetime of the host organism. Self-renewal, by definition, implies active cell proliferation, while preserving an undifferentiated state. Therefore, a commonly used operational definition of stem cell is an undifferentiated, potentially immortal cell, with the capacity of self-renewal and ability to differentiate into multiple cell types (Hime and Abud 2013).

The most primordial stem cells are embryonic stem cells (ESC), which are pluripotent stem cells derived from the blastocyst's inner cell mass. ESC have normal karyotypes, express high levels of telomerase activity, specific surface markers and maintain the developmental potential to form trophoblast and derivatives of all three embryonic germ layers, endoderm, mesoderm and ectoderm (Thomson et al. 1998). ESC pluripotency, understood as the diversity of cell types that can differentiate from them, decreases during embryonic development by a series of sequential fate decisions, leading to committed tissue-specific progenitor cells that can subsequently differentiate into lineage restricted progenies. Adult tissues preserve self-renewing cells, which can give rise to various specialized cell types, termed then adult multipotent tissue- specific stem cells, adult stem cells or somatic stem cells (Zipori 2009). Although it was long thought that the brain was an exception to this phenomenon the pioneer work from Joseph Altman demonstrated otherwise (Altman and Das 1965). The resulting identification of neural stem cells (NSC) present in several areas of the adult brain challenged conservative ideas and has created a new research field dedicated to unraveling the mechanisms of adult NSC self-renewal and differentiation (Ariff et al. 2012). Research over the past 40 years has revealed that NSC can give rise to different types of neural cells: neurons, astrocytes, and oligodendrocytes and recent observations have demonstrated that epigenetic mechanisms play a central role in NSC self-renewal and fate determination under physiological and pathological conditions (Sun et al. 2011; Ariff et al. 2012; Fitzsimons et al. 2014).

The mechanisms leading to neural fate specification from ESC are still being characterized. The initially accepted paradigm was that during embryonic development, specifically gastrulation phase, nascent embryonic ectoderm cells receive signals to adopt a neural fate from mesendodermal tissue, in the absence of which ectodermal cells would differentiate into epidermis. However, later observations were not completely in agreement with this positive induction model, suggesting the existence of a default, autonomous mechanism for the establishment of neural identity from uncommitted ectoderm ESC (Hemmati-Brivanlou and Melton 1994;

Tropepe et al. 2001; Muñoz-Sanjuán and Brivanlou 2002). Therefore, the specification of ESC fate to generate a more committed, less (pluri) potent NSC results from the integration of two sets of signals, one that originates from the cell environment and is affected by systemic factors and a second one which originates from intrinsic signals that operate in a cell-autonomous manner (Edlund and Jessell 1999). Epigenetic mechanisms play a central role in the coordination and integration of these intrinsically different sets of signals and are therefore key for ESC and NSC fate specification towards a more differentiated progeny (Tang et al. 2015).

Importantly, the conditions sufficient for neural differentiation of ESC can be mimicked in chemically defined serum-free, feeder layer-free, low-density culture conditions (Tropepe et al. 2001). Several well established protocols have been described to induce NSC fate from embryonic ESC in culture, followed or not by neuronal differentiation, allowing the development of drug screening assays using ESC derived from human patients suffering severe neurological disorders, such as Alzheimer's, Hungtinton's and Parkinson's diseases and potential regenerative therapies for them (Mountford 2008; Rubin 2008). However, the use of ESC, especially of human origin, has raised ethical concerns (Douglas and Savulescu 2009; Hyun 2014). In this respect, the recent development of techniques allowing the reprograming of human somatic cells into induced pluripotent stem cells (iPSC) and their posterior differentiation into specialized phenotypes has opened new avenues for regenerative medicine and drug screening, which may present less controversial ethical implications (Brown 2013; Sawai 2014). Although the production of human iPSC does not require the use of embryos, a number of ethical issues remain, including consent procedures for somatic cell collection from which iPSC are derived, the potential uses for research, treatment and commercialization and donor privacy (Dasgupta et al. 2014).

The generation of new neurons from resident multipotent NSC takes place in the adult brain principally in two distinct areas, the subgranular zone (SGZ) of the dentate gyrus in the hippocampus, which gives rise to glia and neuroblasts that differentiate into principal dentate granule cells, and the subventricular zone (SVZ) of the lateral ventricle which gives rise to glia and neuroblasts that differentiate into functional olfactory bulb interneurons (Gage et al. 1998; Kempermann 2004). The regulation of adult neurogenesis depends on both intrinsic and extrinsic signals coming from NSC local environments, called neurogenic niches. These niches are composed of various cell types, in addition to the extracellular matrix, including ependymal cells, astroglia, endothelial cells, mature neurons, and immature progeny of adult NSC, which together influence NSC self-renewal and differentiation (Taupin 2006).

Although it is still unclear how adult NSC differentiation is regulated in vivo, it is clear that epigenetic mechanisms are involved in stablishing specific gene expression profiles during differentiation (Ma et al. 2010). Epigenetic mechanisms are defined as the cellular processes that give rise to relatively persistent biological effects such as gene expression stabilization, without alterations in the genome sequence. Currently accepted definitions of the term epigenetic, imply that these persistent effects are preserved across cell division, and thus heritable (Ptashne

2007; Bird 2007). Abiding with these definitions, NSC should be considered as a unique model to characterize and understand gene expression regulatory mechanisms that are truly epigenetic in nature, due to their unique proliferative capacity. Well-characterized epigenetic mechanisms include DNA methylation, chromatin remodeling and transcriptional feedback loops, including non-coding RNAs and histone modifications, however, the heritability of histone modifications has been questioned (Ptashne 2013). Although progress is being made in understanding epigenetic mechanisms, their exact role in the regulation of stem cell self-renewal and differentiation towards a neural progeny remains under intense study. Therefore, in this chapter we will discuss 3 relevant questions, which will help to understand the epigenetic changes taking place in the transition from ESC to tissue specific NSC and their terminally differentiated progeny. In this way, we will discuss epigenetic mechanisms regulating stem cell pluripotency and self-renewal, and the stepwise differentiation from pluri-, to multi-potent, and eventually fully differentiated neural cells.

5.2 Which Epigenetic Mechanisms Are Involved in the Regulation of ESC Self-Renewal, Pluripotency and Differentiation?

Stem cells present several repertoires regarding their differentiating potency. Totipotent stem cells can give rise to the largest variety of cells as they carry the potential to generate a full organism, including the central and peripheral nervous system. Under certain conditions, totipotent stem cells start their differentiation pathway, become more restricted and the variety of cells they can produce is reduced, reaching a pluripotent state. Pluripotent stem cells can give rise to all cells of the organism, including nervous system cells, but they lack the capability to give form and structure to the organism. Following this definition ESC are pluripotent stem cells. As pluripotent ESC progress further into their differentiation pathway, they lose potentiality, become more committed towards specific fates and are therefore known as multipotent stem cells. Multipotent stem cells are capable of producing cells of specific organs, are preserved after embryonic development and are usually defined by the organ from which they are derived from, thus termed tissue-specific stem cells (Gage 2000). Although this progressive loss of potency associated with tissue specificity is well characterized, the precise molecular mechanisms involved in ESC differentiation towards a committed lineage specific stem cell are still been characterized. Highly dynamic transcriptional and epigenetic changes take place during the initial specification of ESC into the three embryonic germ layers and lineage-specific changes in DNA methylation and in histone methylation are present at regulatory elements frequently bound by pluripotency-associated factors in the undifferentiated ESC, suggesting a role in the regulation of pluripotency and differentiation (Gifford et al. 2013). Cell intrinsic signals involving the

transcription factors Oct3/4, Sox2, c-Myc, and Klf4, seem to be essential for ESC pluripotency and thus the nature of the transcriptional and epigenetic machinery involved in preserving this potential is the subject of intense investigation (Takahashi and Yamanaka 2006; Chambers and Tomlinson 2009; Marks et al. 2012).

DNA methylation is an important epigenetic regulator of mammalian development. It has been shown that there is an inverse correlation between the quantity of global DNA methylation and the state of differentiation, with more differentiated cells having lower levels of methylation and undifferentiated cells displaying the highest degree of methylation in regions of the DNA where a cytosine nucleotide is not immediately followed by guanine nucleotide (non-CpG sites) and lower levels promoter methylation in regions of the DNA where a cytosine nucleotide is immediately followed by guanine nucleotide (CpG-rich sites) in ESCs, perhaps to preserve gene expression in the multipotent state (Mohn et al. 2008; Laurent et al. 2010). Particularly, the brain presents a complex epigenetic landscape that changes dynamically with transcriptional activity during development and several neurodevelopmental disorders are associated with genetic alterations in epigenetic regulators (LaSalle et al. 2013). DNA methylation occurs at the cytosine residues, generating 5-methylcytosine (5mC) and this process is regulated by several DNA methyltransferases (DNMTs), responsible for catalyzing DNA methylation. In the simplest model of the dynamic process of DNA methylation, the methyl group is first introduced in the DNA by *de novo* methyltransferases from the DNMT3 family during development, which transfer methyl groups from S-adenosyl-L-methionine to unmethylated DNA targets. DNMT1 recognizes hemi-methylated DNA and maintains DNA methylation of the daughter cell after DNA replication, thereby functioning as a methylation housekeeper (Jeltsch and Jurkowska 2014). DNMT3a or 3b knockdown during embryonic development leads to a reduction in DNA methylation in Oct4 and Nanog and affects the expression of Oct4 and Nanog in pluripotent stem cells, indicating a cooperation of the two DNMTs in establishing methylation patterns in crucial potency genes during development (Li et al. 2007). However, DNMT3a and 3b are dispensable for the reprogramming of somatic cells to iPSC (Pawlak and Jaenisch 2011), suggesting that de novo methylation by DNMT3a and 3b may be required to restrict pluripotency associated with neural lineage commitment and subsequent terminal differentiation (Mohn et al. 2008).

Beyond DNA methylation, other coordinated epigenetic mechanisms regulate the expression of pluripotency genes. The histone H3K4/K9 demethylase Lysine selective demethylase 1 (LSD1) plays a role in embryonic stem cell differentiation. LSD1 is present at enhancer regions acting on Oct4, Sox2 and Nanog, and it may be required for silencing pluripotency genes in ESC. Moreover, LSD1 occupies enhancers of pluripotency genes and reduces the levels of modified histones (methylated; H3K4me1 and acetylated; H3K27ac), hence indicating that LSD1 decommissions enhancers during ESC differentiation through coordinated demethylation and deacetylation events (Whyte et al. 2012). These results suggest that LSD1 may be an important regulator in the repression of pluripotency genes in ESC, associated with the transition towards a more differentiated cellular state.

Other epigenetic regulators, the polycomb group proteins (PcG), are essential for early development in mammals. PcG proteins are transcriptional repressors that help to maintain cellular identity, remodeling chromatin by forming polycomb repressor (PCR) complexes, such as PCR1, composed of several Ring, Phc, Cbx, Bmi1 and/or Mel18 proteins; and PCR2, which is composed of Ezh2, Eed and Suz12 proteins (Schwartz and Pirrotta 2013). PCR2 catalyzes histone H3 lysine-27 (H3K27) methylation through Ezh2, which recruits PRC1. PCR1 has chromatin remodeling activity, thereby silencing gene expression (Schwartz and Pirrotta 2013). In ESC, PCR complexes repress a large number of developmental regulators. Interestingly, more differentiated ESC express more PcG target gene transcripts than undifferentiated ESC, suggesting that PcG proteins play a specialized role in silencing ESC genes associated with differentiation and pluripotency (Boyer et al. 2006). Complementing these results the PRC2 subunit Suz12 binds to a set of developmental genes that have to be repressed to maintain pluripotency and may be primed for activation during differentiation in ESC, indicating that PCR2 is important for the maintenance of ESC pluripotency and repression of differentiation (Lee et al. 2006). However, knockout of Eed, another component of PCR2, induced overexpression of developmental genes and ESC remained functionally pluripotent, suggesting that PCR2 is dispensable for maintenance of ESC pluripotency (Chamberlain et al. 2008). To integrate these apparently contradictory observations Chamberlain and colleagues suggested a positive-only model of ESC maintenance where the positive regulation of a minimal set of pluripotency factors is sufficient to mediate ESC pluripotency and thus the suppression (by PCR2) of factors relevant for differentiation is not necessary. From the three core PCR2 subunits, Ezh2 is the catalytic subunit of the complex and therefore assumed to be key for PCR2 functionality. In agreement with this concept, Ezh2 regulates the expression of factors promoting self-renewal and preventing differentiation of ESC, such as Nanog in pluripotent ESC, and chromatin immunoprecipitation revealed that Ezh2 is directly responsible for H3K27 trimethylation at the Nanog promoter. Importantly, analysis of cell number, cell cycle phase distribution and BrdU labeling indicated that Ezh2 did not influence proliferation, suggesting a possible regulation of the balance between self- renewal and differentiation, by acting on specific differentiation programs (Villasante et al. 2011). Supporting this conclusion, several other subunits of PRC2, including JARID2, MTF2 and esPRC2p48 are highly expressed in ESCs and strongly depleted during differentiation (Zhang et al. 2011). PcG proteins usually work in concert with proteins from the Trithorax group (TrxG) to establish stable expression states of silent and activated genes, respectively, over many cell generations. This phenomenon, termed epigenetic memory, is essential for the correct development and maintenance of differentiated cellular states (Ringrose and Paro 2004; Steffen and Ringrose 2014), and plays an important role in ESC and NSC differentiation.

Histone acetylation and deacetylation are also important chromatin modifications regulating gene expression. Histone deacetylases (HDACs) are highly conserved enzymes regulating deacetylation levels of N-terminal tails of the core histones H2A, 2b, 3 and 4. Deacetylation of these histones leads to tightening of the chromatin

and reduced gene expression. Two relevant HDACs, HDAC1 and 2, work together in mammalian cells and are involved in ESC differentiation, however, HDAC1 seems to play a more relevant role in this process (Dovey et al. 2010). The functional relevance of histone acetylation and deacetylation in the regulation of ESC pluripotency and fate determination will be discussed in more detail in further sections.

Non-coding RNAs are engaged into truly epigenetic mechanism regulating gene expression through interaction with transcription factors and chromatin modifiers (Liebers et al. 2014). This diverse class of RNA molecules, including small and long non-coding RNAs, are key regulators of gene expression and genome stability and in many cases they act by modifying chromatin structure and silencing transcription (Lee 2012; Holoch and Moazed 2015). A complete overview of these RNAs, their functions and mechanisms is beyond the scope of this chapter, therefore we will focus on microRNAs, approximately 22 nucleotides-long small non-coding RNAs, possibly the best characterized members of this family of gene expression regulators. There seems to be a unique microRNA expression pattern in ESC, indicating a specific role for several non-coding RNAs in ESC (Suh et al. 2004). As a particularly interesting example of the complex role of microRNAs in ESCs, the microRNA 290 cluster is important in regulation of differentiation. Inhibition of microRNA cluster 290 resulted in an earlier depletion of Oct4 upon differentiation and, in addition it inhibited mesoderm formation of ESC, by regulating several genes involved in mesoderm formation. The microRNA 290 cluster was also shown to regulate Wnt signaling, a pathway regulating pluripotency and differentiation towards mesoderm in ESC, through inhibition of Dkk-1 (inhibitor of Wnt) subsequently resulting in higher C-myc expression (Zovoilis et al. 2009). Furthermore, the microRNA 290 cluster regulates DNMTs expression, most likely by regulating common transcriptional repressors and thus orchestrating differentiation (Sinkkonen et al. 2008). As a further illustration of the complexity of microRNA-mediated regulation in ESC, the microRNA Let-7 is associated with down regulation of Oct4, Nanog and Sox2 and suppression of ESC self-renewal, while Let-7 is in turn inhibited by members of the microRNA 290 cluster, indicating opposing effects on differentiation and self renewal. Therefore a self-reinforcing loop may exist in ESC, by which the microRNA 290 cluster increases Myc and Lin28, subsequently blocking Let-7, Myc, Nanog, Oct4 and Sox2 during the self renewing state. On the contrary, during the differentiating state, loss of Nanog, Oct4 and Sox2 results in loss of Lin28 and thus upregulation of Let-7, which in turn suppresses Lin28 and Myc, thus preventing up regulation of microRNA 290 cluster and inhibiting downstream targets of Nanog, Sox2, Oct4 and Tcf3 (Melton et al. 2010). Another microRNA cluster, 302–367, gives rise to microRNA 302a-d and 367 from a single transcript, highly expressed in ESCs. microRNA cluster 302–367 is downstream of pluripotency genes Sox2, Oct 3/4 and Nanog, suggesting a possible role for this cluster in ESC differentiation (Card et al. 2008; Barroso-delJesus et al. 2008). Although there are several examples of individual microRNAs playing substantial roles in the regulation of genes involved in self-renewal and differentiation of ESC, the examples highlighted before suggest that the coordinated action of several microRNAs encoded in genomic

clusters and targeting groups of overlapping targets is crucial in ESC fate decisions. Indeed, the convergent action of microRNAs is crucial in NSC during embryonic development (Barca-Mayo and De Pietri 2014).

Overall, in this section we have discussed multiple epigenetic mechanisms involved in the regulation of ESC fate decisions between pluripotency and differentiation. DNMT 3a and 3b cooperate in gene silencing through de novo methylation, PCGs influence the expression of pluripotency, differentiation and self-renewal genes, HDAC1 regulates ESC differentiation and LSD1 regulates enhancer activity at Oct4, Sox2 and Nanog, by coordinately reducing the levels of modified (methylated and acetylated) histones. Finally, several microRNA clusters play a role in coordinating pluripotency and differentiation decisions in ESC. All these mechanisms strongly emphasize the relevance of epigenetic mechanisms, and their interactions, in the regulation of ESC self-renewal, pluripotency and differentiation.

5.3 Which Epigenetic Mechanisms Determine the Transition Between a ESC and a NSC?

As we described in the previous section, a significant amount of research is being performed on the factors regulating pluripotency and differentiation of ESC. However the factors and mechanisms regulating specific neural induction in ESC are less clearly elucidated. In this next section we will try to elaborate on the existing data and discuss the epigenetic mechanism involved.

Although ESC and NSC from a single individual share a common genome, their function, potency and self-renewal are remarkably different. Neural differentiation is essentially unidirectional and brings upon a concurrent loss of progenitor potential and gain of differentiated phenotype. As ESC differentiate, lineage specific genes need to be activated or repressed. After induction of neural differentiation, ESC repress expression of pluripotency genes followed by a coordinated activation of neural progenitor and differentiated neuronal and glial genes, and epigenetic mechanisms play a key role in this process. In fact, ESCs and lineage committed cells present diverging epigenomic landscapes and these landscapes are considered as a read-out of cellular potential. This concept is clearly manifest in ESC, where promoters of lineage-specific genes are marked by bivalent epigenetic signatures composed of trimethylation of both lysine 4 and lysine 27 of histone H3 (H3K4me3 and H3K27me3, respectively). As described before, H3K27me3 is the result of Ezh2 enzymatic activity at inactive gene promoters and acts in opposition to H3K4me3, which is associated with transcriptional start sites of actively transcribed genes (Barski et al. 2007). Therefore, a bivalent histone trimethylation state is considered an epigenetic mark dedicated to preserve lineage-specific regulators in a transcriptionally silent yet poised state, ready for immediate expression upon receipt of an appropriate cue, and thus a powerful epigenetic indicator of stem cell potential. Upon differentiation, bivalent histone trimethylation marks are lost or resolved

into monovalent signatures, characteristic of repressed or active genes in the specific lineage of the progeny, including NSC (Burney et al. 2013). H3K27 methylation at neural specific genes (Olig1, Olig2, Olig3 and Nes) is severely depleted in NSC compared to ESC, and this depletion is established upon differentiation. Jmjd3, a H3K27 specific demethylase up regulated during ESC differentiation, is important for ESC commitment into the neural lineage. Jmjd3 binds promoters of genes known to be expressed in NSC, such as Nestin, Pax6 and Sox1 (Burgold et al. 2008).

Other epigenetic mechanisms play key regulatory roles in the transition to NSC. PcG complexes seem to have a specific role in differentiation of ESC into neural lineage. During neuronal differentiation, PcG targets are much more likely to receive de novo methylation, suggesting that de novo methylation at PcG targets is important for the restriction of pluripotency and neural commitment of ESC (Mohn et al. 2008). Repressor Element 1 Silencing Transcription (REST), is a transcription factor which targets multiple genes in ESC including several neural genes. REST functions through binding to RE1 sites on the DNA and recruiting cooperative factors such as co-REST, mSin3 and epigenetic regulators such as HDAC and MeCP2, thereby repressing target gene expression and in this way regulates the transition from ESC to NSC and later from NSC to mature neurons (Ballas et al. 2005). REST knockdown in ESC resulted in a loss of pluripotency and neural induction, with precocious repression and activation of neural progenitor genes, a delayed expression of pluripotency and an early and sustained peak of neuronal and glial gene expression, indicating that REST coordinates the timely repression of pluripotency and induction of neural differentiation (Soldati et al. 2012).

microRNA regulation plays a role in the differentiation from ESC to NSC. Analysis of ESC lines with neural propensity showed a negative correlation between PAX6 expression and microRNAs of the 371–3 cluster, suggesting an association of this cluster with differentiation towards the neural fate (Kim et al. 2011). The microRNA 302–367 cluster, earlier described as a regulator of pluripotency in ESC, also regulates differentiation towards the neural lineage. Members of the 302–367 cluster promote BMP signaling, which may promote pluripotency by preventing neural induction (Lipchina et al. 2012). The coordinated role of several microRNAs in fine tuning BMP signaling during ESC differentiation is further supported by observations that microRNA 125 is inhibited upon induction of BMP signaling. In the presence of low BMP activity microRNA 125 is not repressed and under these conditions microRNA 125 may promote ESC specification towards the neural lineage by repressing alternative cell fate choice (Boissart et al. 2012).

Although the exact regulatory epigenetic network promoting ESC specification towards the neural lineage has yet to be unraveled, the studies discussed in this section represent relevant pieces of the puzzle. Many of the observations discussed suggest that rather than directly activating neural induction mechanisms, repression of other alternative lineages is crucial for neural commitment of ESC, in agreement with the default mode hypothesis, which states that ESC will differentiate into the neural lineage unless signals instruct otherwise (Muñoz-Sanjuán and Brivanlou 2002).

5.4 Which Epigenetic Mechanisms Determine the Transition Between a NSC and a Differentiated Neuron or Glial Cell?

NSC can differentiate towards neurons or glia cells, mainly astrocytes and oligodendrocytes. In terms of neuronal differentiation, or neurogenesis, during embryonic development multipotent progenitors that reside in the germinal zones along the developing neural tube generate several hundreds of distinct neuron types that compose the adult nervous system. Moreover, in the adult brain, neurogenesis persists in the pro-neurogenic microenvironment provided at specific neurogenic niches. The study of epigenetic mechanisms that regulate NSC commitment into their differentiated progeny is of interest because it allows to understand how the structural adaptation of chromosomal regions may register, signal or perpetuate gene expression states, providing a rationale to understand the concept of cellular memory (Bird 2007; Burney et al. 2013). In this section we will discuss epigenetic mechanisms regulating the transition from NSC towards a terminally differentiated neuron or glia cell.

5.4.1 Neuronal Differentiation

DNA methylation is a likely mechanism through which differentiation can be made terminal and irreversible in NSC and it is a major contributor to the epigenetic regulation of NSC differentiation (Hirabayashi and Gotoh 2010). Inhibition of methylation in well-characterized models of neuronal differentiation results in abnormal cellular morphologies, thus indicating a role for methylation in cell fate determination during NSC differentiation (Hong et al. 2008). In particular, DNMTs play a crucial role in the regulation of these DNA methylation events. DNMT3a is expressed in immature neurons during embryonic development and is increased in post mitotic neurons, suggesting a central role in neurogenesis. During the early neurogenic phase DNMT3a is primarily expressed in the ventrical and subventrical zone of the cerebral cortex. However, in the adult cortex abundant DNMT3a positive cells are detected in the cortical neuronal cell layers, and null or sporadic expression is present in glia cells, suggesting a specific role of DNMT3a in neurogenesis. Nevertheless DNMT3a levels in the adult cortex appeared to be lower than during development, this downregulation may be linked to terminal differentiation and therefore no longer in need of *de novo* methylation by DNMT3a in large areas of the adult brain (Feng et al. 2005). Methylated DNA-binding proteins play a significant role in the regulation of DNA methylation during NSC differentiation. Methyl CpG binding protein 1 (Mbd1) removal resulted in a significant inhibition of neuronal differentiation of NSC, indicating that Mbd1 selectively regulates neurogenesis and NSC proliferation (Li et al. 2008). Another methyl binding protein, Mecp2, is expressed in differentiating NSC displaying neuronal markers but not in

cells displaying astrocyte markers, indicating a specific role in neuronal differentiation. Moreover, Mecp2 suppresses astrocyte gene expression and thereby reduces astrocyte differentiation through binding on a hypermethylated exon1 region of the astrocyte-specific genes S100β and GFAP (Setoguchi et al. 2006). Regarding its direct effects on neuronal differentiation, although Mecp2 may not be absolutely required for neurogenesis, it plays an important role in proper maturation of transitioning neurons and NSC differentiation (Smrt et al. 2007; Tsujimura et al. 2009). Taken together these studies suggested that Mecp2 regulates NSC differentiation towards the neuron fate by inhibiting alternative astrocytic differentiation and promoting neuronal differentiation.

The concerted action of PcG and TrxG proteins confers long-term, mitotically heritable epigenetic memory by promoting and sustaining silent and active gene expression states, respectively (Steffen and Ringrose 2014). The TrxG member mixed-lineage leukaemia 1 (Mll1) is required for neurogenesis in the adult brain. Mll1 deletion is associated with volume reduction in all brain areas where neurogenesis takes place in the adult brain, together with decreased levels of neurogenesis and neuroblast migration. Importantly, Mll1 deletion has no effects on gliogenesis, indicating a specific role in neurogenesis and Mll1 is associated with preservation of H3K27me3 mark in bivalent promoters, thus contributing to bivalent state characteristic of non-commited, pluripotent stem cells (Lim et al. 2009). Emphasizing the important role of H3K27me3, the histone demethylase JMJD3 promotes specific histone H3 trimethyl K27 demethylation in NSC, and this seems to activate pathways towards neuronal differentiation (Jepsen et al. 2007). However, it still needs to be resolved how this activity dependent DNA methylation leads to long term changes in adult neurogenesis (Wu and Sun 2009).

Valproic acid decreases NSC proliferation and promotes neuronal differentiation, probably through its well-characterized inhibition of HDACs. HDAC inhibition induces neuronal differentiation (Hsieh et al. 2004; Balasubramaniyan et al. 2006; Yu et al. 2009), whereas deletion of HDAC1 and 2 blocks neuronal differentiation of NSC (Montgomery et al. 2009), but HDACs also increase embryonic neurogenesis inhibiting BMP signaling (Shakèd et al. 2008). These seemingly contradictory results may reflect distinct roles and expression patterns of HDAC1 and 2 in different brain areas. HDAC 1 is expressed in NSC and glia, whereas HDAC2 is expressed preferentially in neurons. Although HDAC1 is highly expressed in NSC it is downregulated upon neuronal differentiation and HDAC2 is upregulated as NSC differentiate into neurons, therefore suggesting different roles for HDAC1 and 2 in neuronal differentiation (MacDonald and Roskams 2008).

The involvement of microRNAs in NSC differentiation has been extensively documented and several individual microRNAs, among which microRNA 124, 9, 137, 34a, 132 play well characterized roles in the regulation of neurogenesis (reviewed in (Schouten et al. 2012)). In some prominent cases, microRNAs, for example microRNA 124, control the expression of epigenetic regulators and transcription factors with crucial roles in neuronal differentiation of NSC, such as REST. Moreover, microRNA 124 is involved in complex regulatory feedback loops with REST, which binds to a RE1 site present on the microRNA 124 enhancer and

thereby represses microRNA 124 expression. Upon NSC differentiation, REST-induced repression is released, restoring microRNA 124 levels and repressing REST expression via its target small C-terminal domain phosphatase 1 (SCP1, an anti neural factor) (Conaco et al. 2006; Visvanathan et al. 2007). However, in agreement with the postulated relevance of a coordinated action of several microRNAs in ESC differentiation we discussed in previous sections, we will highlight a number of examples that indicate that microRNA clusters play a significant role in the acquisition of neuronal fate in NSCs. In the developing cortex, members of the microRNA 379–410 cluster regulate neurogenesis and neuronal migration by fine-tuning N-cadherin expression, which is essential for maintaining the tissue architecture and stem cell niche (Rago et al. 2014). Another interesting example is presented by the interaction between the Nanog transcription factor and members of the microRNA 17–92 cluster. As discussed in previous sections, Nanog plays a central role in the regulation of ESC self-renewal, as well as in NSCs. The mechanism involves Nanog controlling microRNA 17–92 cluster expression by binding to an upstream regulatory region in its enhancer and maintaining high levels of transcription in NSCs, whereas Nanog binding and expression of the microRNA cluster are lost alongside differentiation into the neuronal fate (Garg et al. 2013). On the other hand, microRNAs from the microRNA 17–92 cluster are crucial for the maintenance of NSC proliferation and modulation of the initial NSC fate decisions to generate neural progenitors in the developing neocortex. Specifically, the microRNA 17–92 cluster is required for maintaining proper populations of cortical radial glial cells and intermediate progenitor cells, both still proliferative populations, through repression of Pten and Tbr2 protein expression (Bian et al. 2013).

In summary, the crucial step from NSC towards a neuronal fate is regulated by several epigenetic mechanisms. In this section, we discussed several studies demonstrating how these mechanisms also influence and interact with each other, providing clear examples of the complexity of epigenetic regulation in NSCs. Yet, a central property of NSCs is their capacity to acquire multiple cellular fates upon differentiation, therefore, we will next discuss the epigenetic mechanisms involved in NSC differentiation towards glia.

5.4.2 Glial Differentiation

Glia cells are the most numerous cells in the brain and play many essential roles. Glia cells are traditionally divided into micro- and macroglia, the latter consisting of astrocytes and oligodendrocytes. Astrocytes are important, among other functions, for metabolic, trophic and structural support of neurons while oligodendrocytes are the myelin forming cells. Mechanistically, differentiation of NSC towards the glia lineage is closely related to neuronal lineage commitment, however, we will discuss several mechanisms important for the specific determination of the glial fate, and for the switch between neurogenic and astrogenic phases in NSC.

As in all NSC differentiation steps discussed before, DNA methylation is crucially important in astrocytic development. DNMT1 and DNMT3a deletion results in an enhanced and precocious astroctytic differentiation during brain development, which is blocked by DNMT1 overexpression. These observations suggested that deletion of DNMT1 results in premature activation of the astroglial differentiation program known to be induced by JAK/STAT pathway (Fan et al. 2005; Wu et al. 2012). This is important because most of the gene promoters in the JAK/STAT pathway contain a STAT binding site which allows for a positive feedback loop, important for the fast upregulation of this pathway during the switch from the neurogenic to the gliogenic phase (He et al. 2005). Furthermore, DNMT1 deletion in NSC results in hypomethylation of STAT binding sites at promoter of astrocytic genes, such as GFAP and S100β, thereby promoting their expression. Under normal conditions, GFAP, Stat1 and S100β are repressed by MeCP2, but when methylation levels are reduced and astroglial differentiation is evident, STAT3 binding predominates over MeCP2. Overall, promoters of glial genes change from a hetero- to an eu-chromatin state, lose methylation marks and MeCP2 occupancy is substituted by STAT3 during the switch from neurogenic to gliogenic fate, demonstrating a key role for DNA methylation, through modulation of the STAT pathway in this transition (Fan et al. 2005).

Additionally, PcG proteins play important roles in astrocytic development as well. The Wnt signaling pathway is involved in NSC differentiation and specification towards neurogenic and oligodendrocytic fates and may be altered in neural progenitor cells derived from iPSC obtained from Schizophrenia patients, suggesting a role in this disease (Azim et al. 2014). Wnt signaling activity promotes neurogenic fate at least partially through activation of neurogenin 1 and 2 (ngn1 and ngn2). However, ngn1 continues to be activated during the astrogenic phase, suggesting a possible intermediate step between Wnt activation and ngn1 expression, during which PcG proteins are engaged. Interestingly, cells in the astrogenic phase are less sensitive to the neuronal differentiating effects of the Wnt pathway and this was associated with reduced H3K9Ac and increased H3K27me3 at the ngn1 promoters. These results indicate that epigenetic regulation of the Wnt signaling pathway involving members of the PCR1 and PCR2 complexes inhibits neuronal differentiation through suppression of ngn1, and promotes neurogenic to gliogenic fate transition in NPC during embryonic development (Hirabayashi et al. 2009). Moreover, PCR2 activity is key for NSC fate decisions. Ezh2 is downregulated during NSC differentiation into neurons and astrocytes, but not into oligodendrocytes, suggesting a specific role in oligodendrocyte differentiation by suppression of neuronal and astrocytic genes (Sher et al. 2012).

Several other epigenetic modulators that induce histone modifications influence gliogenesis. For example, ESET (also known as Setdb1 or KMTE1) is a H3K9 methyl transferase, which induces gene repression in cooperation with KAP1, a corepressor factor also known as Trim28. ESET is highly expressed in proliferating cells of the ventricular zone, but not in cells already committed to the neuronal fate. Moreover, ESET controls the expression of neuronal genes and it is downregulated in genes committed to the astrogenic fate. Furthermore, deletion of ESET induces

regulation of gliogenic related genes, overall suggesting that ESET is important for the induction of neuronal and suppression of gliogenic genes (Tan et al. 2012). Brahma-related gene 1 (Brg1), is a chromatin remodeling factor important for the NSC fate determination. Deletion of Brg1 in mice results in a precocious neuronal differentiation leading to deficits in astrocyte and oligodendrocyte differentiation. Brg1 is involved in the neurogenic to gliogenic switch through repression of neuronal differentiation and permissiveness towards glia differentiation, affecting stem cell maintenance. However, the mechanism of action of Brg1 in this context remains to be fully elucidated (Matsumoto et al. 2006).

The involvement of microRNAs in gliogenesis has been demonstrated using Dicer null mice, in which oligodendrogenesis and astrocytogenesis were disrupted after Dicer ablation, leading to the conclusion that microRNAs play a role in astrocyte and oligodendrocyte formation (Zheng et al. 2010). During oligodendrocyte differentiation several microRNA are coordinately upregulated, including microRNAs 219, 138, and 338 (Letzen et al. 2010; Dugas et al. 2010). The most induced of these, microRNA 219, is necessary and sufficient to promote oligodendrocyte from precursor cells. After microRNA depletion, microRNA 219 induction partially restores oligodendrocyte differentiation by targeting genes important for NSC precursor proliferation (Dugas et al. 2010). Although these studies indicate the importance of microRNAs in gliogenesis, and specifically oligodendrocytogenesis, the topic has been much less studied than microRNA involvement in neuronal differentiation, which warrants further studies to elucidate their specific mechanisms and targets.

The studies discussed before have identified several epigenetic factors playing a role in differentiation towards the astrocytic lineages. However, it is still unclear how this astrocytic fate is maintained. BMP signaling and induced transcriptional suppressors might play a role in sustained neuronal lineage suppression in astrocytes. A possible downstream candidate is NSRF/REST, which is up regulated by Bmp2 in NPC to suppress neuronal genes. Bmp2 induces NSRF/REST expression in NPC through smad1 signaling, but sustained expression is probably established by H3Ac and H3K4me3, indicating that Bmp2-mediated NSRF/REST induction is involved in the establishment and maintenance of astrocytic identity (Kohyama et al. 2010).

In summary, in this section we have discussed several observations describing the involvement of epigenetic mechanisms regulating neurogenesis and gliogenesis. It is clear that DNA methylation, proteins from the PcG, histone modifications and microRNAs all play an important role in controlling NSC fate decisions leading to the formation of neurons or glia cells. In addition, it should be mentioned that neurogenesis and gliogenesis are two processes closely related to each other and that modulation of the neurogenic program has a direct effect on the gliogenic program, possibly through the coordination of common epigenetic mechanisms as those discussed in this section.

5.5 Conclusions and Future Perspectives

NSC hold great promise for future treatment and therapeutic interventions for neurodegenerative disorders, such as Epilepsy, Alzheimer's and Huntington's disease, due to their ability to self-renew for the whole lifespan of the individual and their potential to generate the main neural types, astrocytes, oligodendrocytes and neurons. A significant number of epileptic patients do not react to current pharmacological treatments and may, especially in the setting of medically-intractable epilepsies, experience in time cognitive impairments, behavioral abnormalities or psychiatric symptoms. As a result, in these patients epilepsy may resemble a neurodegenerative disease (Ono and Galanopoulou 2012). Several structural and functional alterations underlie the characteristic hyperexcitatory state of the hippocampus in multiple epileptic disorders. These include loss of hilar interneurons, mossy fiber sprouting, and the birth and integration of aberrant neurons, together resulting in a excitation/inhibition disbalance (Bielefeld et al. 2013). The great potential of regenerative therapy with NSC holds especially for complex epilepsy pathologies, as the incorporation of neurons derived from engineered NSC could be used to overturn the excitation/inhibition disbalance associated with epilepsy. Due to the presence of native NSC, the hippocampus seems a particularly interesting target for such regenerative approaches. Indeed, over the past few years, several strategies have been undertaken in preclinical models of temporal lobe epilepsy. The first attempts in stem cell therapy in the epilepsy field made use of NSCs, ESCs or NPCs that were directly injected into the hippocampus after the induction of SE (Rüschenschmidt et al. 2005; Hattiangady et al. 2008; Waldau et al. 2010). These stem cell populations showed survival, integration and differentiation into both neurons and glia, and were also partially able to control the epileptic phenotype. Another study showed that when transplanting ESC-derived NPCs into the epileptic hippocampus, these preferentially differentiated into GABAergic interneurons, providing possible mechanistic support for the success of stem cell transplation in preclinical epilepsy models (Maisano et al. 2012). The use of GABAergic precursor cells obtained from the medial ganglionic eminence showed similar results, with transplantation able to almost completely rescue the occurrence of spontaneous seizures (Hunt et al. 2013; Cunningham et al. 2014). With the ongoing increasing knowledge of stem cell biology and its regulation, the potential for successful NSC transplantation also increases. The development of human iPSC and human induced neurons (hiNs) from fibroblasts both in vitro and in vivo (Torper et al. 2013; Pereira et al. 2014), which are able to survive transplantation and maintain their neuronal fate in the adult rat brain, have generated the possibility to engineer human adult cells into pluripotent stem cells, providing a broad and interesting technical window for stem cell-based transplantation approaches. Furthermore, with the development of these new techniques that do not require human embryonic stem cells, the common ethical concerns around the use of ESC discussed before may be overcome, providing a potential application for NSC-based regenerative therapies.

Although the studies discussed in the previous paragraph show great potential, stem cell therapy in the epilepsy field is not as developed as with other neurodegenerative disorders, where stem cell therapy already shows great preclinical and evident clinical success (Petit et al. 2014; Grealish et al. 2014, 2015). Cell death is one of the most prominent hallmarks of Alzheimer's disease (AD), further affecting the endogenous NSC pool in the adult brain and impairing its potential regenerative capabilities (Mazur-Kolecka et al. 2006). Accordingly, cell replacement therapies have long been considered a viable strategy for the treatment of AD. Numerous preclinical studies have been conducted in which mouse models of AD were injected with NSC grafts to assess alleviation of AD symptoms (Tong et al. 2014; Kim et al. 2015). While these approaches have shown promising results in terms of cognitive improvement in mouse models of AD, there are still many relevant hurdles to overcome before such cell-based therapies could be translated to humans. One such hurdle is the source of transplantable NSC. Previously, ESC have been considered a readily available source from which NSC could be obtained for therapeutic use, although with quite some ethical controversy. Another, potentially limitless source of transplantable NSC and particularly promising new avenue may be presented by the use of iPSC-derived NSC. As discussed in previous sections of this chapter, current advances in the epigenetics field have provided a careful description of the epigenetic modifications that take place during ESC differentiation towards NSC and subsequent neuronal lineage. While controlled differentiation of iPSCs has yielded NSC that are in most functional aspects similar to endogenous ones, a full picture of their epigenetic landscape has not been fully characterized for AD patients, yet (Israel et al. 2012; Goldstein et al. 2015). In this respect, one critically important caveat still to be addressed is whether iPSC derived from AD patients contain epigenetic traits that represent the disease and could emerge problematic during implantation of NSC derived from them. As such, current research focuses on the molecular characterization of NSC derived from iPSC of AD patients. This approach might also be productive for the purpose of diagnosing AD. Indeed, currently AD is only faithfully distinguished from other non-AD related cognitive impairments in post-mortem analysis. According to the current consensus, there may be significant amount of epigenetic alterations that contribute to the development of AD (Gjoneska et al. 2015). As such, one could hypothesize that potential future treatment and diagnostics may emerge from the careful interpretation of these epigenetic changes. Importantly, with recent advances in the establishment of the human reference epigenomes, patient-derived iPSC can now be analyzed for source tissue "epigenetic memory" as well as compared to tissue and cell specific epigenomes (Roadmap Epigenomics Consortium et al. 2015).

In this chapter we have discussed a significant amount of data demonstrating that many epigenetic mechanisms and components play central parts in the transition from ESC to a committed neural specific cell that can be regulated and specified towards the main neural types. One of the patterns emerging from the literature discussed herein is that epigenetic mechanisms converge and interact with signaling pathways important in NSC self-renewal and fate determination, such as the Wnt pathway, the Notch pathway and BMP signaling. In this respect, epigenetic

mechanisms of gene expression regulation seem to be crucial in fine tuning the expression of several components of these signaling pathways, thus modulating the intensity of the signals transduced, suggesting a paramount role in the coordination between cell extrinsic signals with cell- intrinsic proliferation and differentiation programs. However, the epigenetic network we picture in this chapter still presents many lose ends. In the future, these may be understood better by unraveling common regulators of multiple pathways, which may contribute to understand the complexity of signals that control the final production of a differentiated neural progeny. This is an important concept because the sole enhancement or suppression of lineage specific genes may not be sufficient for full functional differentiation. Most studies presented indicate that a certain epigenetic mechanism could induce pluripotency or differentiation towards a certain lineage. However this pluripotency or differentiation is mostly measured by expression of a limited amount of genes. Thus the network of modifiers and regulators involved in whole transcriptome regulation needs to be extended and mechanisms for controlling the steps towards terminal differentiation (and its reversibility) need to be characterized. Although it is without doubt that epigenetic modifiers are important, the environmental triggers influencing these modifiers are less well characterized. Environmental triggers could have multiple targets and modify the activity of several signaling pathways simultaneously, thereby influencing a variety of epigenetic modifiers and inducing functional differentiation. Further understanding and integration of these mechanisms is therefore important for finding novel therapeutic strategies for application of regenerative medicine to the brain.

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Chapter 6
Mathematical Models in Stem Cell Differentiation and Fate Predictability

Wayne M. Eby and Natalia Coleman

Abstract We review recent mathematical models in stem cell differentiation with a focus on the role of epigenetics in cell fate determination. Gene regulatory networks can be described as a dynamical system. Within this high-dimensional system cell states correspond to attractors. This study spotlights the quasi-potential landscape to represent the transition between cell states as functions of stochastic and deterministic influences. Furthermore, we will investigate how these models apply to the area of neural differentiation, with a focus on the role of the Sonic Hedgehog (Shh), Notch, and Wnt pathways. Finally, we will discuss the epigenetic landscape model in relation to cancer and cancer stem cells.

Keywords Epigenetic landscape • Mathematical model of differentiation • Stochasticity • Neural differentiation • Notch pathway

6.1 Introduction

One of the central problems in stem cell biology is to attain a deeper understanding of the differentiation process and the potential control of cell fate determination. Despite the accumulation of large amounts of data on adult stem cell differentiation in different organs, our understanding of how the adult biological system initiates, directs, and controls the differentiation a stem cell into daughter cells remains limited. This fundamental question has a direct impact on the function of an organism function in both normal and pathological settings.

Mathematical modeling might enhance and enrich our knowledge in this active and significant area of research. The mathematical model represents biological systems or phenomena in two ways: one is an accurate representation in terms of quantities expressible in mathematics, and another one is a prediction, or even in

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silico experimentation. Although accurate representation is valuable by itself, the ultimate goals might be prediction and even in silico experimentation, in terms of equations, mathematical quantities, etc. The use of a mathematical and computational perspective is often based on analysis of large amounts of data, integration of different levels of action, and representation of networks and regulation. In general, systems biology models align with this perspective. Perhaps this is because the epigenetic landscape models are multi-level models of transitions between stable cellular states within a larger dynamical system. The associated energy landscape is based on coordination of activity and state within each cell, and must also coordinate such decisions and events among cells in a tissue.

One of the fascinating aspects of the stem cell research is understanding the role of stochasticity in stem cell differentiation and fate determination. Among other intriguing consequences of this issue, the concept of stochasticity provides interesting ramifications on the stem cell niche composition as an ensemble of all the daughter cells supported/sustained by the stem cells. We will elaborate on the question at what level cells make these decisions and how the stochastic choices of individual cells create the pattern for a higher level of organization, as expressed by Landler (2011).

Our overall goals for the chapter are to present a survey of some of the important models in the area of stem cell differentiation. To address the fundamental philosophical questions of stem cell differentiation and fate determination, we use both biological knowledge and mathematical modeling perspectives. In the context of Waddington's landscape concept and its modern extensions to models of the epigenetic landscape, we will discuss determinism versus stochasticity, and relevant consequences.

We will apply these models to investigate how individual cell fate decisions are made within the context of the larger biological system. We will address the specific questions within stem cell differentiation and fate determination such as epigenetics, gene expression, oscillation, and switching. In general the models should incorporate some of the concepts, machinery, and tools that apply universally to the role of epigenetics in differentiation. In our review, we also will provide insight on how mathematical models applied to specific cases, such as neural differentiation. This issue of neural stem cell differentiation and fate determination is one area of particular interest in stem cell biology. Neural regeneration is the essential question in devastating neurodegenerative diseases such as Parkinson's, Alzheimer's, and others. In the development phase of the nervous system, an integral part is played by the Sonic hedgehog (Shh), Notch, Wnt, and Bone Morphogen Protein (BMP) signaling pathways, and our study of neural stem cell differentiation interacts with interesting issues in these areas. Finally, we will apply epigenetic modeling to cancer stem cells, an important direction of associated research, which can also be profitably studied from the epigenetic perspective and within the context of the major themes of this chapter.

Mathematical modeling attempts to establish links to the underlying biology that can be tested quantitatively and potentially allow expansion of our collective understanding. Ideally the links should be close enough so that discovery within the modeling context can carry over to relevant aspects of the underlying biology, test-

able by experiment. Finally we observe in this review how some of the questions and issues introduced in relation to mathematical modeling can aid in the understanding of the biology. For instance the issue of stochasticity versus determinism in stem cell fate has allowed for extensive cross-fertilization, between the scientific and mathematical perspectives. Particularly in the area of systems biology, a suitable modeling framework, sufficient data, and computational resources can combine to achieve greater understanding.

6.2 Epigenetic Models of Differentiation: Landscape, Stochasticity, and Systems Biology

6.2.1 Introduction and Background in Epigenetics, Stem Cells

In reviewing the types of models that have been developed to address differentiation of stem cells, we begin with a few points of necessary background information. In addition to aspects of stem cells, we also address important background information in the area of epigenetics. In addition we discuss how the systems biology perspective is relevant to this direction in modeling, and we also address these models in relation to switching and genetic and epigenetic regulation.

Some of the fundamental points related to the role of stem cells and their function in the body include their potential for either self-renewal, or for asymmetric proliferation in the formation of precursor cells, and the further differentiation of the precursor cells to form the specialized cells within each body system. A deeper understanding of these functions is fundamental to a more complete understanding of biology, and this research program uses mathematical modeling to help increase knowledge and understanding. Our focus will be on an understanding of differentiation of stem cells, particularly the role of epigenetics, and the use of mathematical models both to represent and to advance knowledge and understanding in these areas. We emphasize that this issue of differentiation is actually a fairly large and diverse topic. We will approach it both from ther perspective of the universal aspects and problems that apply throughout, and we in addition consider specialized cases of neural differentiation in which the specific aspects play an important role. One large distinction within the field of differentiation is the difference between differentiation of adult stem cells through various stages of precursors and beyond, in formation of the fully differentiated cells used within each tissue system, and the differentiation of embryonic stem cells or pluripotent cells in the process of development. Both of these are relevant to the field of regenerative medicine. In the second case of differentiation within the field of development, we find a much larger range of specialized differentiations of cells within developmental processes. Also here we see another underlying difference in concept in the two cases differentiation of adult stem cells and differentiation into cell types within stages of development. Developmental processes have complex sequence of stages of development, each of

which has its own requirements for formation and types of cells to be produced; further these must have some in-programmed course of development that sets up the successive stages—with a very high level of complexity orchestrating all of this. In contrast, in adult stem cells the course of differentiation is fairly static by comparison—either for ongoing support of life, or for homeostasis—although there is a diversity of stem cell differentiation to precursors and successors among different systems and tissues.

In both of these cases we do have some unifying fundamental issues, such as understanding of not only cell fate decision, but also the balance between self-renewal and differentiation, as well as types of cells produced, and how these function together within the cellular niche, as well as the larger biological system. It is a very complicated issue to consider by what means the proper balance is maintained or modulated for homeostasis, or orchestrated as part of a larger program within development. Cell fate models are an important part of these questions, and we may expect them to interact with models of other related questions, including models representing the stem cell hierarchy within the biological system and larger structured population, and also the associated choices of self-renewal versus differentiation, together with our main theme of fate determination for those which differentiate. The survey Eby and Tabatabai (2014) mentions some of the related trends in stem cell modeling, including the relevance of stem cell niche, the relation to signaling and regulatory control within the larger biological system, and the need for models balancing issues of self-renewal, differentiation, and proliferation. We note that the issue of symmetric versus asymmetric division for stem cells also plays an important role in this balance and in organismal control, and this area has been one focus within scientific studies of the role of epigenetics on fate determination. We note that there are direct implications for all of these issues for health and disease, and the epigenetic landscape models of the current chapter are at the center of these same issues. The current chapter also has particular relevance to issues of multipotent and pluripotent cells, either in their original state, or through cellular reprogramming, and we treat some related models. For this issue pluripotent stem cells and for related questions of multipotent adult stem cells, our goal is in part to understand the issue of self-renewal, together with the role of the stem cell niche. Recent research suggests a stable ground state, and we briefly address this issue and its consequences in the associated models.

As differentiation and fate determination correspond directly to biological differences in cell structure, state, and function, the issue of how these differences arise in cells with identical genetic code falls directly within the category classified as epigenetics. See Arney and Fisher (2004) for more information on epigenetics in differentiation. In particular, epigenetics primarily refers to differences in how the genetic code is expressed by applying the machinery of each individual cell. Specifics of means of cellular control include DNA methylation, histone modification, activation or silencing of chromatin proteins, control of gene expression through repressor proteins, and transcription factors. Much of the study of the genes

and their role in the life of an organism has focused on the genetic code, however epigenetics is a critical field which is less fully developed but is currently receiving more attention. However, the role of tuning on and silencing genes Yu et al. (2008) and other aspects of epigenetics have been studied in relation to development of diseases, including cancers, Feinberg and Tycko (2004), where sometimes the genes are present but are not being expressed. As early as the 1940s, innovate work was done by Waddington (1942, 1957) the direction of understanding epigenetics, where he introduced the concept of a metaphorical epigenetic landscape to represent the progression of a cell from a pluripotent state through successive states on its way to its fully differentiated adult cell state. The area of research has experienced a resurgence as biologists are recognizing its importance, and much of the current understanding is based on development and modernization of this landscape concept, as we will see in more detail below. In the article of Huang (Huang 2009a), for instance, the specific approach to epigenetic modeling and the corresponding paradigm makes some of the recent surprises in the field, such as reprogramming and induced pluripotency, to fit nicely into the expected framework. As we review a number of these points below, we will see the value both of modeling and of the proper perspective in understanding stem cell behavior related to epigenetics. Thus, epigenetics is the substance of what me must understand more fully when studying cell states and differentiation, and there are both scientific and modeling bases to understanding and developing this area. In addition to cell states, this course of topics leads directly to important issues in the study of cancer, as has also been recognized in the field, and we will address this point further later. It is also interesting to remark how the epigenetic landscape approach and the underlying relations between genotype and phenotype form an interesting new direction of inquiry in evolution in work such as Moore (2012), Huang (2012a), and Feinberg and Irizarry (2010). In the context of the modeling work we review below in stem cell differentiation, some of the important issues that arise include heterogeneity, multicellularity, and stochasticity, all of which are important current trends in work in epigenetics.

Within the study of epigenetics and its role in both cellular function and fate determination for cells, much of the activity falls within the realm of coordination of differentiation both at the level of the individual cell and among cells within the biological system. As we are studying cell fate determination from the modeling perspective and looking at the role of computation, mathematical modeling, and use of gene expression data in understanding of this issue of differentiation, we are already moving within the realm of systems biology. In fact, systems biology appears to be the natural setting for consideration of these problems, both from the perspective of the role of wide range of players and of scales within differentiation, and also because of the emergence of this larger phenomena of orchestrated differentiation on the organismal level from the multiplicity of reductionist levels, each of which is not able to evidence the overall pattern. Other related issues we will explore include switching, oscillation, and genetic and epigenetic regulation.

6.2.2 Epigenetics, Stem Cell Differentiation, and Quasi-Potential Landscape

6.2.2.1 Models in Differentiation and Waddington's Landscape

We will see that an essential part of the representation of epigenetic processes is an inherent stochasticity, such as described in Kaern et al. (2005), and more generally stochasticity plays a large role in current understandings of cellular function, as can be seen in a wide range of articles, including the recent dissertation Rué (2013). Raser and O'Shea (2005) and Rao et al. (2002) also treat both exploitation and control of intracellular noise in gene expression. Thus the issue of stochasticity will form an important part of our models. In fact this issue of stochasticity relates to an underlying philosophical point that we will address related to cell fate as being primarily deterministic or stochastic, within our models of cellular differentiation. The larger context will be that of a dynamical system, based on the underlying gene network, in which the levels of expression of genes vary stochastically, and an epigenetic landscape will be used to describe passage between cellular states, represented within this dynamical system model by stable states, or attractors. Because of the role of the gene expression network and of the larger biological system in determining, or influencing cellular differentiation, a systems biology approach will eventually be desirable.

We note that the issues of determinism versus stochasticity in cell fate determination relate to an underlying question currently being addressed in the field, and of considerable interest. At some level, we begin to look at this question as a 'false dichotomy,' as in the sentiment expressed by Zernicka-Goetz and Huang (2010), and in fact the models must find a way to balance both the stochasticity from cellular and intercellular noise and determinism from cellular signaling and other influences. Our goal is to address these issues from the underlying philosophical and scientific point of view that allows development of models of epigenetics and differentiation that are universally applicable (and also more generally within the field of epigenetics).

In our initial stages of discussion of the concept of epigenetic landscape, we consider some of the recent challenges to this approach that have arisen in papers addressing recent concepts and advances in stem cell biology from this epigenetic landscape perspective. In particular we mention that Waddington's landscape was initially considered primarily as a metaphor to assist in the understanding of the relation of epigenetics to cell fate determination, and further to serve as a tool in describing and exploring these points. Goldberg et al. (2007) refer to epigenetics as a bridge between phenotype and genotype, and welcome the current modeling and developments in this field. Not only does this approach to epigenetic modeling and cell fate determination interact with the issue of relation between genotype and phenotype, a number of researchers, such as Huang (2012a) and Moore (2012), have emphasized the potential for this perspective as a necessary step for development of deeper theories in development and evolution. We also note there is a direct

link to cancer development and progression, such as in Huang (2012b, 2013) and Huang and Kauffman (2013), which is discussed further in Sect. 6.4. The issues of phenotypic plasticity, robustness, and evolvability are also treated from a different perspective in Kaneko (2009).

In the current work on stem cell differentiation, the concept of an epigenetic landscape is one of the prevailing avenues of inquiry with numerous recent research efforts in areas including questions of its validity, as in Laedwig et al. (2013), attempts to quantify the landscape, for instance in Bhattacharya et al. (2011) and Li and Wang (2013a, b, 2014), and investigations of how the landscape can be utilized to explain various specific aspects of stem cell function, including differentiation, reprogramming, transdifferentiation, pluripotency and self-renewal, signaling, cell-to-cell interactions, and issues of noise, stochasticity, and multi-cellularity. As we develop this landscape approach to modeling epigenetics and its various aspects, our underlying goals will be to use these models for attaining a deeper understanding, for prediction, and to extend knowledge and science. We will also get a view of some limitations and some challenges within this modeling approach. When evaluating the value of this approach to modeling, we will focus on the following five points: accuracy, prediction, potential to bring out new philosophical ideas or issues, comprehensibility, and range, flexibility, and universality.

We begin by considering some of the limitations and drawbacks for the epigenetic landscape approach to modeling of stem cell differentiation that have been noted in recent research papers. In particular we have the paper of Ferrell (2012) in which an alternative landscape model is given in attempt to improve some of the weaknesses noted in the area of modeling of cell fate induction. In addition the paper of Laedwig et al. (2013) notes a number of difficulties with the Waddington landscape and proposes an alternative model of an epigenetic disk.

Another theme in landscape modeling addressed in recent papers, such as Davila-Velderrain et al. (2015), is the issue of dynamism in the landscape. In general our own approach that extensive modeling potential exists for extension of these models and interaction between scientific extension and modeling, coincides with the approach of Rabajante et al. (2015). Based on the issues of stochasticity in cell fate determination, and particularly questioning of what determines cell fate under uncertainty at a junction, we raise the issue of potential stochasticity within the landscape, also addressed by Pujadas and Feinberg (2012), which will be tied into other discussion below. The discussion of Huang (2009b) in relation to induced pluripotency gives a very good example of some limitations in the original concept and how the modernized version can explain experimental observations.

Our perspective also has some affinity with several of the points addressed in Rabajante et al. (2015) regarding such epigenetic models, where part of the discussion advocates consideration of approaches to mathematical modeling that will remain faithful to the scientific results, bring in relevant points from regulatory systems, and where the larger goal is to produce models useful for in silico study of the underlying biology. The underlying approach here of flexibility in approach to modeling, collaborative efforts, temporal changes, and ongoing work toward enough complexity for faithful prediction summarizes some of our underlying principles.

This desire to interact with the science and establish a model that is grounded in the underlying biology, can interact with the science, and help to provide novel perspective coincides with our primary considerations in developing a modeling framework for epigenetics, particularly in relation to stem cell differentiation and fate determination. Furthermore, such models of epigenetics not only apply to differentiation but also apply to many aspects of biology strongly influenced by epigenetics, including cancer, as addressed in Sect. 6.4.

We note that we will follow this same trend of building and improving upon the landscape concept. The underlying concept is both useful for conceptual understanding and appears to coincide with the foundational scientific points, as we observe below. However, the overall value of this approach to modeling will depend on its ability to incorporate new scientific discoveries and new perspectives, as the modeling framework is expanded. Ultimately the goal will be to work with a basis for modeling that explains key aspects of the science and has potential for expansion to include new knowledge and perspectives. Ideally the model could also introduce new insights and relevant questions for the field.

Here we also address a few additional gaps or weaknesses of the standard Waddington's landscape approach to understanding of cell fate determination. These are examples of the types of the issues or gaps related to use of Waddington's landscape in modeling cell state determination, based on reflection of relevant points for modeling. And such gaps or limitations are consequences of inherent limitations of any model in representing this level of complexity. One point of ambiguity (a.) is the point of time and its relation to the landscape, and this is possible from two points, how the cell moves with respect to time and how the landscape itself may vary as a function of time. Another point of ambiguity (b.) is whether the landscape is one identical landscape for many cells, or whether each cell has its own landscape as related to its own epigenetics (and other associated issues). The third point (c.) is directly related to the fate determination and what happens to the individual cells that do encounter the locations where one valley (canal) diverges into two or more, and it is not at all specific what determines which path will be followed and which cell state will be taken. In addition we consider the following:(d.) how does each cell's history up to this point influence or help determine current landscape prospects for fate choice; (e.) what is the proper visualization/understanding for each of the equilibrium points (attractors, as cell states); (f.) what is the total number of attractors for the landscape (as representing transitions between attractors in a high dimensional dynamical system, see below), and how do these correspond to the known cell states? are there any new or unknown attractors? are there any non-standard or strange attractors?; (g.) does the correlation of the (cell state) to (equilibrium or intermediate state) leave our any important/relevant scientific details about the state of the individual cell? Other issues include the role of cell-to-cell interactions in differentiation, including contact relations observed experimentally in differentiating cells in certain stages of development and in the cancer environment, as in Bizarri et al. (2011). After further development of these models and associated themes, we continue this discussion of areas for development of the models, later in Sects. 6.2 and 6.4.

While these questions and issues have been raised, regarding the epigenetic landscape, many other papers have utilized the underlying concept associated with Waddington's landscape in explaining current issues in stem cell differentiation and further as a basis for mathematical modeling in this direction. Our general approach will be to explore what has been done in mathematical modeling in this direction, with an eye toward its potential for further development, and particularly in regard to effective modeling and to the uncovering and addressing of important questions and issues in this field. Thus, ideally the underlying model may facilitate development, new questions, and understanding, while remaining grounded in the underlying scientific issues. In the next section we look at how the epigenetic landscape can be used effectively to model, and particularly how the underlying concept can be used to address the issues raised here.

6.2.2.2 Survey of Epigenetic Landscape Models

In general we want to think of this concept of landscape also as a useful metaphor and to develop the modeling work based on scientific and modeling principles underlying the epigenetics, in particular based on mathematical models in cellular function and based on models of gene expression networks. However, as we go along, we will see that epigenetic landscape does serve as a useful concept in visualizing these relations and the state transitions. As often is the case with mathematics, it will be a considerable simplification, particularly to a lower dimension and with a much lower level of complexity. Nevertheless such simplifications are used throughout mathematics to assist in understanding. Our goal will be to connect this underlying idea of the epigenetic landscape to the actual models being produced in recent research, and the associated issues they are addressing. In addition we will also see how the underlying concept and the issues it helps us to address and explore are helpful in developing these models and in asking important questions.

This modern version of an epigenetic landscape is grounded in science and mathematics, based on levels of gene expression determined by the underlying genetic network dynamics, and formulated as a model in relation to mathematical concepts of attractors within a dynamical system. This approach is expressed in Huang et al. (2005), in which theoretical studies of complex networks are applied to the gene regulatory network to offer the possibility of representing cell states as attractors within a high-dimensional dynamical system. These attractors would correspond to valleys in the Waddington's landscape, although this concept is more general, of much higher dimension, and also scientifically based. The landscape then corresponds to the transition between the stable states. This theory extends from biochemistry and physics where potential landscape is used to study biochemical oscillations within complex systems, as in Wang et al. (2008) and Zhou et al. (2012). The article of Banerji et al. (2013) interprets potential in Waddington's landscape in terms of cellular network entropy, while the work of Wang et al. (2010a) use the interpretation of potential landscape to indicate the arrow of time in (forward) cellular differentiation.

As this concept of cell state attractors and intermediary landscape has gained more acceptance as a quantitative model (rather than only a metaphor), several recent papers have undertaken to quantify the landscape. In the article of Bhattacharya et al. (2011) a means is given to compute an explicit path-integral based quasi-potential, representing the underlying gene regulatory network. The landscape is computed for a representative bistable switch for a mutually antagonistic pair of regulatory genes based on a two-variable dynamical system, with a given set of rate equations representing expression level for the two genes. In addition, there are two alternative computational means to quantify the landscape, Wang et al. (2011a), based on applying the earlier work of Wang et al. (2010b) for constructing a landscape for a nonequilibrium system. This approach is based on exploration of the potential landscape through study of kinetic paths, with a correlation between transition time scales and underlying landscape topology. Also Ao (2004) describes an explicit construction of a quasi-potential function $\boldsymbol{\varphi}$, where $\rho_0(q) = ½ \exp[-\boldsymbol{\varphi}(q)]$ gives the steady state distribution for the network. These three means of computation allow for exploration of underlying landscapes. It is assumed that in most cases these three computation methods will yield landscapes that are qualitatively similar.

Jost (2014) introduces an alternative approach to modeling of landscape based on a computational stochastic model exploring dynamics of epigenetic markings on DNA, which is able to exhibit bifurcations and relevant behavior. Sisan et al. (2012) also make a data-driven stochastic simulation of rates of differentiation on a landscape and predict rates of phenotype formation. A slightly different approach is taken in Paździorek (2014) which studies the effects of stochastic noise on differentiation through a system of Itô SDEs based on a deterministic multistage model. We also mention that recently Li and Wang (2014) have applied the idea of quantification of the epigenetic landscape to explore quantification of paths to cancer and underlying landscapes, a theme to which we return in Sect. 6.4. We also find a fundamentally different approach to modeling of differentiation in the computational simulations of dynamical systems representing gene regulatory networks in Suzuki et al. (2011), which demonstrates oscillatory expression dynamics and chaos provide robustness to differentiation potential. This direct simulation of the gene regulatory network has an added feature of incorporating cell to cell interactions, and is based on the same underlying principles as the landscape, as we discuss further below.

One fundamental point about the epigenetic landscapes relating to its definition as a dynamical system representing levels of expression of the genes within a given cell is the inherent high-dimensionality of such a system. Although many models and representations of such systems may (of necessity) approximate behavior based on dimension reduction, the more complete description of the system involves all associated genes in the state space. In Huang et al. (2005) this view of cell state as an attractor of a high-dimensional dynamical system based on the gene regulatory network is formulated and presented together with experimental evidence of cellular phenotype. Huang has also considered important aspects of cellular differentiation that arise as consequences of the high-dimensionality of the dynamical system and its underlying state space, representing the level of expression for all genes. The

idea of high-dimensionality implies a 'rough' landscape, and in this sense rather than having finely canalized, individual cell states, the rough landscape corresponds to a wider basin of attraction with the potential for numerous substates and stochastic transition between these. In the article of Huang (2007) this issue of substates is addressed in relation to multiple substates in differentiation of endothelial cells classified by hierarchies in location, vasculature, and tissue bed, presented in coherence with characterizing cellular phenotypes by cluster analysis of transcriptome and proteome. Some additional consequences of the high dimensionality of the energy landscape are discussed further in Huang (2009a, 2011) and Lang et al. (2014). We also treat some additional consequences of the high-dimensional system below in relation to stochasticity, heterogeneity and multi-cellularity, and cell state transitions.

One particularly interesting area where the modeling and scientific approaches to this problem interact deals with the issue of levels of oscillation in gene expression dynamics observed among different cell states, and particularly where stem cells observe irregular, or chaotic, gene expression dynamics. In principle, the multipotency associated with more chaotic oscillations still maintain possibility for many states and oscillates between them, while more fully differentiated cells have specialized and fixed an expression pattern. This highly interesting and scientifically based perspective appears to originate in part from the work of Kauffman (1969) with the variety of final states in Boolean networks of genes, and in the work of Kaneko and collaborators in Kaneko and Yomo (1997) and Furusawa and Kaneko (1998, 2001), where a chaos hypothesis is presented. It also meshes very well with the concept of cell states as attractors within the dynamical system based on the gene expression network. The isologous diversification theory of cell differentiation in the first work aligns directly with our next topic of the role of intercellular heterogeneity, as well as stochasticity and genetic noise in the cellular differentiation process and these epigenetic models. It also includes an intriguing picture of epigenetic landscape as intertwining and interplay between many cells, in varying states, on interacting landscapes, which also relates to one of the questions we raised regarding epigenetic landscape and the role of multiple cells. The work of Furusawa and Kaneko (2009) also makes a scientific based illustration of how these issues apply to pluripotency, also reiterated by Efroni et al. (2009).

6.2.2.3 Stochasticity, Noise in Gene Expression, and Role in Landscape Modeling

Many recent papers in stem cell differentiation have focused on the role of stochasticity, and in fact this has become a very popular and important direction for analysis. This follows the trend in scientific studies in stochasticity and noise in gene expression, such as Elowitz et al. (2002) and Mettetal et al. (2006) studying stochasticity in gene expression for a single cell or Pedraza and van Oudenaarden (2005) studying propagation of noise through a gene network, which directly impact the levels of all expressed genes and directly inform the corresponding work in cellular

differentiation. Thus these results arise directly from underlying laws of chemistry and physics, leading to a level of noise, or stochasticity, inherent in gene expression. We mention how this theme coincides with a larger program within biophysics and cellular biology studying the role of stochasticity in cellular function, such as in the recent dissertation of Rué (2013). Papers such as Chang et al. (2008) based on the scientific stochasticity and clonal heterogeneity within cell populations, have established a direct connection to cellular differentiation according to the model of Huang et al. (2005), discussed in Sect. 6.2.2.2, where this oscillation in states actually plays a vital biological role. This interesting perspective claims that oscillation and variation is more than just 'noise', but rather actually an essential part of how cells function and differentiate, as part of their overall design. In fact, a program of research within this field investigates these issues within the underlying concept of the relationship between genotype and cellular phenotype, exploring how these relations play a role in areas of evolution and development.

In fact, the issue of stochasticity versus determinism in stem cell differentiation is addressed in Enver et al. (1998) from the perspective of contrasting the role of (in-cell) stochastic processes to that of (extra-cellular) regulatory processes. This concept of "do stem cells play dice?" is an echo of the famous statement of Einstein regarding quantum uncertainty, and it does suggest there may be profound implications, both philosophical and scientific in this underlying question regarding these basic building blocks of biological systems. Although the scale is not as miniscule as the quantum arena of Einstein's statement, and thus the answer is more easily accessible experimentally, yet at another level more difficult because of complexity of genome, proteome, other 'omics data and as yet not fully described intercellular relations. The research of Süel et al. (2007) has demonstrated through global reduction in cellular noise the strong correlation between noise and differentiation events. At one level this article of Enver et al. (1998) and successive articles answer the question by stating that each differentiation will involve some balance of stochasticity and determinism and that an appropriate model must seek to find the right balance in description of both types of influence. Swain et al. (2002) consider both intrinsic and extrinsic sources for cellular noise. Below we expand further upon sources of the stochasticity, the levels of complexity, and associated issues in stem cell biology.

With all of the attention to the role of noise and stochasticity both in cellular processes, cellular states, and differentiation, the paper of Huang (2009a) in particular has made it a high priority to understand these issues more thoroughly. In fact this paper has an in depth investigation of different types of noise, at different levels within the biological system, and different roles they play in the landscape and in the larger systems theory. This approach aligns well with the larger systems biology perspective discussed further below. In the context of stochasticity, cellular noise, and multicellularity as fundamental aspects of the epigenetic landscape that helps determine cell fate, we mention the article of Johnston et al. (2012) relating mitochondrial variability to cellular noise, and this forms a link to role of metabolism in epigenetics. We note in the remainder of this section how this concept of noise and

stochasticity and its role in differentiation, heterogeneity, and multi-cellularity align directly with this question of stochasticity versus determinism in cell fate modeling.

The question of stochasticity versus determinism in cell fate makes contact with a number of other interesting philosophical issues, in addition to the philosophical and underlying scientific questions we discussed in relation to the physics perspective. The underlying paradigm in understanding of phenotype, in its relation both to organismal function and also to deeper questions of development and evolution, has been based on the genome and (nearly) a one-to-one relationship between genotype and phenotype. One of the main themes of this perspective is the much more complex and biologically based relationship between phenotype and genotype, initially illustrated in the metaphor of the Waddington landscape, and now beginning to be worked out more meticulously, with attention to detail and scientific basis. Although the concept of landscape and epigenetics has been around for some time, it is now beginning to receive more serious attention in relation to current biological problems and needs. In addition to the modeling of epigenetics for its role in stem cells, an important program is developing within this field to extend the understanding of both development and evolution within this framework, as mentioned in Seigal and Bergmann (2002). Although the standard approach to evolution and evolutionary biology is based on stochastics, as well as statistics, this new approach has the potential to transform current approaches, as suggested by Moore (2012), based on stochasticity at a cellular level, and at a means that interacts more directly with potential mutations in the genome and how these would affect expression within organisms. This approach also makes sense from the perspective of the study of inheritance at a systems level and consideration of phenotype, as in Jaeger et al. (2012). We note that evolution sometimes applies a fitness landscape, which could interact with the epigenetic landscape, as described in Huang (2013) and Rabajante and Barbierra (2015). Furthermore the issues of cellular heterogeneity and multicellularity, discussed further below, have a direct correlation to topics of population level diversity, robustness, and variability, studied in relation to evolution.

The perspective of stochasticity coupled with the issue of high-dimensionality in these models allow us to address underlying issues of heterogeneity and multicellularity. The role of stochasticity in phenotype is confirmed in the model of phenotypic heterogeneity produced in Stockholm et al. (2007), where a model for phenotypic switch is compared at intrinsic and extrinsic levels, revealing a phenotypic switch is not triggered exclusively by the local environmental variations, and also dependent on local stochastic interactions. The origin of multicellularity, wherein outliers possessed strikingly distinct transcriptomes is addressed in the articles of Dewitt (2008) and Chang et al. (2008), showing multicellularity is not due only to stochasticity, but also arises from metarandom, metastable states, with slow transitional fluctuations, associated to varying levels in the transcriptome. In the article Huang (2011) three paradigms to help understand behavior of such high-dimensional dynamical systems associated to GRNs, and the issues of stochasticity and a 'rough' landscape associated to high-dimensionality are among the fundamental points for

this understanding. This 'rough' landscape implies many substates within the basin of attraction and potential for stochastic transitions between substates. These points are also addressed in Huang (2010), and this also leads into the related concept of preparatory stages to differentiation, i.e. reversibly pre-differentiations priming toward outlier states and eventually differentiations. Recall the articles of Huang et al. (2005), and Huang (2007) also show some quantitative experimental evidence for such substates based on quantitative gene expression levels, as well as observed behavior. The passage across the landscape in slow, jagged motion is characteristic of high-dimensionality, with transitions between multiple substates. From another perspective such passage may be seen as comparable to Brownian motion with the jaggedness relating to stochastic motion within hidden dimensions. All the evidence of stem cell gives clear display of heterogeneity and multicellularity. Note that even within a cell group that appear uniform in one, several, or even many genes, there could still have wide range of heterogeneity or multi-cellularity in hidden genes—thus differences in cellular behavior—some corresponding to separate substates.

Within recent increased attention to these issues of cellularity and multicellularity, we note the recent work of Veloso (2015) which gives an extensive discussion of multicellularity, developing an underlying theory, associated to issues in epigenetics. The article of Thattai and van Oudenaarden (2004) finds advantages for a heterogeneous cellular population in a fluctuating environment. This important issue of clonal heterogeneity has been represented in the mathematical model of Tuck and Miranker (2010), developed both for modeling heterogeneity of stem cells and also to be applied to cancer stem cell populations, particularly as related to treatment. In this metapopulation model, clonal dynamics are studied in presence of global disturbances and periodic disturbances. The innovative systems based model of Bogdan et al. (2014) focuses on heterogeneity and identifies quiescent, slowly proliferating, and rapidly proliferating subpopulations. This model displays relation of stem cell growth to a multifractal process and quantification of growth rates and dividing times across the multicellular population fits well with experimental data. Another model combining experimental data, quantitative predictions, and cell imaging operates at the systems level, as presented in Herberg et al. (2015), and one underlying goal is to explore both cellular heterogeneity and variations in quantities such as cell to cell adhesion and intercellular feedback in relation to roles in patterning and differentiation.

We note that these immediate consequences of the nature of the dynamical system in substates leads to both heterogeneity and multicellularity. We have just described some of the consequences for stem cell behavior, and others will be found in the references. Some of these do help to explain observed behavior in regard to differentiation and cell fate, as we discuss further below as related to multipotency, pluripotency, and reprogramming. In addition we mention that cellular heterogeneity and multicellularity have important consequences in health and disease and can be used in certain settings to explain resistance of some cells to therapy. In particular, we explore these issues further below in Sect. 6.4 in relation to cancer and particularly resistance of some cancer cells to chemo and radiotherapies. Furthermore heterogeneity has an important relation to theories of evolution, based on a

phenotype and complex systems approach in inheritance, such as that discussed in Siegal and Bergmann (2002), Moore (2012), and Jaeger et al. (2012).

One immediate consequence of these issues we just treated is the potential to understand induced pluripotency in stem cells, as observed in Huang (2009b), in the context of rarity of these events and robustness of the system. The passage through substates as preparation for differentiation, transdifferentiation, or reprogramming helps explain the rarity of these events, also related to stochasticity. Yamanaka (2009), as well explains the inefficiency of reprogramming based on the inherent stochasticity in the models and the underlying process. This perspective combines with works such as Wray et al. (2010) and Hackett and Surani (2014) in which the pluripotent state can be seen to be a ground state and with works such as MacArthur et al. (2008a, b, 2009) and MacArthur and Lemischka (2013) using stochasticity and the perspective of systems biology to explain aspects of induced pluripotency initially puzzling in the stem cell community. Flöttman et al. (2012) produce a stochastically based model describing a Boolean network representing interplay between gene expression, chromatin modifications, and DNA methylation with computing an epigenetic landscape representing somatic reprogramming. Note also the extensive computational landscape study of Li and Wang (2013a), studying reprogramming paths and utilizing sensitivity analysis to identify significant genes. We also recall the clear relation to the perspective of group associated to Furusawa and Kaneko, where highly oscillatory or chaotic gene expression behavior is associated with states of higher potency, and such issues can also be considered with respect to canalization, plasticity, and robustness for evolutionary purposes, Kaneko (2009). Within the context of epigenetic landscape models of fate determination, this ground state of pluripotency is initially stable, but can become destabilized. Its position is (between/above) multiple cell state attractors, and once destabilized there is potential to move to these states. The study of Hanna et al. (2009) interpret cellular reprogramming as a stochastic process, asking what factors would accelerate this process, with the conclusion of cell-division rate being most significant. In Morris et al. (2011) is found a stochastic model based on stochastic differential equations, comparable to Sissan et al. (2012), to represent reprogramming with a goal to further understand quantitative aspects of these state transitions.

One of the modeling approaches suggested in Eby and Tabatabai (2014) related to the issue of describing the population of cells produced by a given group of adult stem cells operating within the niche and following the function of the larger biological system. This modeling approach would include the issue of how the system establishes the appropriate balance between self-renewal, differentiation, and proliferation, and furthermore how appropriate cellular hierarchies are maintained. Within this section we had glimpses relating these issues to issues of individual versus collective action, as addressed in Landler (2011), as well as issues of symmetric and asymmetric division within stem cell hierarchies, all modeled within MacLean et al. (2015). Doumic et al. (2011) also uses a PDE based model of a structured population to represent role of cellular signaling by neutrophils on differentiation and proliferation. See also the interesting recent work of Ridden et al. (2015) where a stochastic population based model concludes regulation of

cellular dynamics at the population level. This stochastic model is based on individually cells remaining maximally non-committed, generating a maximally diverse population. The relation between daughter cells and the proliferation and differentiation of stem cells via feedback signals is also modelled in Sun and Komarova (2012, 2015), using a two dimensional Markov process. Here the mean and variance can be tightly regulated through the model parameters. MacArthur (2014) discusses a recent model based on operations research that allows for adaptation and evolvability. All of these relate directly to heterogeneity and to the role of stochasticity versus determinism in cellular fate determination. Appropriate balance is required for health and homeostasis, as addressed further below.

The deeper level of investigation of the role of stochasticity underlying this section originated from both the underlying scientific studies to distinguish roles of intra-cellular and extra-cellular influences on differentiation and from the modeling perspective considering potential behaviors of a high-dimensional system and consequences for cellular differentiation. The multi-level, systems based investigation of Huang (2009a) in distinguishing sources of noise parallels this underlying question of determining the appropriate balance between various levels of influence in determination of cellular fate. It is at this systems based level that these influences interact, and we can investigate the relations of noise, stochasticity, oscillation, and regulation.

6.2.2.4 Systems Biology and Stem Cell Differentiation

In many regards, systems biology should be seen as the correct framework in which to work on this type of stem cell model. Already with the concepts of landscape and genetic and epigenetic regulation, we are moving into the territory of systems biology. This is an important framework for biology and particularly modeling in biology in which mathematics, engineering, and computation play a large role, and where the underlying perspective is an integrative one of the function of the biological system of a whole, in contrast to the reductionist approach to individual parts. Part of the idea of systems biology is to use the perspective of and tools from systems engineering in the study of systems aspects of complex biological systems. Systems biology includes applications of these principles and the underlying biological relations and interactions to solve problems at various levels within the biological system.

In some sense the idea of Tian et al. (2012) of the applicability of systems biology to cancer, based on application of informatics, modeling, and computation to the underlying heterogeneity, carries over well to the understanding of stem cells and their underlying heterogeneity of potential attractor cell fates within the GRN dynamical system and the corresponding biological system dynamics. Although there are numerous differences in these settings, systems biology applies well to the understanding of both fields, and furthermore there is enough analogous biological activity so that the underlying programs have many areas of overlap. We revisit these in Sect. 6.4 from the perspective of cancer as an attractor state that has

evolved into the underlying biological system, as found in Huang et al. (2009), a systems based discussion of cancer attractors. We mention that the papers of Ao et al. (2008, 2010) also address this concept from the larger perspective of systems cancer medicine. At this point we recognize how the study of both fields is directly aligned with the underlying goals and concepts of systems biology—both from the sense of use of modeling and computation, and from the sense of putting together all the relevant information from different scales to more fully answer the biological questions, especially ones that are both important and elusive. We remark that in addition to the science, technology, and computing aspects of the larger systems biology program, the mathematical side and modeling side of these programs is also a critical aspect. The development of landscape and associated models of differentiation and epigenetics is one important aspect of this larger program. In addition we remark that this systems approach has been very useful in reaching the current level of understanding in stem cell activity, both based on experimental and scientific evidence and from the perspective of models of the underlying systems. One example of cellular informatics applied to understanding stem cell development is the article of Glauche et al. (2009), where a wide range of informatics are applied to this issue.

We mention in passing that the systems view is also important in understanding tumor growth and cancer development, and the article of Huang et al. (2009) establishes a direct connect to cellular attractors and epigenetic landscape. The article Creixell et al. (2012) emphasizes particularly the relation of cancer attractor states and molecular states in relation to signaling networks as a grand challenge in cancer biology. The review of Aebersold et al. (2009) outlines the importance and perspective of this larger cancer research program and addresses issues including cellular pathways and relations to developmental biology, computational models, use of quantitative data, and personalization of treatment based on specifics of progression. Much more has been done with cancer research both from this systems biology perspective and from this epigenetic landscape perspective, and we review some relevant points in Sect. 6.4.

One important aspect of systems biology is the concept of emergence, in the sense that there phenomena which arise at the systems level that cannot be identified or analyzed on any of the reductive levels. However at the systems level, such phenomena emerge in the interaction between various reductionist levels. In study of this epigenetic landscape modeling, there will be emergent phenomena that must be seen from the systems level to understand fully, and among these is the full role of noise and stochasticity in differentiation, as described in Huang (2009a). The epigenetic landscape will ideally put together relevant information from many different scales, including chemical level noise/oscillations, cell to cell contact, external cell signaling, as well as noise at different cellular levels and from different sources within in the gene regulatory network. We also emphasize the close relation of the systems perspective to the issue of noise versus regulation and for the contribution of the different levels of noise. Furthermore, the systems level is also good place to address diverse issues that could affect differentiation or interact in epigenetics issues, such as cell-to-cell interaction. Note that the issues of dynamic

landscape, changing parameters, noise in landscape, metabolism and environment influences, also have the potential to be absorbed into the models at the systems level. These areas that we have identified as potential directions for expansions of the models have also been identified in other sources, such as Pujadas and Feinberg (2012) and Davila-Velderrain et al. (2015). We also remark on the interaction between levels of analysis in Herberg et al. (2015) where the image-based analysis interrelates with cellular heterogeneity and collective decisions. The work of Crespo, et al. (2013) provides combinatorial- and topological-based tools for investigating differential roles of genes within a network and the impact on differentiation. Similarly Bogdan et al. (2014) relates the population level choice of differentiation, proliferation, or self-renewal to systems level choices made at the cellular level. Garcia-Ojalvo and Martinez Arias (2012) apply the concept of statistical mechanics to this same issue. MacArthur et al. (2008a, 2009) address associated issues from the systems view.

In our review of recent models in cell differentiation and epigenetics, we observe the emerging use of models of cell states as states in an underlying dynamical system and the concept of quasi-potential landscape in representation of transitions between these states. While this emerging approach to the epigenetic landscape model is fully within the realm of systems biology, in the following we will also dive more deeply within this field, as we explore issues of stochasticity versus determinism, switching, oscillation, and regulation through genetic pathways, and how these play a role in the epigenetic landscapes and associated models. In using this perspective, we join the authors of a number of papers in this field who have proposed systems biology as the underlying framework for study of these problems. Halley et al. (2008, 2009) are good examples of the overlapping between various issues including gene expression, regulatory network architecture, and external signals, as well as the role of the niche, that can be addressed with these issues.

The article of Peltier and Schaffer (2010) finds an extensive role for various aspects of systems biology in study of stem cell differentiation, integrating modelling with experimentation to understand complex means of cellular signaling and genetic regulation including areas of modeling requiring intricate feed-forward loops, feedback loops and cross-talk between pathways to determine cellular fate. The hope is that such methods can advance understanding of cellular fate determination and stem cell biology. The dissertations of Scharpe (2010, 2014) are good examples of tools of systems biology that can be applied to these issues. Fagan (2011) has also recognized that the mathematical modeling framework of landscapes associated to stem cell differentiation and epigenetics is well suited to the systems biology approach, and clearly this relationship is based on the underlying genetic regulation making contact with the cellular machinery of expression in epigenetics. Fagan also mentioned the known relevance of systems biology in connecting phenotype to genotype, and even anticipated the potential use of this systems biology approach to epigenetic landscape in understanding of evolution. Clearly the relevance of heterogeneity, noise, and stochasticity in cell differentiation makes systems biology even more relevant, as do the need to include models of switching and regulation, as pointed out by Huang (2010, 2011). All of this grows naturally out of our view of cell states as states $S = [x_1, \ldots, x_N]$ in a genetic network, where both the attractor states and the paths between states are determined by the underlying

network, as described above, and in Huang (2010). With the epigenetic landscape as a model of interaction between various levels of regulation, the systems perspective becomes an essential tool in combining these influences, such as in Lei et al. (2014), where a model of cross-talk between genetic and epigenetic regulation, is applied to collective stem cell dynamics.

The article of Roeder and Ratke (2009) describes recent collaborative work in stem cell biology in which the underlying systems biology perspective served as a unifying theme. This article focused on the influence of computational and mathematical methods, and another unifying theme was heterogeneity of stem cells and stem cells populations, one of our themes from the previous subsection, together with the theme of the role of external signaling, which we address in the next subsection. One intriguing point form this article relates to experimental results in between the standard view of the epigenetic landscape and attractor cell states. In this work of Roeder for a system of ODEs analyzing heterogeneity in the effect of Nanog on murine ES cells, it was still to be determined whether there was noise based fluctuation between two coexisting attractors or one oscillatory attractor. Other work in Roeder and Ratke (2009) emphasized the underlying ideas of dynamical systems and non-linear dynamics, addressing the need to explain transitions between attractors (as cell state), including differentiation, transdifferentiation, reprogramming, and de-differentiation. Also other work in Roeder and Ratke (2009) addresses theoretical representation of cell state transitions and cell fate decision with underlying goals to fully describe the structural relationships between the components.

The paper of Huang (2010) advocates the systems biology point of view for proper perspective and understanding in epigenetic landscape issues and the space of inter-attractor trajectories, based on the underlying gene regulatory network. As a deeper understanding of how these transitions are made and what paths may be followed are fundamental points in this area of research, this is a deeper point worthy of study. Furthermore this principle is echoed in the Peltier and Schaffer (2010) which concludes by describing the need to apply the systems biology perspective in applying the necessary level of complexity, all relevant genes, for understanding stem cell fate choice.

Note also there are definite connections with previous section and the roles for stochasticity, multiplicity of states and greater sensitivity. Note there is an inherent link here to the systems biology approach in the study of cancer, which has in fact become an active research area. We will return to investigate this point further in Sect. 6.4. Note that the epigenetic landscape has important aspects of signaling and external control, in addition to the potential for multiple levels of influence and interaction, particularly when viewed from the systems view. Ideally the epigenetic network should incorporate all relevant issues, which are based on aspects of epigenetics and cellular function. Some of these themes are extended below, including in the next section addressing aspects of regulation and signaling.

6.2.2.5 Switching, Oscillation, and Genetic and Epigenetic Regulation

In the issue of developing models that find the appropriate balance between stochasticity and determinism, we have mostly focused on the stochasticity side. However, the side of determinism, as related to switching, cellular signaling, and genetic

regulation can play an important and sometimes dominant role in cell fate determination. In visualizing gene expression networks as a complex dynamical system in which the cell states are equilibria and the transitions between states are represented by a quasi-potential landscape, there is then a direct media for interaction of means of genetic and epigenetic regulation as integral parts of these models. In this subsection we want to look closer at how this may be done and to review some existing models in this direction including some models of switching, cellular signaling, and regulatory networks. While our overall goal is to fit all of these topics within the modeling framework we have been addressing, we also want to consider the issues of how these critical aspects of differentiation may be represented within this framework.

We note that in epigenetic modeling related to stem cell fate determination we include a wide range of behavior including pluripotency, multipotency, self-renewal, and various aspects of differentiation associated to different stages in development. In some of these stages there are certain well known mechanisms we would also like to address within our modeling context. For instance in development, morphogen gradients are often used for direction of cell state determination. In Sect. 6.3 we discuss further the use of a Sonic hedgehog (Shh) morphogen gradient to control differentiation within development of the neural tube, as in Dressoud et al. (2008) and Panovska-Griffiths et al. (2012), and we look at some associated models, including one representing Shh and Wnt antagonism in neural tube formation. Several others address interaction of pathways. Furthermore, an associated strategy in development and mechanism of differentiation is use of oscillation, and here the Notch pathway is well known for its importance in development and its fundamental use of oscillations. One very well known use of oscillation to direct important events of development is that of Hes1 in somitogenesis, in which the temporal chemical oscillations are converted into spatial oscillation in formation of vertebra, also leading eventually to other important developmental events, including neural. One review of some of the important functions of Hes1 and the Notch pathway is given in Tabatabai et al. (2012), and other pathways are similarly known to be critical in guiding the events of development. We will pick up this theme of Notch signaling further in our review of neural differentiation models in Sect. 6.3. This connects directly with the observations of Kobayashi and Kageyama (2010, 2011) in which Hes1 oscillations lead to heterogeneity in embryonic stem cell fate patterning. Issues of Hes1 and Notch signaling are particularly interesting in relation to context dependent signaling, an example of the underlying complexity of developmental processes. In the article of Cau and Blader (2009) it is emphasized that Notch signaling plays a variety of roles, including lateral inhibition, binary switch, depending on the context, and in many cases it acts cooperatively with other pathways in cell fate specification, while most of these are only beginning to be investigated. This underscores the complexity of these events and the underlying relationships.

In the context of oscillations, switches, or morphogen gradients that adjust the landscape and direct cell differentiation, we present a couple representative examples. For instance the article Momiji and Monk (2009) presents a model of the

Notch-pathway based on oscillation, and its role in neuronal differentiation. On the other hand a model based on a morphogen gradient can be found in Balaskas et al. (2011), giving the gene regulatory logic for a Sonic hedgehog signaling gradient in the vertebrate neural tube. We revisit this issue with further specific examples in the section on neural differentiation below.

Here we look at bifurcation dynamics for bipotent progenitors, as well as variations and switches. Just as in the previous subsections we considered other necessary issues and accommodations to develop the model and make it more complete, our modeling concept and perspective must also certainly address these points. Our goal in this section is to address how they can be included and to survey some related models. Beginning with bifurcation dynamics for bipotent progenitors, we have Huang et al. (2007) where auto-stimulation and cross-inhibition leads to stable attractors, as well as an uncommitted metastable state of multilineage priming. The article of Andrecut et al. (2011) treats the issue of extension binary and multistate conversion in cases of a degenerate manifold where the gene circuit varies from the standard model used, and they suggest further development related to a wide range of potential reaction dynamics. An important variation on bifurcation dynamics for a bipotent progenitor includes a wide range of potential behavior from asymmetry, to perturbations away from symmetry, to a dynamical landscape, and models corresponding to these issues are developed. Variations on the bifurcation dynamics in the case of a symmetric inhibition is considered in Li et al. (2015), where different forms of lateral inhibition, especially asymmetry or symmetry breaking perturbation, lead to different forms of bifurcation dynamics.

In cases where gene regulatory networks act to direct differentiation toward one cell fate, it is good to have a model to represent this type of switching behavior. In the paper Cinquin and Demongeot (2005) models the classes of regulatory networks where genes can behave as a multi-switch, directing differentiation toward a certain cell type. An example of use of a switch is for embryonic stem cells between self-renewal and differentiation, found in Chickarmane et al. (2006) yielding from several bistable feedback loops a bistable switch activated by environmental signals. A related model is that of Chickarmane and Peterson (2008), where a computational model is developed for reprogramming of a cell through directed perturbations. The potential landscape for the toggle switch presented in Kim and Wang (2007) and the global landscape is explored with respect to changes in rates protein synthesis, binding, and degradation. For an interesting study of interaction between determinism and stochasticity, Artyamov et al. (2007) address how stochasticity can create a bimodal response in a simple model based on competing positive and negative feedback loops which is otherwise deterministic. In the context of dynamic landscapes and how a toggle switch may change with respect to a changing landscape, the study of Verd et al. (2014) considers how calculation and visualization in the low dimensional case can give rise to interesting behaviors including transitions, pursuits, and captures. Finally, the article of Rabajante and Talaue (2015) is a concurrent decision-making model (CDM) representing a more complex interaction network with lateral antagonism, and this model can generate oscillatory behavior from asymmetric interaction.

The article of Raj and van Oudenaarden (2008) describes how the cellular level is the meeting ground for stochasticity related to biomolecular interests and the precision of outside coordination of development, much as we have represented within our discussion on epigenetic landscape. Furthermore, this article emphasizes how a large stochastic burst is capable of changing the output in a bistable or multistable switch. These authors furthermore emphasize how this field of stochasticity in gene expression is still in its infancy, and much remains to investigate in relation to cell to cell variability, its relations to different levels of noise, and aspects of cellular biology. In reference to the role of oscillations and combining of multiple signaling, the modeling approach of Arenas et al. (2006) may prove relevant. In the area of differentiation, the epigenetic landscape gives one important modeling approach wherein these two levels of influence can meet and work out their relative influences. Within this context, it is important to recall the systems biology perspective discussed above and the level of complexity of the underlying genetic network.

6.2.3 Conclusion

The underlying question of stochasticity from biomolecular processes versus determinism from regulatory processes in fate determination of cell state has a complex answer involving layering of different levels of cellular noise. However, these competing influences meet within the landscape for cellular differentiation, with the potential for interesting interaction within this modeling framework. Nevertheless we have seen that a complete and accurate representation from this perspective still requires much further development. We consider some associated issues in use of quasi-potential landscape to represent transition between cell states as stable states in a dynamical system. One point to be developed more fully relates to stochasticity and what actually determines individual cell fate in cases of stochasticity. Deep enough analysis may be able to undercover some hidden cause(s) underlying the stochastic rule. For instance, Zhou and Huang (2011) investigate the cell fate near a branch point. Other points such as the role of cell-to-cell contact, metabolism, environment, and related issues are addressed further below. One particularly interesting place of further development for such models relates to the connection between the perspective of Kaneko and chaotic dynamics for gene expression and multipotency and the perspective of the epigenetic landscape. From the perspective of direct modeling of gene expression, there was important work in dynamical systems simulations by Suzuki et al. (2011) at the GRN level, with interesting results. It is definitely valuable to establish a link to the modeling and computation of epigenetic landscapes, based on GRNs.

Here we have focused on development of tools for modeling in epigenetics with particular focus on stem cell differentiation and fate determination. One of our underlying goals has been to produce flexible tools that apply universally to the study of stem cell differentiation and fate determination, while maintaining a

modeling framework wide enough to incorporate all issues arising in specific situations. We have approached this problem by addressing this issue of epigenetics from a mathematical/physical perspective based on our underlying interest in cell state, with a view toward representation of differentiation. This approach follows from the recent large amount of work with epigenetic landscapes and based on the biological reality of a gene expression network, represented as a dynamical system. Furthermore one underlying goal has been expansion of the concept of epigenetic landscape to adapt to the new scientific discoveries and issues as they arise in the field. It appears this epigenetic landscape approach may be wide enough to fulfill our underlying goal of incorporate important issues that arise in this new and developing field. However ongoing development will answer how well incorporates new discoveries or theories and synthesizes the remaining complexities. Up to this point we have focused primarily on this perspective of use of tools in epigenetic modeling with universal application, but we now want to change perspective from the general to the particular. To better understand this field of modeling and its problems, possibilities, and intricacies, it is necessary to look from the other side of the issue, focusing on specific models that have been developed for specialized cases of differentiation. Here one goal will be to see what aspects of these models might arise from relevant pathways, switches, signals, morphogen gradients, cell to cell interactions, etc. We particularly want to see what aspects of genetic and epigenetic regulation apply to various forms of differentiation and what form the epigenetic landscape models will take. To focus our attention, we focus on one specific type of stem cell differentiation, neural differentiation, which we pursue in the next section.

6.3 Mathematical Models in Neural Differentiation

6.3.1 Introduction

This approach to modeling of cellular fate determination based on epigenetic landscapes also plays an important role in understanding and modeling within the field of development. In fact, we can interpret development as a finely orchestrated chain of differentiations of cells into the required form and following the required patterns, though at a very high level of complexity. However, at the basic level each of these cellular differentiations within the program of development is based on these same principles underlying epigenetics and cellular differentiation. The tools were developed to be generally applicable to epigenetics and cellular differentiation, and we hope they can apply as generally as possible. We also note that Waddington's original metaphor of epigenetic landscape was designed to work very well in this context, beginning with one totipotent cell and through each of the successive stages. The extent to which the tools apply in the study of cellular differentiation at different stages of development will be an important measure of their usefulness. We mention in passing that the issue of the distinction between genotype and phenotype inherent in this type of model is also of central importance in development.

While the field of modeling in stem cell fate determination is a new field in which knowledge is very limited and much remains to be studied, it is also a field of seminal importance and where important work has been done, particularly in areas of critical importance, such as modeling of hematopoiesis and leukemia, or in neural differentiation, both areas where extensive work has been done. Perhaps the area of greatest interest and most effort in modeling has been in the hematopoietic stem cells, which are the most active among adult stem cells, and also associated with leukemia. We mention models such as those of Roeder and Glauche (2006) and Lei (2011), and furthermore development and modeling and in this area are ongoing. We will focus instead on the area of neural differentiation.

Within this area of neural differentiation we will consider a variety of models, from differentiation of adult neural stem cells into neural progenitors and to further differentiation into neural lineages and also to include various cases of neural differentiations within development, including development in vertebrate neural tube, neural crest development, ventral spinal cord development, and brain development. Also as differentiation is one side of the larger stem cell program, such models will also address the related issue of modeling in mechanisms that inhibit differentiation to progenitors but rather promote self-renewal of the stem cells. According to Doe (2008), understanding of issues of self-renewal vs. differentiation in neural stem cell development is critical for advancement in areas of embryogenesis, cancer biology and brain evolution. The differentiation of neural stem cells to progenitors is important for a variety of reasons from application of the neural cells in regenerative medicine to understanding of roles of signaling networks and understanding of neural development. Here we can extend our efforts to understand genetic and epigenetic issues in specialized models and how they may relate to the above epigenetic modeling framework. The article of Lilja et al. (2013) emphasizes the importance of understanding histone acetylation and methylation, aspects of epigenetics, for a more complete understanding of neural development. This area of neural differentiation has great potential because of the role of genetic and epigenetic regulation in the existent models, and the varieties of models that are available the are influenced by several genetic pathways, including Shh, Notch, BMP, and Wnt, and their interactions. Here we will see a range of behaviors including switching, oscillation, morphogen gradients, context dependent signaling, and the interaction of multiple pathways.

Although this area of neural differentiation is both valuable and interesting, this is an area of limited knowledge, and much more remains to be discovered. Suzuki et al. (2011) note how the need for greater understanding of differentiation of stem cells is of fundamental importance for advancement of developmental biology. In the area of embryonic development, Zaraisky (2007) has observed how the induction of neural differentiation based on signals from adjacent tissues has made recent in advances in understanding the bone morphogen protein (BMP) signaling, its relations to FGF and Wnt cascades, and associated mathematical modeling for spatial self-organization. More generally, there are a variety of aspect of differentiation of embryonic stem cells in the development of the nervous systems that we will explore, along with differentiation of neural stem cells into neural progenitors. In the article of Stern (2005) it is emphasized that understanding of the developmental process of neural induction requires more complex models, as the biology depends on a complex cascade of sequential events and on cooperation between different

siganling pathways. Modeling in neural development offers us an opportunity to explore a variety of forms of signaling influencing differentiation, including use of a morphogen gradient in the Shh pathway, the role of oscillations of Hes1 in heterogeneous differentiation within the Notch pathway, and others, including interactions between pathways, such as interaction of Notch and Wnt or the interaction of Wnt versus Shh in patterning of neural tube.

6.3.2 Neural Differentiation Models

In our brief overview of some of the neural differentiation models currently available, we have an interest to see how the genetic pathways play a regulatory role in neural differentiation and how this appears in the models. We focus on three pathways: Notch, Shh, and Wnt, each of which is highly significant in this area. The following subsections will treat models involving Notch and Shh. Finally, we look more in depth at one specific model in the Notch pathway, important in differentiation into neural cells, astrocytes, or oligodendrocytes, based on computation of the epigenetic landscape in Sect. 6.3.4, the final subsection of Sect. 6.3.

We begin, however by mentioning an important model involving differentiation of stem cells into neural progenitors on a scaffolding, an area important to production of cells for regenerative medicine. In Howk et al. (2012) and Howk (2010), a computational based mathematical model is applied to interleukine-6 (IL-6) for differentiation of neural progenitor cells in vitro. This model is composed of a system of ODEs, based on reaction pathways for surface activated IL-6, and for intracellular diffusion factor J. It follows from a sequential treatment by surface receptors, followed by soluble receptors. The model shows good agreement with experimental data, and a sensitivity analysis of the parameters agree with known sources as the intracellular differentiation factor J and the JAK-STAT pathway having the most significant effect on differentiation of neural progenitors. The pathways Notch, Shh, Wnt, and BMP have a significant influence on embryonic development generally, in in Sects. 6.3.3 and 6.3.4 we observe some models related to the influence of these pathways in neural induction, neural patterning, and/or neural differentiation.

However, we mention here one significant model within the Notch pathway and its role in differentiation. The model of Pfeuty (2015) studies Notch-Hes1 signaling in relation to connections to cell cycle dynamics and to differentiation of multipotent cells into various lineages. The model supports the concept of the importance of oscillatory dynamics both for gene expression and for regulation. We revisit this model in the context of other models in Notch signaling and neural differentiation below. Next we consider another model based on interaction of several other important pathways. The model of Tecarro et al. (2009) is significant in relation to interaction of genetic pathways in relation to neural induction. This basic model is based on a system of ODEs, and it is able to display important relations between the BMP-Smad1 pathway and the MAPK pathway activated by FGF. The relevance of these interacting pathways during development in neural induction is addressed in Pera et al. (2003).

Finally we finally consider a few models in neurogenesis that are stochastically based. Recall, differentiation itself is considered to a large extent stochastic, based on cellular noise, and it is to be expected to find good stochastic models, especially as accumulation of data makes us aware how ubiquitous stochasticity is, Johnston (2012). The model of Barton et al. (2014) is also interesting because it relates to the role of asymmetric division in establishing the balance between self-renewal and differentiation of neural progenitors. The consequent model is stochastic and depends only on the concentration of the factor mNumb in the cell. Furthermore, we have another model representing development of the ventral spinal cord for which boolean modeling reveal regulatory connections orchestrating the process. Lovrics et al. (2014) produces a model of gradient mediated morphogenesis, as is conducted by Shh, Wnt, and BMP based on modeling with a Boolean network representing the transcription factors downstream of Shh.

6.3.3 More Details on Some Models Utilizing Signaling Pathways and Genetic Landscape

6.3.3.1 Role of Morphogen Gradients; Antagonism of Shh-Wnt Gradient in Neural Tube Development

Sonic hedgehog (Shh) is the best studied of the proteins within the mammalian signaling pathway family known as hedgehog. Shh plays important roles within development, and particularly in tissue patterning. As the primary action of Shh in influencing cellular differentiation in tissue patterning is through morphogen gradients, we begin with related considerations and models. A good introduction to mathematical models in pattern formation by morphogen gradients can be found in Shvartsman and Baker (2012). A model for a morphogen gradient such as Shh is formed based on the source–diffusion–degradation models. The model of Cohen et al. (2014) provides a theoretical framework for Shh morphogen gradient and in fact produces a flexible model broadly applicable, based on locating the neutral point in the gradient, either side of which altering the Gli binding affinity has opposite effects on gene expression. A model of the Shh morphogen is produced in Saha and Schaeffer (2006), that illustrates how this morphogen gradient can be a determining factor in cell fate. Furthermore, the model demonstrates how Patched and Hh inhibiting protein promote degradation and limit signaling range. The article Saade et al. (2013) addresses the tightly controlled balance between self-renewal and the Shh morphogen gradient is needed to maintain stem cell identity in the developing spinal cord. In the model of Balaskas et al. (2011), a spatially and temporally changing gradient of Shh signaling is interpreted by the regulatory logic of a downstream transcriptional network. The model demonstrates the morphogen interpretation is an emergent property of the transcription network, and this provides robustness to the process of tissue patterning.

An important role is played by Shh in interaction with other pathways in tissue development, including dorso-ventral patterning of the vertebrate neural tube, where an important role is played by both the Shh and Wnt pathways and their interactions. Here Shh acts as a morphogenetic agent, and also interacts with Wnt and BMP pathways. Joksimovic et al. (2009) offer the example where Wnt antagonism of Shh facilitates midbrain floor plate neurogenesis. Furthermore Hagemann and Scholpp (2012) reveal how in thalamus development, the three signaling pathways Shh, Wnt, and Fgf meet and interact in a strictly controlled manner. Tang et al. (2010) show a delicate balance between Wnt/beta-catenin and Shh signaling mechanisms in the progression from progenitors to dopamine neurons. In neural tube formation, for instance in the article of Ulloa and Marti (2010) treating the antagonistic role of Wnt and Shh, it is shown that the patterning of Shh is antagonized by Wnt through production of Gli3, which silences Shh. Part of the goal of studying the interaction of these pathways in the neural tube is to better understand their interactions in tissue formation, and furthermore it is suggested that mathematical models could be developed in this direction to support the data.

Shh plays multiple roles in stem cell regulation, and it can function as a genetic switch, such as the epigenetic switch in medulloblastoma development described in Shi et al. (2014). Positive and negative feedback loops endow the Shh network with the ability to switch fate choices at a threshold Shh concentration. The article of Lai et al. (2004) addresses the feedback loops with Gli and Patched which form this genetic switch and gives a computational model based on a system of ODEs modeling the interactions of the genes. This model is used to study switching behavior, and is also considered as a representative of other similarly functioning switches. Genetic regulation is an important theme in cell state determination, and we observed multiple means by which Shh plays a role and multiple pathways of interaction. Next we look at the Notch pathway, which plays a different role, also important in signaling for cellular fate determination.

6.3.3.2 Notch Signaling in Neural Differentiation Models

Of the many models for neural differentiation reviewed above, a large number of them were based upon the role of Notch signaling in determining neural fate decision. This is consistent with the role of oscillations and with the fundamental importance played by the Notch pathway in development, and these models give us a good opportunity to explore the role of genetic signaling within stem cell fate models. Furthermore we recall that the stochastic modeling approach of Sect. 6.2.2.3 included a scientific example from Kobayashi and Kageyama (2010, 2011) in which Hes1 oscillations have a direct impact on the heterogeneity of cell fates for embryonic stem cells, also discussed in Shimojo et al. (2008). A number of these models fall within the category of switches and oscillations, overlapping with models and issues addressed in Sect. 6.2.2.5. Cornell and Eisen (2005) address how in neural crest formation Notch first acts by lateral induction to establish neural crest,

then by lateral inhibition in diversifying its cell types. Furthermore a few of the models include interaction with other networks, which is also important for inclusion in our modeling framework, and Cau and Blader (2009) addresses the variety of roles Notch can play in cell fate determination. Of added interest within the field of development is the paper of Agarwal et al. (2009), which addresses the well known developmental phenomena of context dependent signaling. The prospect of finding appropriate models to represent this level of complexity should also be seen as an additional challenge for the development of our epigenetic modeling framework. As stated in the article of van Ooyen (2011), neural development exhibits an intense level of complexity, especially in interchange between gene changing cellular environments, and many of these issues will have to be understood at the systems biology level, as we also addressed in Sect. 6.2.2.4. As addressed above, complexity in signaling and interaction with other pathways are aspects of development in the Notch pathway, and Andersson et al. (2011) consider versatility within Notch, discussing variation of signal strength, and addressing how diversity and complexity relate to cross-talk with signaling systems, such as the Wnt, hypoxia and BMP pathways. Nevertheless, we agree with the proposal of development of mathematical and computational models for this purpose, which is in fact our main theme.

Beginning with various approaches to modeling of Hes1, within the Notch pathway, and its role in the self-renewal and differentiation of neural stem cells, we address various models of allied topics. Shimojo et al. (2008) demonstrates how a system of SDEs can be used in modeling Hes1 oscillations within the Notch pathway and their role in self-renewal of neural precursors. The paper of Heron et al. (2007) is an applied tool for accurate estimates for parameters in such systems of SDEs representing regulatory networks, as applied to a set of experimental data in the realm of Hes1 expression. Barrio et al. (2006) treat this same problem of regulatory oscillation of Hes1 using the tools of discrete stochastic delay modelling and simulation. The manner of complex, transient oscillations observed within Notch during neural development can be modeled in Momiji and Monk (2009) which applies a model with local feedback loops in a model of the Notch pathway, based on lateral inhibition. This model is able to represent a variety of patterns to control system behavior, including in-phase and out-of-phase oscillatory modes, intermittent oscillations, and oscillation death. A model for the Delta1/Notch1 pathway based on a system of differential equations representing interactions in the gene network is constructed in Kirparissides et al. (2011) to study the propagation of the signal and mediators for neural differentiation. Representing neural progenitors by a stable limit cycle, the model requires a dramatic change in order to transition to differentiation.

The next group of models in Notch signaling deal with various aspects of higher complexities sometimes found in signaling. The first of these deals with trans-activation and cis-inhibition within Notch signaling, and this model of Wang et al. (2011a, b) has dual roles for trans- and cis-signaling, allowing oscillation in neural progenitors, asynchrony between adjacent cells arising from cis-inhibition. Another complexity within Notch signaling is represented in the model of Formosa-Jordan

and Ibañes (2014), which investigating cell states arising from the combination of cis-signaling with additional Notch signaling sources. This model demonstrates competition within Notch signals and displays the complexity associated to multiple sources sharing the same receptor. To address the issue of switching from oscillatory to sustained expression in neural fate decision, observed in development, the update model of Barton and Fendrik (2013) has the property that slight differences in Notch expression can lead to sustained oscillations in one neighboring cell while oscillations terminate in the other. The model of Pfeuty (2015) confirms the need for oscillations in genetic regulation of differentiation, and furthermore it also confirms the concept from Sect. 6.2 of the relation between multipotency and high levels of oscillation within gene expression, which is partially silenced as part of fate specification. Much more has been done in modeling of the role of oscillations within Notch, and a related topic for further development will be interaction with other pathways. The next section applies epigenetic landscape in modeling of one specific neural differentiation, also related to Notch and regulated by Hes5, allowing for exploration of varying levels of noise.

6.3.4 Model of Neural Differentiation Utilizing Epigenetic Landscape

In this section we review briefly a recent neural differentiation model that is based on computations using an epigenetic landscape. This model brings together many of the themes we have considered in our discussion, and it illustrates the applicability of the epigenetic landscape as a modeling tool. Furthermore this model addresses some interesting points related to cellular reprogramming, as well as the themes of stochasticity and dynamism in the landscape. This work continues the theme we addressed above relating to the role of the Notch pathway in development within the nervous system. This model represents commitment of neuron lineage through lateral inhibition, in which neighboring cells are inhibited through the Delta-Notch pathway.

We focus on the article of Qiu et al. (2012) in relation to its role in bringing together themes of neural differentiation through the models of epigenetic landscape. The model produced constitutes an advancement in that it gives a first example of a two-step bifurcation landscape model of cellular differentiation. The authors claim to study neural differentiation in relation to the wealth available numerical data, and the produced model is evaluated in relation to experimental data. This model involves interaction of a network of genes in regulation of the differentiation into neuron, astrocyte, and oligodendrocyte cells. The authors establish a minimal network of genes, focusing on the five factors Mash1, Brn2, Zic1, Hes5, and Olig2, within a slightly larger network of twelve factor, also including Scl, Stat3, A1dh1L, Myt1L, Sox8, Brn2, and Tuj1. In the first step Mash1 for neural induction and Hes5 for glial induction are mutual inhibitors. In the second step, the cells that had differentiated in the glial direction are further differentiated into

astrocyte or oligodendrocyte. The authors based their epigenetic landscape based on stochastic differential equations using the computational methods of Wang et al. (2011a, b), rather than the other computational approaches of Bhattacharya et al. (2011) or Ao (2004). Although this two-step model extends the concept of previous one-step models, the two-step landscape is obtained by gluing of the associated landscapes, rather than producing a holistic two-step landscape. The model produced is a system of SDEs, with twelve variables to represent the twelve genes listed above and with Gaussian noise terms. One advance made in this model is diagramming of paths for differentiation, as well as retrodifferentiaion and transdifferentiation, along the landscape. Furthermore the authors find different paths for differentiation and retro-differentiation, first for differentiation between neural precursor and neuron or glial cell, and in the second stage for differentiation between glial cells and astrocytes or oligodendrocytes. The cause for the differences in forward and reverse paths is identified to be the additional curl force in the dynamics of the quasi-potential system. Another point of interest of this model in relation to the theme of dynamic transformations of the epigenetic landscapes is a numerical exploration of the models using varying levels of noise. The impact on the landscape is given, where it lowers the levels of the barriers between cell states, which is explored in relation to scientific data.

6.3.5 Conclusion

By addressing models of epigenetics and its role in the fate determination of cells in the previous section, and by addressing models related to the roles of genetic pathways, including Sonic hedgehog (Shh), Notch, and Wnt in directing the fate determination of cells, we are naturally led to consider certain additional related issues regarding modeling of cell fate determination. Both of these play a role in understanding of cancer pathways and cancer stem cells. In fact the consideration of mathematical models in cancer stem cells and their relation to attractors in epigenetic landscapes and to genetic pathways directing the fate determination would be in itself an area worthy of further in depth study. This issue of epigenetics in understanding of differences in cell fates relates directly to formation of cancer cells from regular cells and the associated role of epigenetics, particularly from the systems biology perspective. In fact these models of epigenetic landscape and associated issues that we visited in the first section are also fully aligned to pathways of cancer and states of cancer as attractors within these landscape models, and these are fully valid applications of the study of epigenetics and the associated models. As we will observe in some of the models below, the subfield of epigenetics related to cancer pathways and the development of cancer is a major area of research with numerous important papers and models.

6.4 Epigenetic Models in Relation to Cancer Pathways and Cancer Stem Cells

6.4.1 Introduction

In this study of epigenetic modeling of differentiation of stem cells, many of the underlying themes and ideas relate directly to interesting topics in other areas of biology. We note, in particular, important relations to aspects of modeling in cancer, one of our other modeling interests. Such a larger range of relations is to be expected, as epigenetics is an important new area, experiencing development, which is important throughout biology. Furthermore, the role of epigenetics in cancer is one that has gained significant attention, as described for instance the Baylin and Jones (2011) article reviewing work epigenetics in cancer in the last decade. One important interaction of epigenetics in cancer relates to silencing of genes, as in Yu et al. (2008). We will observe how the epigenetic landscape perspective treated in Sect. 6.2, including attractor states, noise and stochasticity, and the systems approach, have all been considered in relation to cancer and cancer stem cells, noting some of the new perspectives they bring. We furthermore raise a few questions in relation to other areas of interest.

One interesting perspective connects cancer to gene expression and epigenetics via the theoretical approach to cellular differentiation of Kaneko and Furusawa we observed earlier, based on chaotic dynamics in gene expression, as in Furusawa and Kaneko (2001, 2009, 2012). Here the perspective is that cells in earlier stages of Waddington's metaphorical landscape, with higher potency and less commitment to a specialized adult lineage, will exhibit different patterns of gene expression with a wider range of oscillations robustness, and levels of chaotic expression. It is easy to make a connection to what happens in cancer cells, based on genetic instability, less structured gene expression, and higher levels of cellular heterogeneity.

In fact, from this perspective of dynamics of gene expression, Kaneko was able to anticipate in Kaneko (2011) the perspective of cancer attractors as non-robust network states at a distance from the normal epigenetic states of gene expression. Furthermore this perspective explains the accumulation of mutations in cancer cells, due to both oscillatory dynamics in gene expression and lack of canalization and robustness to noise, both in contrast to normal cell states. Both of these anticipate the perspective we will observe later in Sect. 6.4.2.1 in the culmination of the work in applying the theory of epigenetic landscape and cancer attractors to the issues of cancer cells and cancer stem cells. In fact numerous recent studies in cancer medicine have focused on this issue of heterogeneity of tumors, which in fact have exaggerated levels of heterogeneity, to an unhealthy level.

Heterogeneity of cell types within cancer, in fact increased levels of heterogeneity, is a well-known cancer property, carefully studied both for the progression of this

disease and for better understanding of its source. Recent cancer research has also focused on this issue of cellular heterogeneity, both at a theoretical level of understanding tumor cells and for the practical side, as it relates to response of cancer to treatments, as discussed in Fisher et al. (2013). These issues can easily be related to the study of multicellularity and its relations to stochasticity in gene expression, together with the associated epigenetic landscape, as addressed in Sect. 6.2.2.3. This is the prevailing perspective underlying the approach taken by Huang in his recent work extending the epigenetic landscape to apply to cancers as attractors Huang et al. (2009), and we investigate further the associated work below. Furthermore there is a related direct connection to multi-cellularity, heterogeneity, and stochasticity addressed in the article of Miller-Jensen et al. (2011) related to its role in disease and drug resistance, and these researchers find that the slow random transitions between activity and inactivity of a gene are due to chromatin remodeling, and other aspects of epigenetics.

Of the issues of genetic regulation, signaling, and oscillation which we observed played a role in stem cell differentiation, we can observe closely associated issues that play a role in cancer, as well. For instance, the genetic pathways Notch, Shh, and Wnt that we investigated in stem cell differentiation also are well to known to have an important role in relation to cancer. We mention the work of Hendrix et al. (2007) which observes effects of embryonic stem cells and the local microenvironment on reversion of the metastatic phenotype, as part of the program to search for convergence of embryonic and tumorigenic pathways for use in therapies. This relation between overlapping genetic pathways and their roles in both ontology and oncology is also a common theme in current cancer research, including in the works Kim and Orkin (2011) and Karamboulas and Ailles (2013). The distinction between these two types of cells is, at least in part, to be found at the epigenetic level. It may be expected that further development of the associated models may help increase understanding of these issues, and the differences in the role of these pathways in cancer and stem cells. Within this framework we also address the role of metabolism, both for stem cells and for tumor cells. Here the two differ significantly, and there are significant issues in development and spread of cancer that relate to metabolism. As part of extension of the epigenetic model to address this critical issue of cancer, we intend to develop further the issue of metabolism and the role it plays, in the biology and in the models. In the study of D'Anselmi et al. (2011), the development of cancer is connected with cellular membrane restructuring coupled with transformation to tumor metabolism, suggesting a significant shift in phenotype.

We have just observed how the issues visited in this chapter provide a number of overlapping perspectives of interest from which we can consider the issue of cancer. In this section we will look at these topics in a little more detail, outlining why these problems are interesting and how they lead to areas of further research and modeling. The background from which we approach the subject is the model of the epigenetic landscape and how these tools provide new tools and a fresh perspective for exploring these issues. We will outline some of the models that have been developed, as well as other issues that could be addressed in areas of differentiation therapy of cancer stem cells, the role of the genetic pathways of Notch, Shh, and

Wnt, the role of metabolism both in epigenetics and in cancer models. In particular, modeling can profitably be done in each one of these areas. We are particularly interested in aspects of metabolism and how these can be incorporated into the epigenetic landscape models.

6.4.2 Epigenetic Models and Cancer

6.4.2.1 Epigenetic Models, Cancer Pathways, and Cancer Stem Cells

We mentioned in the section on stochasticity a direct link between stochasticity, multicellularity, and heterogeneity and the heightened cellular heterogeneity observable in cancer cells. This point is also reiterated by Kaneko (2011), relating stem cells and cancer cells based on gene expression profiles and stability, as relates to the genetic instability of cancer, where this genetic instability and the associated behavior are hallmarks of the disease. From this viewpoint of stability within gene expression and epigenetics, the differences in plasticity for stem cells and cancer cells suggests a relationship, Lotem and Sachs (2006). This relates directly to the line of research taken by Huang and colleagues in application of this direction of modeling to the issue of cancer, beginning with such works as Huang et al. (2009) and Huang and Ingber (2007). In addition, Hernandez-Vargas et al. (2009) studied epigenetics in cancer stem cells. A significant program of research has emerged in this direction and the concept of a cancer attractor is addressed in multiple settings.

We also note there are some a priori reasons to link cancer and stem cells within a field of research, independently of the advances and connections just mentioned. For instance, existence of cancer stem cells which appear to fuel the tumor growth has also become a significant area of research in recent years. Differences in the cell membranes are detectable for such cancer stem cells, and it can also be displayed that these cells are resistant to treatment. Targeting of cancer stem cells is an important direction in current cancer research, and the dissertation of Youssefpour (2013) investigates behavior cancer stem cells through a multispecies continuum model that accounts for protein signaling factors produced by cells in lineages and nutrients supplied by the microenvironment. Simulations from this model emphasize the value of combination therapy on tumors, and use in conjunction with experimental data suggests direct differentiation of stem and non-stem cells. It makes sense to ask about differences in epigenetics for cancer stem cells, and aspects of this underlying approach are addressed in Sect. 6.4.2.3 on the role of metabolism both for cancer cells and as related to epigenetics, and in Sect. 6.4.2.4 on the relation of EMT (endothelial mesenchymal transition) to important areas of cancer and as related to epigenetics.

One of the underlying points in the development of epigenetic landscape is the concept of a cancer attractor, which arises naturally when thinking of cell states as attractors in the landscape associated to the underlying dynamical system. There are a number of studies extending this work of Huang et al. (2009) viewing cancer

states as attractors within the epigenetic landscape. It is relevant that cancer attractors are thought to be far away from the main part of the epigenetic landscape where the healthy cells reside, in a pathological state. Then two associated issues are addressed: first the nature of the landscape and consequences, and second concepts of how to return to the regular landscape. Associated to the pathological state of cancer, one large issue here relates to canalization, and the poor canalization and coarsely grained landscape are directly related to both tumor heterogeneity and also to development of resistance to therapies. In articles such as Brock et al. (2009), the development and progress of cancer is also directly related to landscape qualities. In concept the defect in the landscape is related to evolution, in articles such as Huang (2012b), as the standard landscape with well-defined channels is ascribed to evolution. On the other side, a number of studies also address the issue inheritable epigenetics, and the relation of the epigenetics and landscapes.

It is not surprising that closely aligned models and approaches can help to address the issue of cancer stem cells. The underlying issue of stochasticity associated to state transitions in an epigenetic landscape is dealt with differently in Gupta et al. (2011), also leading to a conclusion of a wide heterogeneity among cancer cells, as well as inherent transitions to a cancer stem cell state. This issue of phenotype conversion is also addressed in Zhou et al. (2014b), which addresses the population dynamics. In a related issue, Cabrera et al. (2015) addresses the plasticity of cell types within a tumor, including bidirectional conversion between stem and non-stem tumor cells, contributing to the high level of heterogeneity. The model of Zhou et al. (2013) illustrates the need for considering bidirectional cell conversions as part of mechanisms of cancer cell heterogeneity, and associated impact for understanding the underlying dynamics. These microenvironmental signals and cellular interactions with tumor niche are other aspects of cancer to be modelled, and associated issues are raised below.

These issues lead naturally to the issue addressed in Huang and Kauffman (2013), how to escape the tumor attractor, which gives an epigenetic analysis related to cancer drug development. These also directly contribute to response of tumors to therapy and radiotherapy. These issues are addressed in the articles Huang and Kauffman (2013) and Pisco and Huang (2015), which gives an alternative view of how certain therapies can produce strengthened versions of the cancers. Associated to the article of Fessler et al. (2013), addressing cancer stem cell dynamics and progression to metastasis, is the issue of the microenvironment, known to be of critical importance in cancer. This issue in dynamics of cancer stem cells and progression to metastasis is directly related to additional issues we need to explore in epigenetic cancer modeling. Sections 6.4.2.3 and 6.4.2.4 address address issues of metabolism and EMT, their role in cancer, and relations to epigenetics. Perhaps there are additional weaknesses of the current state of the landscape models, including representation of the role of the microenvironment, and associated issues including EMT and metabolism.

6.4.2.2 Pathways, Cancer Stem Cells, and Differentiation Therapy

As we observed in the previous discussion on systems biology, this is an important approach to science and medicine currently undergoing much development, such as in Aebersold et al. (2009), that applied equally well in areas of cancer and stem

cells. In fact we observed some relations between these two settings above, and these issues are developed further in the current section. With the advent of investigation of the role that cancer stem cells play in tumor growth and development, there have been efforts to use this knowledge in treatment of cancer, including the targeting of tumor cells and the approach of differentiation therapy.

Furthermore there is an accumulation of evidence relating to overlap between the genetic pathways that control stem cell fate and pathways involved in cancer, in particular Wnt, Notch, and Shh. In particular, these are dysregulated in cancer, and it is hoped that these pathways may provide novel approaches to treatment, including differentiation therapy. In Ruiz i Altaba et al. (2002) the issue of origin of tumor stem cells was treated from the perspective of genetic and signaling pathways similar to developmental programs, with results suggesting that inappropriate activation of Shh and Gli transcription factors could lead to onset of a tumorigenic program, in turn suggesting these as targets for therapies. This issue of the role of gene regulatory networks within the development and progression of cancer is an extensive area of research, and the concept that cancer involves a deregulation of one or more genetic pathways is generally accepted. We mention the article Wang et al. (2007), which addresses the program studying cancer-associated genes from a systems biology point of view, and discusses integration of protein and signaling networks. Finally, in the context of differentiation therapy associated to targeting of stem cells mentioned above, understanding of associated pathways, including Shh, Wnt, and Notch, is critical to this promising approach to therapy, Han et al. (2013).

In an initial paper based on modeling the disruption of quorum sensing, Agur et al. (2010) gave a simple model of tumorigenesis for mammary stem cells. Research in this direction led to the study of Agur et al. (2011) in which the role of Dkk1 in the Wnt pathway is explored through a simple quorum sensing model, suggesting that Dkk1 treatment may be a robust method for eliminating cancer stem cells. A successive study on the Wnt signaling pathway by Kogan et al. (2012) suggests combinational therapy of sFRP and Dkk. In addition the research and modeling of Bao et al. (2013) considered cancer related variations on Wnt signaling, and concludes that cancer cells will mutate in order to maintain a normal level of Wnt signaling. The study of Matsuda et al. (2014) addresses the means of self-renewal and differentiation of CSCs within the CSC niche. This in vitro experimental study reveals that the same factors secreted from the CSCs promote self-renewal through Notch signaling and regulate differentiation lineage. In this brief overview of these critical issues, we do get a sense of some possible interactions and connections in these deeply interrelated fields. There is a very large research program to develop these further and work out details and implications.

As a specialized area within this overlap between systems biology of cancer and systems biology of stem cells, we want to understand how epigenetics relates to development of cancer and particularly to the formation of cancer stem cells. Within the context of the epigenetic landscape and its role in cell fate determination, an important goal of this section is to apply the epigenetic landscape and associated tools to this issue. Understanding epigenetics of stem cell differentiation in relation to carcinogenesis is one such goal, addressed in Yamada and Watanabe (2010), which

also explores the epigenetic regulations of differentiation of tumor cells and particularly the relation to cells capable of tumor initiation. Cancer stem cells are a primary area of concern in the treatment of cancer, and Hernandez-Vargas et al. (2009) reviews the evidence for epigenetic mechanisms in formation of cancer stem cells, emphasizing relevant regulatory networks, particularly the deregulation of self-renewal and pluripotency mechanisms, and their potential deregulation in cancer.

6.4.2.3 Interaction of Metabolism and Epigenetics; Role of Hypoxia in Cancer and Epigenetic Perspective

Just as with some of the finer points relating to issues discussed above in modeling of cellular differentiation, the epigenetic landscape we have developed so far cannot completely address the issues of cancer, cancer attractors, and cancer stem cells. Clearly it does tell us a great deal and offers many valuable new perspectives. However central points to cancer, such as the role metabolism and the microenvironment, have not been addressed. Furthermore the related issue of formation of cancer and how the cells move into that part of the landscape is largely absent from the current models. Our goal here is to look at how metabolism is an integral part of our understanding of cancer, both in formation and development, and to discuss its inclusion in the models. Thus we join Menendez et al. (2014) in calling for an adjustment to the epigenetic landscape approach that includes the role of metabolism in epigenetics. This area is one of critical importance and must be included in the models to represent this aspect of both cancer biology and stem cell biology. The article of Menendez and Alarcón (2014) looks forward to new advances in precision in cancer treatment from the metabo-epigenetic perspective.

There is a direct link of cancer to metabolism, both in formation and in subsequent developments. It is also certain that metabolism has a direct impact on epigenetics, such as observed in relation to the role of metabolism in epigenetics and cell fate, Kida et al. (2015), who also found metabolics to play a role in reprogramming of somatic cells to iPS cell. The article of Johnston et al. (2012) addresses how mitochondrial variability introduces noise and stochasticity at the cellular level, thus affecting the epigenetic landscape in the nature discussed above. Shaughnessy, et al. (2014) discusses the role of mitochondria and signaling mechanisms in environmental impact on cell and epigenetic regulation. Lu and Thompson (2012) also address metabolic regulation of epigenetics.

Clearly the combination of these two factors implies that the role of metabolism on epigenetics should be explored in relation to these models. In regard to cancer and cancer stem cells, Muñoz et al. (2012) discusses relevant epigenetic alterations in DNA methylation, histone modifications, and miRNA in relation to CSC regulation. The article of Menendez and Alarcón (2014) defines the concept of 'metabostemness' in relation to the metabolic alterations and epigenetic rewiring found in CSCs. We mention two additional points related to important aspects of metabolism in cancer biology: hypoxia and ROS. The need for inclusion of effects of metabolism in epigenetic landscapes is reiterated in Hitchler and Domann (2012),

where the issues of metabolic remodeling within a cell's epigenetics is addressed in relation to redox regulation of the epigenetic landscape in cancer. The issue of production of ROS in both stem cells and tumor stem cells is addressed in Zhou et al. (2014a), where it describes pathologies related to disruption of the tight control of this production in stem cells. Furthermore in tumor stem cells the aberrant production of ROS leads to tumor progression. The article of Muñoz et al. (2012) addresses the role of hypoxia and hypoxia-inducible factors in progression to CSCs and potential development of metastasis. For hypoxia, there is also a bridge to EMT that we address briefly in Sect. 6.4.2.4. We mention in closing that the interaction between epigenetics and metabolism is another instance of systems biology, as addressed in Sect. 6.2.2.4 above, and it is worthwhile to consider how this can fit into the larger program, such as Hood and Tian (2012) and Hood et al. (2012a, b).

6.4.2.4 Role of EMT in Cancer and Metastasis, Epigenetic Perspective

The endothelial-mesenchymal transition (EMT) has been implicated in cancer as a key event in cancer cells which confers the ability for a tumor to metastasize. During EMT, which also occurs regularly during development and wound healing, the endothelial cells lose cell-to-cell adhesion through E-cadherin repression. In cancer cells this provides increased capability for invasion, and these cells can then break through the basement membrane and enter the bloodstream. Currently researchers are interested in better understanding how EMT performs a role both in the transition of states to the cancer stem cell state and in preparation of cancer cells for metastasis, such as Tsai and Yang (2013). Particularly, in relation to the role of EMT in metastasis, we are interested to further develop the landscape models, and our interest relates directly to these issues, particularly the role of metabolism, hypoxia, and signaling pathways. In looking at these in relation to epigenetics, we are also interested in the role of transcription factors (EMT-TF) known to repress transcription of E-cadherin, or others which increase its transcription. This issue is important in relation to drug resistance, as well as in development of stem-like properties. In one of the earliest works applying the concepts attached to the epigenetic landscape models to the problems of cancer, Huang and Ingber (2007) address the role of EMT and the alteration of cell mechanics in relation to non-genetic bases of cancer and progress toward metastasis, also addressed in Cabrera et al. (2015) in relation to plasticity. As EMT has been indicated to be involved in acquiring drug resistance, it is interesting to compare and contrast with the discussion of the epigenetic landscape in relation to drug resistance, Huang (2013) and Pisco and Huang (2015), as addressed in Sect. 6.4.2.1 above. Note that recent evidence suggests that cells that undergo EMT gain stem-like properties, giving rise to CSCs, suggesting possible similarities.

Just as mentioned in Eby and Tabatabai (2014), an intriguing possibility related to the concept of a cancer stem cell is the idea of stem cell niché in relation to metastatic cancer, and the concept of metastatic cancer based on analogy with a group of stem cells freed from the locality, for which the niché is also able to travel

to another locality. Clearly both the EMT and its reverse process the MET (mesenchymal-endothelial transition) play an important role, associated to metastasis. Based on where we have arrived in this study, we can again see the overlapping of a number of important issues in relation to EMT and metastasis. Clearly there is a relation of EMT to epigenetics, with extensive epigenetic changes coinciding with the change in phenotype, also addressed in Malouf et al. (2013). There are also clearly associated adjustments in metabolism, in relation to the discussion of Sect. 6.4.2.3, and in fact hypoxia induced EMT has an epigenetic basis, which Wang and Wu (2015) are studying to determine targets for therapy. This new tool of the epigenetic landscape may be applied to investigate these intriguing questions in relation to metabolism, epigenetics, and EMT. In particular we would like to look at quantification of the associated epigenetic landscape and investigate how metabolism and regulation affect this landscape. In particular one underlying goal of this extended modeling effort will be to learn more about metastatic process.

In relation to the role of metabolism in epigenetics addressed above, and particularly in relation to hypoxia and redox processes, current research show a role for hypoxia and redox processes in EMT. The study of Salnikov et al. (2012) found that in the increased invasion and metastasis found in tumor cells undergoing EMT induced by hypoxia, it was the stem-like cells in the CSC^{high} category that acquire high potential for migration and are likely responsible for invasion and metastasis. Although tumor hypoxia can induce EMT, the study of Prasanphanich et al. (2014) found that the after the extensive reprogramming in EMT, the new phenotype is resistant to antioxidant perturbation. As also mentioned in Pujadas and Feinberg (2012), epigenetics associated with EMT should be further studied and corresponding models developed, especially in relation to its importance in both ontology and oncology.

6.5 Conclusions and Additional Questions

We have reviewed how the current research in epigenetics is represented well by an extension of the concept of epigenetic landscape based on underlying scientific concepts representing the underlying regulatory networks as a dynamical system, in which various cell states can be represented as attractors. The issue of how the transitions are made between cell states within this model is represented by a quasi-potential landscape, expanding the underlying landscape metaphor of Waddington to incorporate current scientific knowledge and mathematical theories. Models of stem cell differentiation fall within this realm, in connecting the underlying principles of biology to mathematical tools that are universal to cell differentiation and to epigenetics more generally. We also recognized how this modeling perspective was expansive enough to accommodate the current level of scientific knowledge, and furthermore provided means for insightful questioning and discovery, particularly within the area of noise and stochasticity in cell function, cell states, and multicellularity. We also recall the importance of the systems biology perspective and the relation of landscape to issues of oscillation, regulation, and switching.

This field of epigenetic modeling appears to be in its early stages, and much development can be expected in the future in this expansive and promising field. It appears this underlying approach to epigenetic modeling has much potential and also allows connections to a number of important areas of biological research. Furthermore, within the context of the underlying systems biology approach and background, the machinery appears to have potential to mesh with goals of the larger research program. Although much remains to be developed in this direction and the level of complexity of the larger program is vast, there is also significant potential. Furthermore an underlying strength of this modeling concept is the nature by which it both appeals to creativity and imagination and leads to valuable predictions, perspectives, and results. A few quotes from Einstein seem appropriate to describing this potential. "Imagination is more important than knowledge. Knowledge is limited. Imagination encircles the world." Although there is much more not known about this model and field than what is known, it does offer a channel for creative thought and development. Furthermore, it offers numerous paths for exploration and development, and ideally can accommodate information learned by experiment. "A theory can be proved by experiment; but no path leads from experiment to the birth of a theory." What remains to see is how the epigenetic landscape of the model becomes more fully described and what paths unfold from the present.

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Chapter 7
Organ and Appendage Regeneration in the Axolotl

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Abstract Regeneration is a remarkable feat of biology. It requires an organ system – most often consisting of many different cell types – to stop its specialized function and step back in ontological time. Regeneration overcomes the general understanding that development is a one-way street. We now know that there is great variability in regeneration capacity across phylogeny, likely because animals need to have some mechanism in place to survive injuries or diseases that it will encounter. Some animals, including humans, meet this need by closing the wound as quickly as possible and making due with the deficit. Other animals, such as the axolotl described here, instead regenerate the damaged or missing tissue.

Ambystoma mexicanum, commonly known as the axolotl, is uncommon among vertebrates because of its superior regenerative abilities. References of their unique abilities cross from the scientific to the main stream media, but they are also useful animals for understanding the mechanisms that regulate regeneration. As axolotls are tetrapods that can breed all year-round and accept grafts between adults, they possess some major advantages that make them uniquely suited to be animal models of regeneration. In terms of appendage regeneration, there is currently a lack of animal models for complex regeneration. The two dominant models are zebrafish, which regenerate caudal fins throughout life, and *Xenopus* frogs, which can regenerate limbs and tails early in development. Although these systems have provided important insights into vertebrate regeneration, the axolotl limb is particularly well suited to study adult appendage regeneration. Axolotls are capable of regenerating complete adult limbs that are morphologically similar to human limbs using endochondral ossification. In contrast, the zebrafish dermal caudal fin skeleton has no

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mammalian counterpart, and the fin skeleton regenerates by direct ossification from mature osteocytes (Sousa et al. Development, 138(18):3897–3905, 2011; Knopf et al. Dev Cell 20(5):713–724, 2011). *Xenopus* regenerates early in development, but its ability to regenerate patterned skeletal structures is absent in adulthood. Therefore, the axolotl is among the best vertebrate models for adult joint regeneration.

Although axolotls have been studied for almost 200 years, only recently have technological advances helped revive the axolotl into a model organism in modern regeneration biology (Voss et al. Cold Spring Harbor Protoc 2009(8), 2009). The axolotl is becoming a chosen model for regenerative biology because it can regenerate more completely than any other vertebrate in the majority of organs studied. Modern genomic tools are available including microarray analysis (Monaghan et al. J Neurochem 101(1):27–40, 2007; BMC Biol 7, 2009; Biol Open. 2012; Campbell et al. Dev Dyn: An Off Publ Am Assoc Anat 240(7):1826–1840, 2011), RNAseq (Monaghan et al. BMC Biol 7, 2009; Stewart et al. PLoS Comput Biol 9(3):e1002936, 2013; Knapp et al. PLoS One 8(5):e61352, 2013), a genomic map (Smith et al. Genetics 171(3):1161–1171, 2005a), genomic sequence data (Smith et al. BMC Genomics 10:19, 2009), and bioinformatic databases (Smith et al. BMC Genomics 6:181, 2005b). Functional testing of genes is also available through the generation of transgenics (Sobkow et al. Dev Biol 290(2):386–397, 2006; Monaghan and Maden, Dev Biol 368(1):63–75, 2012a; Khattak et al. Nat Protoc 9(3):529–540, 2014; Whited et al. Proc Natl Acad Sci U S A 109(34):13662–13667, 2012), knockdown of genes by morpholinos (Schnapp et al. Development 132(14):3243–3253, 2005; Zhu et al. Dev Biol 370(1):42–51, 2012), over-expression of genes by electroporation (Mercader et al. Development 132(18):4131–4142, 2005) and viruses (Whited et al. Development 140(5):1137–1146, 2013; Khattak et al. BMC Dev Biol 13:17, 2013), and cell tracking by tissue grafting between GFP and white axolotls (Nacu et al. Cold Spring Harbor Protoc 2009(8), 2009). With this array of modern tools, married with the qualities that have made the axolotl a subject of research for hundreds of years, the axolotl system has become a powerful model to dissect the mechanisms that regulate development and regeneration.

Here, we will highlight what is known about the axolotl's regenerative abilities and discuss the mechanisms that regulate regeneration of each organ system. It is generally assumed that the axolotl has the ability to regenerate most if not all of its tissues, but a survey of tissue regeneration has yet to be performed in this animal model. We will focus upon the regenerative capacity of the axolotl, but it is necessary to include examples of regeneration in the newt, *Xenopus laevis*, and zebrafish because in some aspect these species have been studied in more detail than in the axolotl model.

Keywords Axolotl • *Ambystoma mexicanum* • Cardiac regeneration • Nervous system • Limb regeneration • Spinal cord • Wound healing • Immune system • Extracellular matrix

7.1 Axolotl Limb Regeneration

Though the axolotl possesses many extraordinary regenerative capabilities, its ability to fully regenerate amputated limbs is among its most striking and well-studied. Urodele salamanders are the only vertebrates capable of regenerating limbs throughout adulthood, and thus this process has been the subject of scientific study and fascination for more than two centuries (Spallanzani 1769). Though many of the molecular mechanisms underlying limb regeneration remain poorly-understood, past studies have nevertheless elucidated the major steps and some underlying mechanisms of the process.

Axolotl limb regeneration occurs in a series of stages that are morphologically and transcriptionally distinct (Voss et al. 2015). Amputation of the limb induces rapid vasoconstriction, which serves to minimize blood loss from the injury. Clotting is very rapid, as is epidermal migration and closure of the wound site. So long as excess bone is trimmed and kept from protruding from the wound site, wound closure will occur within 24 h post-amputation. Over the next several days, the wound epithelium thickens and forms a structure called the apical epithelial cap (AEC), which intimately contacts the mesenchyme in the absence of the dermis. This AEC is a crucial component of limb regeneration- if it is prevented from forming or replaced with fully-thickened epidermis, regeneration does not take place (Tassava and Garling 1979; Loyd and Tassava 1980). It is believed that the AEC secretes a host of factors, including metalloproteases, which are necessary for breaking down the extracellular matrix (ECM) of the underlying tissue, thus permitting and guiding cell migration and accumulation in the mesenchyme (Thornton 1960; Yang and Bryant 1994; Yang et al. 1999; Lévesque et al. 2007). Once a critical mass of dedifferentiated cells accumulates below the wound epithelium, the next stage of regeneration is initiated and a proliferative mass called the blastema is formed. Although the overall rate of limb regeneration largely depends on the size of the animal, the switch from wound healing and cell accumulation to blastema formation generally occurs at around 10 days post-amputation (DPA). Once the blastema has formed, cell cycling and proliferation increases dramatically (Loyd and Tassava 1980) as blastemal cells divide rapidly and promote the outward growth of the regenerating structure.

7.2 Characteristics of the Blastema

Though it appears to be a homogeneous population of near-identical cells, the blastema is in fact comprised of local (within 1–2 mm of the wound site (Butler 1935; Butler and O'Brien 1942)) dedifferentiated cells arising from several different tissue types, including fibroblasts, Schwann cells, and satellite cells (Monaghan and Maden 2012b). This last cell type represents a crucial distinction between axolotls, which are members of the family Ambystomatidae, and newts, which are members

of the family Salamandridae. Though newts and axolotls bear superficial similarities and are both studied for their regenerative capabilities, the families in fact diverged 145 million years ago (Zhang and Wake 2009) and certain differences have been noted in their regenerative mechanisms. Of note is the fact that while satellite cells dedifferentiate and contribute to the blastema in the axolotl, in newts it appears that myocytes themselves dedifferentiate (Sandoval-Guzman et al. 2014). Caution is thus advised when applying findings from the newt to the axolotl, and vice versa. However, one feature that remains constant across both examples of limb regeneration is the fact that all blastemal cells are lineage-restricted: that is, once regeneration is nearly complete, they re-differentiate back into their tissues of origin. Thus, satellite cells eventually differentiate into myocytes while Schwann cells will only become Schwann cells. The lone exception is that of fibroblasts, which can differentiate into either fibroblasts or chondrocytes (Kragl et al. 2009). Dedifferentiated fibroblasts make up a disproportionately large percentage of the regenerating blastema (Muneoka et al. 1986), and their plasticity allows the limb to fully regenerate even if all skeletal elements are removed prior to amputation (Thornton 1938; Foret 1970). Although the mechanisms behind this cellular "memory" remain unknown, one can safely qualify the blastema as a collection of generally lineage-restricted, dedifferentiated cells arising from multiple tissue types.

7.3 Nerve Dependence and Molecular Mechanisms of Limb Regeneration

One curious characteristic of blastemal formation and growth is the fact that it is nerve-dependent. If the limb is denervated prior to or shortly after amputation via simple transection of the brachial nerves, the wound heals over without incident but formation of the blastema does not occur and regeneration does not go forward. This phenomenon has been the source of scientific curiosity and study since it was first discovered in the early 1800s (Todd 1823), and nerve dependence is in fact found in numerous examples of regeneration and wound healing across phylogeny. From starfish arm regeneration to mammalian ear punch healing, an intact nerve source appears to be imperative for proper regenerative growth (Kumar and Brockes 2012). Though the molecular mechanisms underlying this nerve dependence remain poorly-understood in the axolotl, a host of studies spanning the past half-century have done much to elucidate the roles of these nerves during limb regeneration.

Nerves heavily invade the wound epithelium within several days after amputation, and they appear to be critical for the maintenance of the AEC, as early denervation eventually results in the collapse of the AEC and the impairment of blastema formation. Nerves further appear to be necessary for the maintenance of blastemal proliferation. Denervation once the blastema has formed starkly reduces the proliferation of blastemal cells (Tassava et al. 1974; Maden 1978; Goldhamer and Tassava

1987), though it does not appear to affect cell differentiation or limb patterning. Thus, denervation well after blastema formation (at around 15 DPA) actually induces the formation of a miniature, fully-patterned limb (Schotté and Butler 1944; Singer and Craven 1948; Powell 1969). Axolotl peripheral nerves are themselves capable of regeneration and will in fact rapidly regrow after denervation. Consequently, studies involving limb denervation must be careful to re-denervate every 7–10 days and cannot last longer than approximately 20 days, after which re-denervation is effectively impossible and regeneration of the limb goes forward. This characteristic represents another divergence between newts and axolotls, as amputated newt limbs do not recover from brachial nerve transection and will not regenerate even up to 72 days post-denervation (Liversage and McLaughlin 1983).

A series of elegant experiments performed by Marcus Singer in the 1950s characterized the nature of nerve dependence in the regenerating axolotl limb. Singer found that a certain number of nerve fibers is necessary for regeneration, although this amount varies depending on the site of amputation- if the number of nerves present falls below a specific threshold, regeneration does not take place. Singer also found that the critical function of the nerves does not involve action potentials or neurotransmitter release, nor does it require sensory or motor neuron innervation in particular. Instead, nerve support of the wound epithelium and blastema appears to be trophic in nature (Singer 1952, 1964). These experiments suggest that nerves- and the dorsal root ganglia, implantations of which are capable of rescuing regeneration in denervated limbs (Kamrin and Singer 1959; Tomlinson and Tassava 1987; Goldhamer et al. 1992)- release factors which are critical for inducing blastema formation and maintaining blastemal proliferation. The precise identities of these factors remain largely unknown, although evidence has been gathered in support of many- including neuregulin-1 (Wang et al. 2000), transferrin (Kiffmeyer et al. 1991; Mescher et al. 1997), fibroblast growth factors (Satoh et al. 2011), and anterior gradient protein (Kumar et al. 2007). Identification of these critical nerve-derived factors thus constitutes a major topic of regenerative science moving forward.

The early invasion of macrophages into the wound site is also critical for limb regeneration. Total macrophage ablation prevents regeneration and induces aberrant fibrotic deposition in the wound site (Godwin et al. 2013). Limb regeneration like all forms of axolotl regeneration is a totally scar-free process that occurs with minimal collagen deposition (Seifert et al. 2012; Levesque et al. 2010). Instead of collagen, a dynamic network of fibronectin provides loose structure for the regenerating blastema (Maden and Keeble 1987; Rao et al. 2009). It is therefore possible that macrophages, which peak in number at approximately 4 DPA (Godwin et al. 2013), are necessary for maintaining a permissive regenerating environment early after injury. Further studies have also demonstrated that macrophages play a role in clearing senescent cells from the wound site and blastema during regeneration (Yun et al. 2015). It is thus believed that macrophages have multiple crucial functions in axolotl limb regeneration, and the study of these functions remains an ongoing process.

7.4 Limb Patterning and Mammalian Appendage Regeneration

One extraordinary characteristic of axolotl limb regeneration is the fact that perfect regeneration occurs regardless of the site of amputation. Thus, amputation at the shoulder will result in regeneration of the entire limb, while amputation distal to the elbow joint will regenerate only distal tissues. Considerable research has therefore been devoted to elucidating the molecular underpinnings of axolotl limb patterning. Studies have found that fibroblast-derived blastemal cells demonstrate a "memory" of their initial position (Kragl et al. 2009), and this position is in some way expressed on the cell surface, as proximal cells cultured in vitro reliably engulf distal cells (Nardi and Stocum 1984). These cells are thus capable of detecting discrepancies in the proximal/distal and dorsal/ventral axes and subsequently intercalating any missing structures, though the stability of this positional memory varies and can be reprogrammed in early and distal blastemal cells if they come into contact with more stable proximal cells (McCusker and Gardiner 2013). A host of studies have demonstrated that retinoic acid (RA) plays crucial roles during limb patterning. Application of exogenous RA early after amputation induces its expression in (Monaghan and Maden 2012a) and proximalizes (Niazi et al. 1985; Keeble and Maden 1989) fibroblast-derived blastemal cells, completely erasing distal positional memory and suggesting that positional cell memory is in some way maintained via a proximodistal retinoic acid gradient throughout the limb (Scadding and Maden 1994). Meanwhile, the salamander-specific cell surface protein Prod1, which is upregulated in the blastema in response to RA (da Silva et al. 2002), may mediate cell adhesion differences. Prod1 ablation impairs proximal cell engulfment (da Silva et al. 2002) and overexpression of the protein proximalizes distal blastemal cells (Echeverri and Tanaka 2005). The molecular mechanisms of limb patterning remain under investigation, and as salamander limb patterning is itself a topic vast enough to fill an entire chapter, numerous reviews have covered the process in greater detail (Stocum and Cameron 2011; McCusker and Gardiner 2014; Mariani 2010; McCusker et al. 2015).

The axolotl's extraordinary ability to fully regenerate amputated limbs sets it apart from mammals and virtually all other vertebrates outside the urodeles, but some regenerative mechanisms are conserved across phylogeny. Mammalian appendage regeneration is very limited in scope: mice can regenerate only the tips of their digits (Borgens 1982) while human children are capable of regenerating amputated fingertips so long as the wound is not immediately sealed after injury (Illingworth 1974). Like axolotl limb regeneration, mammalian digit tip regeneration relies on a heterogeneous mix of lineage-restricted progenitor cells (Lehoczky et al. 2011) and is disrupted if there is no intact nerve source, although nerve dependency in this case appears to affect tissue patterning more than cell proliferation (Rinkevich et al. 2014). However, nerve dependency during mouse ear hole punch regeneration appears to align closely with salamander nerve dependency, as denervation of the ear prevents blastema formation and induces necrosis (Buckley et al. 2012). This over-

lap further underlined by the conserved presence of *msx1*, a homeobox-containing gene that is highly upregulated during both mammalian digit tip (Reginelli et al. 1995; Allan et al. 2006) and axolotl limb regeneration (Koshiba et al. 1998). Axolotl limb regeneration thus shares some essential similarities with mammalian digit tip regeneration, and consequently it offers an enticing model for the study and advancement of regenerative medicine in mammals.

7.5 Nervous System Regeneration

Axolotls have the striking ability to regenerate their central nervous system after multiple injury paradigms including tail amputation, spinal cord transection, spinal cord crush, and brain injury ((Chernoff et al. 2003; Chernoff 1996) for reviews). Considering the variability of CNS morphology and injury types, the axolotl's ability to regenerate after so many different injuries is remarkable. Reasons for the axolotl's regenerative capabilities are unknown, but traits including ongoing widespread adult neurogenesis, a lack of a glial scarring after injury, and the overall retention of embryonic characteristics likely contribute. However, axolotls are not unique in their regenerative ability as teleost fish, other salamanders, and lizards can regenerate CNS axons in the tail, while embryonic birds and mammals can regenerate CNS tissue to some extent ((Chernoff et al. 2003; Tanaka and Ferretti 2009) for reviews).

7.6 Brain Regeneration

Brain regeneration has been demonstrated mainly in the telencephalon (Kirsche and Kirsche 1964a, b; Richter 1968). The axolotl brain looks similar in structure to the embryonic mouse brain. Neural stem cells with radial processes send processes out to the pial surface throughout the animal's life. These neural progenitor cells proliferate throughout the uninjured adult axolotl nervous system, with the highest level of proliferation occurring in the telencephalon (Maden et al. 2013; Richter and Kranz 1981), and differentiate into NeuN+ neurons. Injury induces extensive proliferation of neural progenitor cells in ventricular zones, which then migrate and differentiate into several different cell types such as mature and immature neurons, astrocytes, and radial glial cells (Maden et al. 2013). Ongoing neurogenesis in regions capable of regeneration may provide an essential pool of active progenitor cells required to mount a regenerative response. Interestingly, these proliferation trends are not unique to axolotls. The human brain has active adult stem cell niches in the subventricular zone and subgranular zone of the hippocampus (Eriksson et al. 1998; Spalding et al. 2005). In addition, injury in adult rats induces extensive proliferation capable of ameliorating the damage produced by stroke induction,

though the environment surrounding the lesion normally prevents successful healing (Arvidsson et al. 2002). It is intriguing to think that the availability or induction of neural progenitor cell proliferation could enhance regenerative ability in mammals.

7.7 Spinal Cord Regeneration

Regeneration of the spinal cord can occur anywhere along the rostro-caudal axis of the animal after spinal cord crush, transection, or tail amputation. Tail amputation is the simplest and most repeatable injury model and does not have detrimental effects such as paralysis. Therefore, it has become the most widely adopted model for CNS injury. Tail amputation is followed by regression of the spinal cord by ~0.5 mm from the amputation plane accompanied by a rapid immune response with an influx of leukocytes to the wound site. Over the first few days, cell death of neurons near the amputation plane is apparent along with degeneration of white matter (Monaghan et al. 2007; Zhang et al. 2003). By 3 days after amputation, neural progenitor cells have migrated to generate a terminal bulb at the caudal end of the spinal cord, which is surrounded by blastema cells. By approximately 7 days after injury, cell proliferation of neural progenitor cells and axon regeneration of spared neurons ensues in a caudal direction. The migration and proliferation of the neural progenitor cells lining the central canal is a conserved mechanism for all animals that retain the ability to regenerate their spinal cord (Gaete et al. 2012; McHedlishvili et al. 2007). The cells about 500 um proximal to the tip of the damaged spinal cord give rise to all of the central and peripheral nervous system components of the regenerated tail (McHedlishvili et al. 2007, 2012). Most neurons in the regenerated spinal cord arise from new neurogenesis, although a subset of neurons in the new cord arise from translocation of differentiated neurons located proximal to the injury site (Zhang et al. 2003). The importance of the neural progenitor population for regeneration was clearly demonstrated using CRISPR/Cas 9-mediated genomic ablation of the important neural stem cell gene Sox2, which showed that axolotls with nearly 100 % Sox2 deletion had defective spinal cord regeneration (Fei et al. 2014). This finding is extremely important in our search for the differences between molecular mechanisms of mammals and axolotls, as even conditional Sox2 knockouts cause developmental problems in mice (Ferri et al. 2013). Future knockout studies are likely to elucidate the genetic networks that are essential for axolotl CNS regeneration.

Spinal cord regeneration after tail amputation is considerably different than spinal cord transection or crush because it occurs within a regenerating tail blastema. Furthermore, tail amputation necessitates the regeneration of multiple tissues types beyond the neural tissue, grows only in a single direction, and is nerve dependent (Holtzer 1956). Both injury models rely upon the proliferation of existing stem neural stem cells of the spinal cord, which are analogous to the mammalian radial glia seen only during development (Tanaka and Ferretti 2009). Axolotl spinal cord transection may thus provide the most beneficial insights into possible therapies for mammalian CNS injury. After transection, the terminal bulb is generated by 5 days

post injury and rejoins the proximal to distal stumps by 15 days post injury. Axon regeneration begins between 7 and 15 days and axons cross the lesion site by 21 days (Hui et al. 2013). Axon tracing studies after spinal cord transection have demonstrated that the regenerated portion of the spinal cord is thinner than the uninjured portion at 6–12 weeks post-transection, but eventually – sometimes up to 23 months after transection – all of the axonal connections are reestablished (Clarke et al. 1988; Davis et al. 1990).

The axolotl's ability to regenerate its spinal cord may be explained by a combination of pro-regenerative events that overcome inhibitory signals. For example, in contrast to mammals, axolotls do not form a glial scar after spinal cord lesion. Regeneration of the CNS in mammals is impaired by the formation of a glial scar comprising of astrocytes and inhibitory proteoglycans (Fawcett and Asher 1999). This barrier is thought to be initially beneficial because it prevents the spread of damage throughout the system. However, it becomes detrimental when axons begin sprouting across the injury site and are stopped by the glial scar. Similar glial cell migration after injury exists in salamanders, but these processes establish a permissive environment that promotes CNS regeneration (O'Hara and Chernoff 1994; Zukor et al. 2011). A brief spike in apoptosis might enable lower vertebrates to eliminate injured cells and prevent the spread of tissue damage (Sirbulescu and Zupanc 2009). Furthermore, expression of matrix metalloproteinases, which chew up extracellular debris, may limit the deposition of scar tissue and provide necessary remodeling of the extracellular matrix (Chernoff et al. 2000). Mammals also express myelin proteins such as Nogo-A, Oligodendrocyte myelin glycoprotein, and myelin-associated glycoprotein whose release after injury may inhibit axon regeneration (Yiu and He 2006). Regenerative species possess each of these molecules so it is not the presence or absence of these molecules in lower vertebrates that imparts regenerative ability (Hui et al. 2013; Shypitsyna et al. 2011), although their presence does not interfere with healing and regeneration. Lastly, upregulation of axon growth-inhibiting molecules such as Semaphorin 4D also limits regeneration in the mammalian CNS (Moreau-Fauvarque et al. 2003). Interestingly, comparisons of microRNA expression between regenerating axolotls and spinal-lesioned rats showed that *miR-125b* is downregulated in axolotls during regeneration. Overexpression or inhibition of *miR-125b* inhibits proper axolotl spinal cord regeneration and increasing *miR-125b* levels in rats decreased Semaphorin 4D, which improved functional recovery after spinal cord lesion (Macdonald-Obermann and Pike 2014). Altogether, several factors may contribute to the axolotl's ability to regenerate. Functional studies that test the role of each of these factors should elucidate the mechanisms that enable CNS regeneration.

7.8 Scarless Wound Healing

Axolotl skin facilitates cutaneous gas exchanges and protects the body from external damage. The axolotl can regenerate wounded epidermis, dermis, and dermal organs without forming scar tissue, and this ability is present throughout its

lifespan. This is in contrast to mammals, which repair skin wounds via accumulation of fibroblasts in the area and replacing the original architecture of the skin with a dense collagenous matrix, ultimately resulting in loss of elasticity, pigmentation, and sensation (Martin 1997; Seifert and Maden 2014). This is an imperfect solution that does not repair the integrity of the tissue, but instead acts to keep pathogens from entering the body. The scar-free wound repair process in the axolotl provides us with the means to understand what may lie beyond these limits. Deciphering the molecular mechanisms that are responsible for axolotl skin regeneration after injury will not only grant us aesthetically pleasing healing but may also allow for complete functional recovery beyond fibrotic scar formation.

Both mammalian and axolotl skin is composed of an epidermis and dermis which are separated by the basal lamina/membrane. In both classes, a hypodermis is also present beneath the dermal layer adjacent to the muscle and connective tissue. Mammalian wound repair of the skin starts with bleeding and platelet accumulation and is followed by fibrin clot formation. Next, cytokine-directed migration of immune cells to the wound bed causes inflammation. Once the immune cells clear the debris of dead or damaged cells, new tissue formation starts with the proliferation and migration of keratinocytes from the edges of the wound to re-epithelize underneath the scab. Mammalian re-epithelialization takes up to 10 days (Gurtner et al. 2008). Fibroblasts deposit mainly collagen as they remodel the new ECM and scar tissue forms as a result. In contrast, in the axolotl there is minimal bleeding and no scab formation upon comparable injury. Immune cell migration and inflammation is minimal. This is followed by re-epithelialization that is 5–10 times faster in comparison to mammalian tissues (Ferris et al. 2010). Re-epithelialization occurs in a vortex motion (Tanner et al. 2009). Once the remodeling of the extracellular matrix is complete, there is minimal collagen deposition and the wound is repaired with a total lack of scar formation.

Full thickness excisional flank wounds are induced by removing the epidermis, basement membrane and the dermis of the skin. Upon this excisional skin injury, the axolotl completes scar-free wound healing in 80 days (Levesque et al. 2010; Seifert et al. 2012). The axolotl is the only adult tetrapod that can heal full thickness excisional flank wounds scar-free (Seifert et al. 2012; Levesque et al. 2010), while only the fetal stage of other animals shows a similar regenerative capacity (Seifert and Maden 2014; Gurtner et al. 2008; Adzick and Longaker 1992). Anurans have some ability to regenerate dermal tissues, but also retain features of a mammalian-like fibrotic scar (Bertolotti et al. 2013; Yannas et al. 1996). The aquatic axolotl has a mucogenic epidermis whereas the terrestrial axolotl has a keratinized epidermis. Of interest for translational work, the keratinized epithelium of the terrestrial axolotl resembles mammalian skin composition (Seifert et al. 2012; Page et al. 2009). Surprisingly, scarless wound healing also takes place in the adult terrestrial axolotl (Seifert et al. 2012), albeit at a slower rate. Overall, it is important to note that in comparison to mammalian wound healing in both the aquatic and the terrestrial axolotl, hemostasis and re-epithelialization occur faster, inflammation is lower, and ECM deposition is delayed, granting scarless wound healing (Seifert et al. 2012). Given the compositional similarities, the metamorphic/terrestrial axolotl has great

potential to aid in our understanding of the molecular and cellular events that enable the physiological outcome of scar-free wound healing.

7.9 Extracellular Matrix Formation and Matrix Metalloproteases

Fibronectin, collagen, and tenascin-C are ECM components which are synthesized by fibroblasts. The ECM composition determines the outcome of how a wound will close. Thus, the expression levels and timely deposition of these components are tremendously influential in promoting scarless wound healing. During mammalian wound healing, there is a transient deposition of fibronectin, which is later replaced by collagen and localized tenascin-C deposition at the edges of the wound bed. Contrastingly, in the axolotl, tenascin-C is abundant across the wound bed and maintains high levels throughout the dermal regeneration. Additionally, collagen deposition in the axolotl follows a pattern similar to that of mammalian wound repair. However, alongside the continuously elevated levels of tenascin-C, fibronectin levels are much lower in the wound bed resulting in a different ECM composition that is capable of scar-free healing (Seifert et al. 2012).

Upon early deposition of these ECM components to the wound bed, the mammalian wound is repaired mainly by fibrotic tissue. In contrast, there is a delay of ECM deposition in the axolotl. This phenomenon is attributed to the matrix metalloproteases (MMPs) that are abundant during the healing process. These proteases inhibit new ECM formation by degrading ECM components (Seifert et al. 2012). In the axolotl, MMP1, MMP19, MMP28, MMP9 and MMP3/10a & b are highly expressed before and during the re-epithelialization process and aid in keratinocyte migration from the edges of the wound bed. While MMP1, MMP19 and MMP28 expression is returned to baseline levels following the completion of re-epithelialization, MMP9 and MMP3/10a & b maintain high levels of expression. MMP2 expression is also elevated after re-epithelialization is complete. When treated with broad-spectrum MMP inhibitors, the axolotl excision wounds are unable to re-epithelialize (Ferris et al. 2010). Thus, in order for a successful re-epithelialization, keratinocyte migration is greatly dependent on MMP activity. MMP9, MMP3/10a & b and MMP2 are conserved proteases capable of degrading fibronectin and are also present in the mammalian tissue. Besides fibronectin degradation, high levels of MMP3/10a & b and MMP2 in the axolotl degrade collagen type I and II, and the persisting activity of these three MMPs may be considered important mechanisms of the scarless wound healing process. Fetal mammalian tissue, which is capable of scarless healing, also presents higher levels of these three MMPs when compared to the adult mammalian tissue during wound healing, further supporting the importance of MMP activity (Namazi et al. 2011; Li et al. 2006).

Furthermore, ECM composition and substrate stiffness play an important role in the rate of re-epithelialization. When skin explants from terrestrial axolotls were

cultured on collagen beds wound closure was completed. However, wound closure was unsuccessful when explants were challenged with ECM components of fibronectin, laminin, or tenascin (Huang et al. 2015). These findings once again support the importance of MMP function in degradation of ECM components during scarless wound healing and the avoidance of fibrotic scar formation. In addition, the substrate stiffness on which the explants were cultured also had a reverse effect on the plasticity of the epithelial cells, as it slowed the rate of re-epithelialization (Huang et al. 2015).

7.10 Molecular Mechanisms of Scarless Wound Healing

The molecular mechanisms of scarless excisional wound healing are not yet fully understood. In mammalian wound repair, myofibroblasts that express α-SMA trigger inflammatory, angiogenic, and fibrotic factors and also orchestrate fibrotic tissue formation (Wynn 2008; Bellayr et al. 2010). Given this specificity, α-SMA is used as a fibrotic marker in mammalian tissues. Therefore, α-SMA expression was investigated in the axolotl to determine myofibroblast involvement in the wound healing process. α-SMA expression was not detected at the site of post excisional skin injury (Levesque et al. 2010). Only minor α-SMA activity was observed in the basement membrane in later stages of wound healing. These findings overall indicate a lack of myofibroblast activity and a subsequent lack of fibrotic tissue formation in the axolotl, which results in scarless wound healing.

It is also known that TGF-β1 signaling triggers the production of α-SMA expressing cells in mammals and is crucial for wound healing (Desmouliere et al. 2005). Increased TGF-β1 secretion from macrophages initially stimulates ECM components but later leads to scar formation in mammals (Bellayr et al. 2010). Contrasting with adult mammals, fetal models exhibit lower TGF-β1 signaling and thus exhibit scarless wound healing (Zgheib et al. 2014). A recent study showed that axolotl TGF-β1 expression was elevated 1 h post injury in the surrounding epidermis of an excisional wound but ceased 4 days post-injury. Specifically, TGF-β1 was initially expressed by the migrating epidermis, and later only by the epidermis and the new ECM (Levesque et al. 2010). Similar expression patterns of TGF-β1 have also been seen in fetal mammalian models (Martin et al. 1993). Thus, we can conclude that TGF-β1 signaling results in fibrosis in adult mammals but does not affect the scarless wound healing of the axolotl due to its transient pattern of expression. Further investigation of the regulators of this pathway may be crucial in understanding scarless wound healing.

In addition, the molecular limits of scarless wound healing in the axolotl were established when excisional wounds were challenged with the drug bleomyosin and scar formation was subsequently observed (Levesque et al. 2010). The resulting fibrotic tissue was composed of accumulated fibronectin that thickened the epidermis. The regeneration of the basement membrane was also impaired upon bleomyosin treatment. Even though it was established that the axolotl possesses the necessary

mechanisms to retain fibrosis in the presence of a chemical inhibitor, the collagen deposition was minimal, whereas collagen plays a major role in mammalian scar tissue. In addition, there was no increase in α-SMA expressing cells, even though fibrotic tissue was present (Levesque et al. 2010). To further understand regenerative factors, a study isolated AmbLOXe from limb blastemas. AmbLOXe is highly similar to the mammalian LOX gene and is a lypoxigenase that mediates the inflammatory responses important for wound healing. Transfecting human cells (human osteosarcoma and keratinocyte cell lines) with AmbLOXe lead to increased cellular migration (Menger et al. 2011). These findings suggest that utilizing regenerative factors from the axolotl may also enhance scar-free wound healing in mammalian systems.

The adult axolotl is an outstanding model to study scar-free wound repair. The axolotl not only provides us with a convenient translational model, but it also utilizes existing conserved mechanisms for regeneration, therefore providing relevant insight into scarless wound healing. The metamorphic/terrestrial axolotl is especially promising given its skin composition and its resemblance to mammalian tissue. Overall, scarless wound healing is affected by many factors. Rapid re-epithelialization, high activity of matrix metalloproteases which delay new ECM formation, high levels of tenascin-C and a lack of collagen deposition which aid in remodeling new ECM, lack of α-SMA expressing myofibroblasts, transient TGF-β1 signaling, and low inflammatory responses are all contributing factors to scarless wound healing in the axolotl. Further studies will reveal how conserved mechanisms are regulated differently in the adult axolotl compared to mammals, with the ultimate goal of orchestrating molecular and cellular mechanisms of wound healing in order to generate the complete recovery of healthy, non-fibrotic skin in mammals.

7.11 Cardiac Regeneration

Regeneration of the heart myocardium is observed to some extent in most vertebrates including zebrafish (Poss et al. 2002), mice (Porrello et al. 2011), frogs (Rumyantsev 1966), and newts (Oberpriller and Oberpriller 1974). It is commonly believed that lower vertebrates can recover function throughout life while mice can only regenerate up to 7 days after birth (Porrello et al. 2011). Although only three studies have experimentally demonstrated myocardial regeneration in the axolotl, the evidence supports that axolotls can regenerate adult myocardial tissue. Regeneration of the cardiac tissue after 10–15 % partial ventricular amputation is demonstrated in all three of these studies (Flink 2002; Vargas-Gonzalez et al. 2005; Cano-Martinez et al. 2010). In the axolotl, the completeness of regeneration has not been assessed yet. In newts, it is known that cardiac regeneration occurs without any presence of a scar (Witman et al. 2011). Continuous BrdU labeling from 14 to 21 days post injury in axolotls showed that the majority of BrdU+ cells were found within 75–125 μm of the injury site. In addition, co-staining with cardiomyocyte-specific antibodies showed that 74.3 % of epicardial cells and 12.8 % of

cardiomyocytes were BrdU+. Some BrdU+ cells were found as far as 750 μm away from the injury site suggesting a widespread proliferative response after cardiac injury (Flink 2002). Most functional recovery occurs within 30–90 days post injury, which is preceded by cardiomyocyte proliferation (Cano-Martinez et al. 2010). Co-labeling of proliferative markers with cardiomyocyte markers suggest that cardiomyocyte dedifferentiation drives the regenerative process (Flink 2002; Vargas-Gonzalez et al. 2005; Cano-Martinez et al. 2010), which is supported by the fact that adult ventricular cardiomyocytes in newts can readily proliferate in vitro (Mercer et al. 2013; Nag et al. 1979; Tate et al. 1989). Furthermore, genes involved in the embryonic cardiogenic programming including Hand2, Nkx.2, Gata4, Islet1, and Gata5 are all upregulated during newt cardiac regeneration with a significant proportion of Gata4 and Islet1 expression co-localizing with cardiomyocyte markers supporting the likelihood of cardiomyocyte dedifferentiation (Witman et al. 2011).

Based on the evidence, it is likely that axolotls regenerate both by a local injury response (epimorphic) and a widespread organ-wide (compensatory) mechanism. A similar mechanism of cardiomyocyte proliferation was observed in endogenous cardiomyocytes during zebrafish regeneration after ventricular cryoinjury (Sallin et al. 2015). Moreover, cellular lineage tracing in zebrafish have shown that dedifferentiation of resident cardiomyocytes generate the new myocardium in zebrafish (Kikuchi et al. 2010; Jopling et al. 2010; Zhang et al. 2013), which also seems to be the case in the regenerating neonatal mouse (Porrello et al. 2011, 2013; Mahmoud et al. 2013). Although a more comprehensive analysis of heart regeneration is required in amphibians to elucidate their mechanism of regeneration, it is intriguing to think that all animals with the capability to regenerate heart myocardium do so using the same basic mechanisms.

7.12 Early Wound Healing and the Role of the Immune System

Regeneration across all tissue types is a complex process dependent on inflammation, tissue remodeling, and tissue formation. Blood and its circulation plays a major role in orchestrating these events by closing off the initial wound and carrying necessary factors to the wound site. Here, we will cover what is known about the axolotl's clotting factors and the immune system's role in regeneration, with a particular emphasis on the role of leukocytes for this process.

7.13 Clotting Factors

An early step in regeneration is the formation of a clot mediated by cleavage of plasma fibrinogen by thrombin. The clot not only closes off the wound to prevent infection, but may also provide activating factors required for regeneration. Axolotl blood clots within several minutes after amputation, and the wound epidermis

surrounds the clot within 12–24 h depending upon the size of the wound (Sobkow et al. 2006). It is possible that clotting factors released early after injury may be an inductive signal. Indeed, a thrombin-generated ligand is known to induce newt myotubes to re-enter the cell cycle in culture (Tanaka et al. 1997), supporting the hypothesis that thrombin is required for regeneration. In the regenerating newt lens, thrombin activity is present and required for regeneration (Imokawa and Brockes 2003). Thrombin seems to be associated with regenerating tissues, but it is not known whether it is sufficient to induce a regenerative response. Furthermore, the thrombin-mediated ligand or its downstream targets have not been identified, making it difficult to know what role it plays during regeneration. Regardless, clotting is associated with the early injury response and therefore is a prime candidate for inducing a regenerative response.

7.14 Inflammation and the Immune System

The axolotl immune system is comprised of an innate immune system and a rudimentary adaptive immune system. Axolotls are deemed relatively immunodeficient due to the fact that they only produce two immunoglobulin classes (IgM and IgY) – neither of which are anamnestic – and overall humoral and cytotoxic responses are slow or non-existent (Tournefier et al. 1988; Chen and Robert 2011; Godwin and Rosenthal 2014). IgM is produced by lymphocytes within the spleen around 7 weeks post-fertilization, though IgY is not detected until the axolotl reaches 7 months old (Fellah et al. 1989). It has been surmised that inflammation and immunomodulation may be implicated in regenerative ability because there is an inverse relationship between the maturation of the immune system and capacity to regenerate (Mescher and Neff 2005; Harty et al. 2003; King et al. 2012). Inflammation is an initial response to wounding. Upon injury to mammalian tissue, cytokines direct immune cells to the area of the assault, thus creating inflammation and inducing scar formation (Wynn 2008). Contrastingly, the larval axolotl lacks neutrophils and macrophages that migrate to the wound bed, and the adult axolotl has a low number of neutrophils found in the wound bed (Levesque et al. 2010; Seifert et al. 2012; Ziegels 1971). This reduced inflammatory response correlates with scar-free wound healing of the axolotl (Seifert et al. 2012), which is reminiscent of the scar-free wound healing capabilities of fetal mammals (Namazi et al. 2011). This is supported by studies which have shown that after 24 weeks of gestation, onset of a high inflammatory response is consistent with low regenerative capacity and scar formation in mammals (Yates et al. 2012; Adzick and Lorenz 1994; Xue and Jackson 2015). Despite this clear inverse correlation, the molecular mechanisms behind how the immune cells affect regeneration is poorly understood.

Macrophages specifically seem to be important in the regeneration process. Their roles are unclear, but they likely contribute through the phagocytosis of debris following initial injury, breakdown of extracellular matrix (ECM) and promotion of its reconstruction, release of pro- and subsequently anti-inflammatory cytokines,

and mobilization of stem cells. Depletion of macrophages in the axolotl limb immediately after injury prevents blastema formation, and depletion of baseline-level macrophages 15 days post injury will prolong total regeneration time (Godwin et al. 2013). In mouse bone marrow studies, macrophage depletion caused hematopoietic stem cells to egress from the niche due to a loss of paracrine homeostasis factors such as Cxcl12 (Chow et al. 2011). In zebrafish, depletion of macrophages also leads to defective caudal fin regeneration (Li et al. 2012). Macrophage depletion has also been implicated in prevention of heart regeneration in the adult axolotl, though if they are required for merely debris clearance, cytokine signaling, or a greater paracrine role is still not clear (Pinto et al. 2014). Overall, the ability of macrophages to alter their environment physically and through molecular signaling is clearly essential to tissue regeneration, though the extent of these mechanisms are not yet fully understood. While the immune system is implicated in many aspects of regeneration, the entire blood lineage itself shows a high level of regenerative capacity in the axolotl. Ablation of the liver or spleen by targeted irradiation of the adult axolotl will lead to anemia and eventually death (Lopez et al. 2014). Furthermore, the liver and spleen may provide different niches as the HSPC populations of the spleen are 1000-fold enriched in lymphoblastic populations compared to the periphery of the liver (Lopez and Scott 2015).

7.15 Conclusion

Based upon the examples in this review, axolotls utilize multiple mechanisms to coordinate a regenerative response, but it is clear that with time the animal will fully regenerate its limb, tail, spinal cord, and heart. In all injuries models studied, injury initiates a local infiltration of leukocytes, which is associated with migration of cells nearby the injury. Migration of cells is followed with a sustained proliferation of progenitor cells that arise either from dedifferentiation or recruitment of local adult stem cells, most commonly located near the injury site. Differentiation of progenitor cells is a recapitulation of development of each organ, coupled with enhanced growth until the injured tissue is replaced. The molecular processes that initiate a regenerative response or regulate when an organ stops growing are critical questions that need to be elucidated to in order to understand the regenerative process.

The axolotl's regenerative abilities are not limited to its brain, spinal cord, nerve, tail, heart, limb and skin regeneration. The regenerative ability of other organs are highlighted in Table 7.1. Considering the broad regenerative ability that has been described here, it is likely that most if not all organs and tissues can regenerate at some point in their lifetime. For example, it was previously thought that axolotls could not regenerate their lens. This idea was recently overturned, showing that for a duration of 2 weeks after hatching/Stage 44, axolotls possess the ability to regenerate the lens from the ventral or the dorsal iris or simultaneously from both (Suetsugu-Maki et al. 2012). Overall, given its wide variety of organ and appendage regenerating capabilities, the axolotl is a compelling model for the study of adult

Table 7.1 Table includes original citations that describe the regenerative ability of each animal group

Tissue	Species	All citations
Retina	Ambystoma– intermediate	Keefe (1973), Stone (1950)
	Newts – yes	
Brain	Ambystoma– yes	Kirsche and Kirsche (1964b), Parish et al. (2007), and Minelli and Del Grande (1974)
	Newts – yes	
Spinal cord	Ambystoma– yes	McHedlishvili et al. (2012, Butler and Ward (1965, 1967), Egar and Singer (1972), and Piatt (1955)
	Newts – yes	
Lens	Ambystoma– up to 2 weeks post hatching	Eguchi (1963), Suetsugu-Maki et al. (2012), and Collucci (1891)
	Newts – yes	
Jaw & teeth	Newts – yes	Ghosh et al. (1994), Goss and Stagg (1958)
Heart	Ambystoma – yes	Oberpriller and Oberpriller (1974), Flink (2002), Vargas-Gonzalez et al. (2005), and Cano-Martinez et al. (2010)
	Newts – yes	
Lungs	Unknown	
Liver	Ambystoma – yes	Williams (1961), Heberlein (1930)
	Newts – yes	
Spleen	Newts – yes	Garavini (1977)
Gall bladder	Unknown	
Pancreas	Newts – intermediate	Vethamany-Globus and Liversage (1973)
Stomach	Unknown	
Intestine	Newts – yes	O'Steen and Walker (1962), O'Steen (1958), and Grubb (1975)
Kidneys	Newts – no	Scadding and Liversage (1974)
Testis	Newts – yes	Flament et al. (2009), Uchida and Hanaoka (1949), and Bois and Beaumont (1927)
Ovaries	Unknown	
Lateral line	Ambystoma – yes	Jones and Corwin (1993), Jorgensen and Flock (1976), Stone (1933,1937), and Jørgensen and Flock (1976)
Tail	Ambystoma – yes	Holtzer (1956), Iten and Bryant (1976)
	Newts – yes	
Limb	Ambystoma – yes	Schotté and Butler (1944); Iten and Bryant (1973), and Chalkley (1954)
	Newts – yes	
Skin	Ambystoma – yes	Seifert et al. (2012), Levesque et al. (2010), and Ziegels (1971)

Note that the references are not comprehensive. "Newt" includes references to *Cynops pyrrhogaster, Triturus cristatus, Pleurodeles waltl*, and *Notophalamus viridescens.* "Ambystoma" includes references to *Ambystoma maculatum* or *Ambystoma mexicanum (axolotl).* Missing values indicate the organ has not been studied in all animals

tissue regeneration. A broad survey of organ regeneration should provide much insight into the common and divergent mechanisms that regulate regeneration.

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Chapter 8
Development and Regeneration of the Vertebrate Brain

Brian Key

Abstract The vertebrate brain is hierarchically assembled about orthogonal axes using organizing centers that control cascades of signaling events. The reiterative generation of these centers at defined times, and in precise spatial locations, leads to the conversion of a contiguous and homogenous epithelial sheet into the most complex biological tissue in the animal kingdom. The critical events orchestrating the construction of a "typical" vertebrate brain are described. Attention is focused on specification of major brain regions common across the vertebrate phylogeny, rather than on the differentiation of constituent cell types and specific cytoarchitectures. By uncloaking the complex spatial interactions that unfold temporally during the build of the vertebrate brain, it becomes clear why regeneration of this tissue following injury is such a challenging task. And yet, while mammalian brains fail to regenerate, the brains of non-mammalian vertebrates, such as teleosts, reptiles and amphibians, can successfully reconstitute brain tissue following traumatic injury. Understanding the molecular and cellular bases of this remarkable regenerative capacity is revealing the importance of developmental programs, as well as exposing unexpected roles for extraneous processes such as inflammation. Recent discoveries are now fuelling hope for future therapeutic approaches that will ameliorate the debilitating consequences of brain injury in humans.

Keywords Morphogen • Development • Neural plate • Neuroepithelium • Regeneration • Development • Brain

8.1 Introduction

In this Chapter I identify and interrogate the principal developmental processes that lead to creation of a "generic" vertebrate brain. In doing so, I draw upon an extensive literature involving a number of experimental model systems across the vertebrate phylogeny, including teleosts (ray-finned fish) and tetrapods (amphibia, reptiles,

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aves and mammals). Given that there are, at least, 30,000 species of teleosts occupying a plethora of environmental niches it would not be surprising to find diversity in developmental programs. One strategy that reduces the complexity of this problem is to first identify the key features of the vertebrate brain that distinguishes it from invertebrate brains and then restrict discussion to the development of these characteristics. For purposes of comparison, I have adopted Insecta (fruit fly) and Cephalopods (octopus and squid) for my model invertebrates, both because of their relatively complex brains (Boycott 1961; Hockner et al. 2006; Hartenstein et al. 2008; Boyan and Reichert 2011), and because they are not chordates, since they have a closer relationship to vertebrates (Bourlat et al. 2006, 2008; Pani et al. 2012). Comparative analyses expose, at least, three principal defining features of the vertebrate brain: (1) relatively large expansion of the brain in comparison to the remaining nervous system; (2) contiguity of the brain as a unitary structure; and (3) longitudinal modular organisation. The common link between each of these structural characteristics is the anterioposterior axis.

While trying to avoid Sisyphean arguments regarding measures of intelligence and brain size across widely divergent species (Roth and Dicke 2005), the anterior enlargement of the vertebrate brain has generated both an increase in neuron number (increased processing power) and regional specialisation of cytoarchitecture (allowed evolution of specialized functions; *e.g.* cortical functions in Mammalia). The contiguity of the brain and its axial organisation also probably facilitated the emergence of hierarchical control mechanisms of function with progressive elongation of the brain providing additional complexity in neural control of behaviour (Quartz 2003). The anterior axial enlargement seems to have been made possible by evolution of the myelin sheath (Hartline and Colman 2007; Zalc et al. 2008). The insulating properties of this lamellated membrane allowed rapid conduction of neural activity with relatively small axon diameters. This property enabled both more neurons per unit brain volume and an expansion in brain size. Since conduction speed of action potentials increased with myelination, rapid motor behaviours necessary for survival were still possible despite transmission over the relatively longer axial distances associated with larger brains. This trade-off between axon diameter and neural conduction speed and neuron size ultimately restricts the final number of neurons and hence limits the processing power of the invertebrate brain.

While there is modular organisation of the brain in both Drosophila (Hartenstein et al. 2008) and Cephalopods (Shigeno et al. 2001a, b; Yamazaki et al. 2002), these modules develop within dispersed lobes as small clusters of neurons derived from a single neuroblast (neural stem cell) (Boyan and Reichart 2011). As these lobes expand they merge to form a brain containing a small ladder-like arrangement of interconnecting axon pathways. Each of ~100 neuroblasts produce a stereotyped lineage of projection and local interneurons on each side of the brain. These neuroblasts are patterned early in development by anterioposterior and ventrodorsal cues. This patterning ensures that specific stereotyped lineages arise from each neuroblast.

What distinguishes the vertebrate brain is that its modular form is generated by morphogen gradients acting within a contiguous neuroepithelial sheet (Temple 2001; Nakamura et al. 2008). The reiterative and sequential appearance of signaling centers (secreting these morphogens) at precise spatial locations along the anterio-posterior axis within the neuroepithelium leads to a complex hierarchical organisation of the vertebrate brain.

Following these specification events, modules in the vertebrate brain are selectively wiring together, initially about the longitudinal axis, into functioning circuits. Most importantly for humans, consciousness relies on both hard wiring between brain regions (*i.e*. structural connectivity) as well as the level of electrical activity flowing between these regions (*i.e*. functional connectivity) (Sporns 2013). The initial development of structural connectivity provides the anatomical scaffold for subsequent functional connectivity and hence, the behavioural and cognitive ability of the human brain. Currently, there is considerable interest in the possible causative role that defects in structural connectivity play in neurodevelopmental and neuropsychiatric disorders, such as autism and schizophrenia (Dennis and Thompson 2013). Simple structural abnormalities, such as a decrease in the size of the internal capsule and anterior commissure, are being investigated as possible prognostic tools in schizophrenia (Wobrock et al. 2009; Choi et al. 2011; Levitt et al. 2011) and preterm infants (De Bruine et al. 2013; Northam et al. 2012). However, at present the evidence is equivocal. Given the clinical implications of this work, there have been strong calls for more extensive experimental studies and, in particular, the need for better animal models to understand the genetic basis of purported structural defects in axon tracts (Cao et al. 2014; Wang et al. 2015; Wheeler and Voineskos 2014).

Understanding the general principles of vertebrate brain development provides an entrée into regeneration in this neural tissue. I will restrict my discussion to regeneration of specific brain regions rather than to cellular mechanisms such as axon regeneration, adult neural stem cell homeostasis and neuroplasticity involving axonal and dendritic arbor remodelling and synaptic turnover. The focus will be on the ability of specific brain regions to regenerate and repair following injury, as this trait is therapeutically relevant for human brain injuries caused by stroke, tumours, pathologies and brain surgery.

8.2 Classification/Definitions

In this section terminologies and concepts associated with the gross morphology of the developing vertebrate brain are described. Basic embryology is introduced to assist in the understanding of the morphogenetic role of molecular signaling events. In addition, pertinent issues regarding the actions of morphogens are discussed since these molecules play a fundamental role in vertebrate brain development.

8.2.1 *Conservation of Brain Structure Across the Vertebrate Phylogeny*

Fish and humans shared a common ancestor approximately 400 million years ago. The brains of these species have had an extraordinarily long time to diverge in order to adapt to new environments. And yet, even after this long time, the brains of modern-day fish and humans share many common design features. While fish and humans do not look very much alike, they still have brains that are built upon three major subdivisions: the forebrain (prosencephalon), midbrain (mesencephalon) and hindbrain (rhombencephalon). The forebrain is most anterior, the hindbrain is most posterior and the midbrain lies in between along the longitudinal axis of the brain. Many textbooks describe a generic vertebrate brain arising from a neural tube consisting of three primary vesicles (each one giving rise to fore-, mid- and hind-brain). However, this idea has long been know to be a myth that arose from early studies that lacked sufficient mammalian embryonic material (Streeter 1927). While the chick brain merges from vesicle swellings of a closed neural tube, the human brain subdivisions are separated by sulci in the neural folds during the mid-fourth week of gestation, before the tube has closed (Bartelmez 1923; O'Rahilly and Gardner 1979). Later fate mapping studies in fish, amphibian, chick and rodents confirmed that the major subdivisions of the brain emerge during the neural plate stage (Garcia-Lopez et al. 2009). After formation of the neural tube in humans, only a single prominent rhombencephalon swelling is observed (Bartelmez 1923). While the prosencephalon appears as a slight anterior ballooning of the neural tube within a few days of anterior neuropore closure, it is a full week before the prosencephalic vesicle is clearly visible (Fig. 8.1)

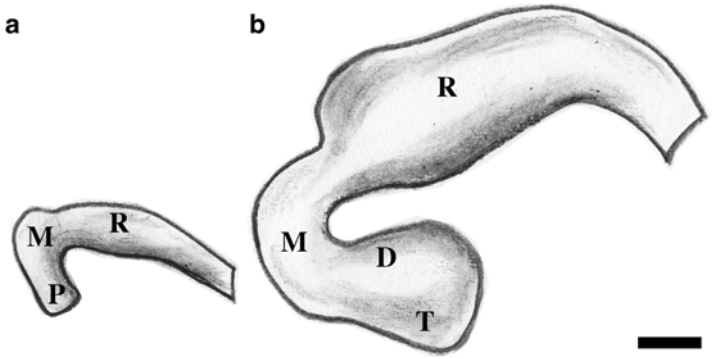

Fig. 8.1 Gross morphology of early developing human brain. The outlines of the human brain were traced from images of human embryos (Yamada et al. 2010) at (**a**) ~32 days (Carnegie stage 13) and (**b**) ~38 days (Carnegie stage 16). Carnegie stage 13 is a few days after anterior neuropore closure. During this narrow window of time there is an exponential growth in the size of the brain with only minor observable change in the overall differentiation of brain regions. It is noted that the early brain does not possess the large balloon-like primary vesicles observable in chick embryos. Swellings in the forebrain (T and D) and hindbrain (R) only appear in the sixth week of development. *P* prosencephalon, *T* telencephalon, *D* diencephalon, *M* mesencephalon, *R* rhombencephalon. The scale bar is 1 mm

(Yamada et al. 2010; ten Donkelaar et al. 2014). The mesencephalon does not exist as a vesicle in humans but is readily defined by the cranial flexure of the brain.

While there is considerable variation in the appearance of swellings/vesicles across vertebrate species, the initial subdivisions typically give rise to progressively more anatomically defined regions during subsequent development. The prosencephalon will form the telencephalon and diencephalon; the mesencephalon will form the tectum and tegmentum; and the rhombencephalon will form the metencephalon (cerebellum) and mylencephalon (medulla oblongata). The forebrain exhibits a progressively more complex anatomical patterning, cytoarchitecture and neural connectivity during vertebrate evolution (Kuranti et al. 1998; Barreiro-Iglesias et al. 2008; Moreno and Gonzalez 2011). For a detailed understanding of brain structure across taxa, the reader is referred to an article and references within it by Ann Butler (2000a). Butler emphasizes the major role development plays in generating the diversity of brain morphologies in Craniates (chordates with heads). In humans, the ventral telencephalon gives rise to subpallial nuclei such as the basal ganglia (striatum and pallidum), amygdala and septal nuclei while the dorsal telencephalon gives rise to the cerebral cortex and olfactory bulb. During development, the human cerebral vesicles expand like a balloon before the cortical plate folds to form gyri and sulci. In ray-finned bony fish, the telencephalon gives rise to the pallium (rudimentary homologue of the cerebral lobes) and a subpallium (homologous to the ventral telencephalic region sin tetrapods as noted above). Rather than expanding under the pressure of cerebral spinal fluid as in chicks and humans (Desmond and Jacobson 1977; Del Bigio 1993), the pallium of these fish appears to simply evert as a sheet of neural tissue without forming a vesicle with a central ventricular cavity (Butler 2000b). Others, however, propose that pallial formation in the ray-finned fish occurs via complex eversion involving folding and migration (Mueller et al. 2011) and/or bulging and pushing movements (Folgueira et al. 2012). Nonetheless, the lack of a central ventricular system completely surrounded by pallium most likely contributes to the primitive nature of the fish pallium (*i.e.* lack of massive expansion and differentiation of multiple discrete and highly interconnected subregions) in comparison to the dorsal telencephalon of tetrapods (particularly mammals). This becomes clear when examining the telencephalons of lobe-finned bony fish (such as the African lungfish) that are closely related to tetrapods and possess an evaginated telencephalon with a central ventricular system, as in amphibia. The dorsal pallium in these fish possesses a primitive cortical-layer layer of neurons referred to as the pallial cortex and the pallium is relatively enlarged in comparison to ray-finned fish (Northcutt 2008).

Despite 400 millions of separation, both the mid- and hindbrains of fish and humans have maintained gross structures and functions that are highly conserved in these two disparate species. The medulla in the hindbrain contains motoneuronal pools in both fish and human that control reflex respiratory movements (Ballintijn and Roberts 1976; Kumral et al. 2011; Sultan et al. 2011; Smith et al. 2013). The cerebellum, which arises developmentally from the hindbrain in both fish and humans, shares similar microcircuitry homology and is involved in the control of motor learning in both species (Rodrigeuz et al. 2005; Bell et al. 2008; Hashimoto

and Hibi 2012; Manto et al. 2012). The midbrain in fish principally consists of the optic tectum and tegmentum (Goodson and Bass 2002; Kittelberger et al. 2006; Kittelberger and Bass 2013) that are homologous to the superior colliculus and tegmentum, respectively, in mammals. Interestingly, the gross morphology of the midbrain optic tectum is preserved from fish to human, which is consistent with its preserved function in vision, such as orienting behaviour (Torres et al. 2005; Sauleau et al. 2008) and eye saccades (Sereno et al. 2006). In some fish, the tectum is also involved in specialized functions such as modulating electric organ discharges in the weakly electric fish (Zupanc and Horschke 1996). The motoneuronal circuitry stimulating eye movements is present in the hindbrain cranial nerve nuclei while control regions are spread across the reticular formation and periaqueductal gray matter in hindbrain and midbrain tegmentum in both fish (Perez-Perez et al. 2003; Angeles Luque et al. 2005) and humans (Linzenbold et al. 2011; Terao et al. 2013). It has recently been demonstrated in, at least, one species of fish that uses vocal communication, that the tegmentum is also associated with processing of vocal-auditory information which is consistent with the role of this midbrain region in other vertebrates, including humans (Esposito et al. 1999; Kittelberger and Bass 2013).

8.2.2 Organizers Secrete Morphogens that Pattern Vertebrate Embryonic Tissue

The sub-regionalization of the embryonic vertebrate neural tube into the anterior brain and posterior spinal cord involves secreted molecules known as morphogens. The function of morphogens is usually concentration dependent; that is, their actions vary as their extracellular concentration decreases at sites remote from the cells secreting these molecules. The term morphogen initially referred to those molecules that cause changes in the shape or gross structure of tissues. Growth factors were instead considered to act locally to regulate cell differentiation. However, as discussed below, this nomenclature is not particularly rigid, given that many molecules initially characterized as growth factors were later found to also behave as morphogens.

Morphogens are secreted by patches or fields of cells called “organizers”. The first of these organizers to be identified was the dorsal lip of the blastopore in amphibia, which became known as the Spemann-Mangold organizer. This discrete cluster of cells patterned the dorsoventral axis of the mesoderm (Key 2003). The Spemann-Mangold organizer was able to induce gastrulation and to generate a secondary body axis when grafted into ventral endoderm of blastula-stage amphibian embryos. Later this organizer was shown to be under the influence of an adjacent patch of endoderm that became known as the Nieuwkoop center (also referred to as the “organizer of the organizer”) (Harland and Gerhart 1997).

Morphogens are able to generate multiple phenotypic effects in a single tissue because of variable responses of cells to different concentrations of these molecules

(Green et al. 1992). For example, if a morphogen is secreted by an organizer and diffuses through the surrounding neuroepithelium it can establish a concentration gradient within the extracellular space of that tissue. The steepness of this gradient will be determined not only by the amount of morphogen secreted, but also by its physiochemical properties of diffusion, its degradative rate and the nature of the extracellular space *e.g.* size of intercellular channels and presence of adsorptive or non-adsorptive substrates. By manipulating these various properties it is possible to radically alter the distribution of a morphogen within a tissue (Lander 2007; Yu et al. 2009). For instance, if the diffusion rate is slow, and the degradative rate is high, the concentration gradient will be steep and narrow.

If the cellular response to a morphogen changes at different extracellular concentrations, then cells close to the source will clearly behave differently to distant cells. Furthermore, if the response is not linear, but instead dependent on the morphogen reaching some threshold level, then a smooth gradient of morphogen could easily lead to the tissue being subdivided into distinct subpopulations. Lewis Wolpert from University College London has proposed the "French flag" model to explain patterning with sharp boundaries. In this model, a tissue is believed to be partitioned into stripes of cells (like the red, white and blue stripes of the French flag) which all respond similarly to a particular range of morphogen concentrations (Wolpert 1969). Consequently, a smooth gradient of morphogen converts a seemingly homogenous field of cells into domains with sharp boundaries, whose shape depends on the threshold responses of the cells.

8.2.3 Principles of Morphogen Action

In the early vertebrate frog embryo, morphogens act by diffusing through embryonic ectoderm for distances up to 250 μm (about 15 cell diameters) (McDowell et al. 1997; Harvey and Smith 2009). The extent of this diffusion is modulated by interactions with the cell surface and with extracellular matrix molecules such as heparan sulfate proteoglycans (Selleck 2006). The sugars on these molecules are notorious for their ability to bind to morphogens/growth factors such as Wnts and FGFs. By binding to these sugars, morphogens are known to increase their potency and to lengthen their half-life. In this way morphogens are able to maintain their actions long after their expression has ceased. The activity of morphogens is also controlled by the expression of inhibitory molecules that bind to and prevent the morphogen from interacting with its receptor.

How morphogen gradients are interpreted by cells remain to be fully understood. The binding of morphogen to its receptor can either simultaneously activate multiple signaling pathways, which regulates the expression of numerous downstream genes, or they can act through sequential signaling pathways which also leads to differential gene expression. Deciphering the underlying mechanisms should facilitate the development of drugs that influence the action of morphogens. The morphogen response is typically determined by both the concentration of the morphogen

and the length of time a cell is exposed to the morphogen (Green et al. 1990; Gurdon et al. 1995; Dyson and Gurdon 1998; Gurdon and Bourillot 2001). Only a several fold increase in either concentration, or duration of exposure, is necessary to change the fate of a cell during development. By either lengthening the time an organizer expresses a morphogen, or by changing the half-life of the molecule (perhaps by altering the extracellular environment), it is then possible to change the response of cells within the morphogen field. The availability of unbound receptors is not normally the rate-limiting step in the response of a cell to morphogen. At physiological morphogen concentrations, receptors are never saturated. For instance, *Xenopus* blastula cells have about 5000 activin receptors at their cell surface, and yet, a response can be achieved with only a 2 % occupancy rate. The limiting factor for the ability of a cell to respond to a morphogen appears to be the extracellular concentration of the morphogen. Increasing the concentration three-fold can result in a three-fold increase in the occupancy rate of the receptors. The occupancy rate can also be increased through lengthening the time receptors are exposed to the morphogen. The level of transmembrane signaling is proportional to the level of occupancy of the receptors. Interestingly, despite high receptor-ligand affinity, there is always ligand that remains unbound and free to diffuse in the extracellular space. This turns out to be an important property of morphogen-receptor interactions. Since cells near the source of the morphogen do not absorb all the morphogen, it allows morphogen to diffuse and influence more distant cells in the field.

It is important that the action of a morphogen is restricted to discrete populations of cells in order for normal morphogenesis to occur. Although a morphogen may spread over 250 μm, its action needs to be limited to a specific zone in order for regional subspecialisations to arise. Thus, within a field of cells, boundaries are often formed to ensure that a morphogen is restricted in action. The "French flag" model suggests that a linear gradient is converted into stripes by the fact that cells within a field possess different thresholds of activation for signaling events. Consequently, distinct sets of genes are expressed at low and high concentrations of morphogen. It is hard to envisage how precise boundaries are achieved in this model. It would be expected that stochastic variability in the response of neighbouring cells would prevent the formation of distinct and sharp boundaries. And yet, in the nervous system there are many examples of such borders between domains of cells (e.g. Hox gene expression in the mouse rhombencephalon). What seems likely is that there is some form of feedback regulation that ensures boundary formation is sharp. Discrete tissue boundaries may arise because one set of genes, turned on by a morphogen at high concentration, acts to inhibit expression of other genes in nearby cells. Thus, at high concentration a morphogen activates gene A, which in turn inhibits genes B and C. At lower morphogen concentrations gene A is not activated which allows expression of gene B, which in turn inhibits gene C. At the lowest concentration of the morphogen only gene C is activated. However, in order for this scenario to achieve very sharp boundaries, gene B must then inhibit genes A and C, and gene C must inhibit gene B. In this way, both reciprocal inhibition and differential threshold responses to morphogens act together to ensure very sharp boundaries are maintained between compartments within a field of cells. The

maintenance of boundaries is often reinforced by differential expression of cell adhesion molecules and chemorepulsive molecules so that compartments do not disappear after morphogen expression is downregulated. In some cases, specialised boundary cells either physically maintain the boundary or act as organising centers that further stabilise gene expression (see the IsO in Sect. 8.3.4.1 below).

8.3 Principles/Mechanisms

8.3.1 The Neural Plate Is Established During Gastrulation

During early development, the vertebrate embryo proper is either sphere shaped (e.g. fish and amphibia) or flattened (birds and mammals). In the former case, one-half of the embryo will be a compact hemisphere of endoderm while the other half will be a sheet of ectoderm surrounding a fluid-filled cavity (called a blastocoel) (Fig. 8.2a). In the latter case, the two sheets of ectoderm and endoderm will be directly apposed to form an embryonic disc. Whatever the gross morphology, interactions between the ectoderm and endoderm result in the induction of a third tissue type called the mesoderm (Key 2003) (Fig. 8.2a). In fish and amphibia (the animal models that are the most characterised), the mesoderm arises in the equatorial region of the embryo. The mesoderm is patterned along a dorsal-ventral axis by the morphogens secreted by the Spemann-Mangold organizer (Fig. 8.2b). The Spemann organizer also secretes morphogens that assist in specifying and patterning the

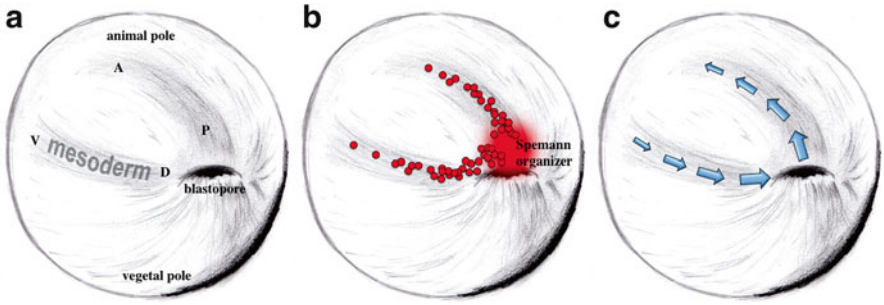

Fig. 8.2 Mesoderm induction and gastrulation in the amphibian embryo. (**a**) The early amphibian embryo is divided into animal and vegetal hemispheres. The opposite ends of these hemispheres are referred to as poles. The mesoderm emerges as an equatorial band of tissue at the interface of the animal and vegetal hemispheres. The dorsal end of the mesoderm is defined by a pore (referred to as a blastopore). The future neural plate arises in the animal pole. Its anterior end is closer to the animal pole while its posterior end is nearer the blastopore. (**b**) The Spemann-Mangold organizer (located in the dorsal lip of the blastopore) secrets morphogens that diffuse along the plane of the mesoderm and presumptive neural plate and pattern both of these tissues. (**c**) The mesoderm migrates into the blastopore (following the direction of the arrows) and will eventually lie beneath the ectoderm of the animal pole (future neural plate)

anterior-posterior axis of the neural plate (Fig. 8.2b). The mesoderm migrates inside the embryo, through a depression called a primitive node or a blastopore (Fig. 8.2c). This mesoderm migration is known as gastrulation and is led by the dorsal mesoderm (which is equivalent to the Spemann-Mangold organizer). The dorsal mesoderm will migrate under the epithelium of the animal hemisphere towards the future anterior end of the embryo, while the ventral mesoderm will eventually reside at the posterior end of the embryo. The dorsal mesoderm forms the prechordal plate, which is a triangular-shaped cluster of mesoderm that demarcates the presumptive head region of the embryo. This plate leads a long narrow chord of mesoderm called the notochord that will ultimately form a portion of the intervertebral discs of the vertebral column. The trajectory of this migratory midline mesoderm defines the anterioposterior axis of the embryo.

8.3.2 Vertical and Planar Induction in the Nervous System

Much of our understanding of the importance of the migratory mesoderm in inducing the over lying ectoderm to form neural plate comes from a series of elegant transplantation experiments conducted using amphibian embryos in the early part of the twentieth century (De Robertis 2006). Holtfreter in the 1930s inhibited the mesoderm from entering the embryo by altering the extracellular salt concentration. In the presence of high salt he found that the mesoderm migrated as a sheet away from the embryo in a process known as exogastrulation. While these embryos developed mesodermal and endodermal derivatives they failed to develop morphological signs of neural tissue (Ruiz I Altaba 1992). Moreover, several different investigators have shown over a number of years that when pieces of early gastrulating mesoderm, or dorsal lip of the blastopore, were co-cultured with the animal pole ectoderm, neural tissue was induced. The prechordal plate is formed from the dorsal mesoderm or Spemann-Mangold organizer and is capable of inducing anterior neural tube while the later migrating notochord is ventral mesoderm and induces only posterior neural tube. Together, these results led to the idea that the gastrulating mesoderm was secreting inducers that not only induced neural tissue, but also patterned the tube, in a process known as vertical induction. While these results also raised the possibility that there were distinct inducers for the anterior and the posterior neural tube, recent experiments indicate that morphogen gradients differentiate the anterior from the posterior neural tube (see below).

Over a number of years, Ray Keller developed and perfected a method of culturing the dorsal mesoderm and the adjacent ectoderm to address the question of morphogen action in the early embryo (Sater et al. 1993). A linear explant assay that prevented gastrulation of the mesoderm, but maintained normal morphological relationships (unlike in the exogastrula experiments of Holtfreter) enabled Keller to show that planar induction was operating. In the absence of gastrulation (and hence vertical induction), the ectoderm was found to not only express neural specific markers, but also markers indicative of correct anterioposterior patterning of the

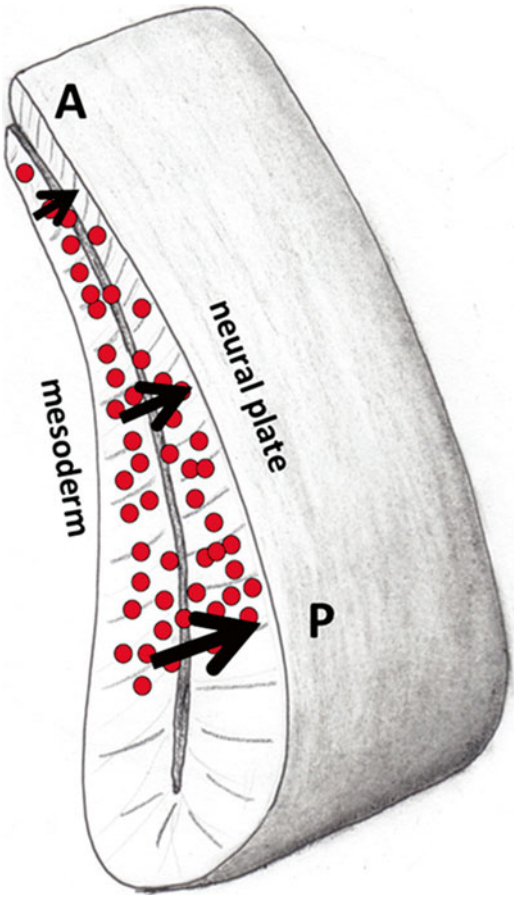

Fig. 8.3 Vertical patterning of the neural plate. As the mesoderm is migrating inside the embryo under the overlying ectodermal epithelium it is secreting morphogens (which diffuse vertically into the neural plate). These morphogens (*e.g.* Wnt8) are present at lower levels in the dorsal mesoderm and hence allow the anterior neural plate to express anterior brain markers. The higher morphogen levels in the ventral mesoderm pattern the posterior regions of the neural tube. The size of the *black arrows* indicate the relative level of the morphogen signaling

neuroectoderm. Thus, induction and patterning of the neural tube involves both planar signals secreted from the organizer (that diffuse through the plane of the ectoderm prior to gastrulation; Fig. 8.2b) as well as vertical signals released from the migrating mesoderm during gastrulation (Fig. 8.3).

8.3.3 Molecular Basis of Neural Induction and Patterning

The default state of differentiation of the amphibian animal pole was originally believed by Spemann and Mangold to be epidermis (Hemmati-Brivanlou and Melton 1997). This idea was readily accepted given that skin-like tissue arose from animal poles cultured in isolation (Slack and Forman 1980; Slack 1984; Melton 1991). However, the concept of the default state was subsequently found to be

context specific, since isolated animal pole can differentiate into neuroectoderm when the receptor for BMP-4 is blocked by over expression of a dominant negative form of this molecule (Hawley et al. 1995). Since BMP-4 is ubiquitously expressed by the animal pole (Hemmati-Brivanlou and Thomsen 1995) it seems that the default state of epidermis arises because this tissue secretes BMP-4 that acts in an autocrine loop to prevent differentiation into neuroectoderm. These results shed light on an earlier finding from Johannes Holfreter that animal pole explants from salamander (*Amblystoma punctatum*) uniquely dissociate during *in vitro* culture and, in doing so, spontaneously generate neural tissue (Holtfreter 1944). Similar observations were later made in Jonathon Slack's lab in the 1980s when he showed that axolotl animal pole explants sometimes spontaneously formed neural tissue (Slack 1984) and that dispersed *Xenopus* animal pole cells differentiated into neurons rather than epidermal cells (Godsave and Slack 1989, 1991). In these dispersed cultures, the local level of BMP-4 was probably not high enough to activate its receptors and induce epidermal differentiation, which led to default neural differentiation.

The subsequent hunt for a neural inducer in amphibians turned into a search for molecules that blocked the function of BMP-4. Richard Harland revealed in the early 1990s that Spemann's organizer, as well as the later developing prechordal plate and notochord, selectively expressed Noggin, a BMP-4 antagonist (Smith and Harland 1992). This molecule probably diffused from Spemann's organizer within the plane of the animal pole ectoderm prior to gastrulation. Later it also diffused vertically as the prechordal plate and notochord migrated anteriorly under the overlying animal pole ectoderm. In both cases, it induced neuroectoderm by inhibiting BMP-4 from binding to its receptor (Zimmerman et al. 1996). While Noggin induced neuroectoderm, this tissue only expressed anterior brain characteristics and not hindbrain and spinal cord markers (Lamb et al. 1993). These results indicated that other molecules were necessary to pattern the posterior regions of the emerging neural tube.

Two other molecules, Chordin and Follistatin, were subsequently identified that also possessed the ability to neuralize the animal pole. De Robertis reported that Chordin was expressed by the organizer as well as by the prechordal plate notochord. He further revealed that ectopic expression of this molecule in ventral blastomeres induced a second anterior neural axis (Sasai et al. 1994). More importantly, when endogenous Chordin was knocked down using translation-blocking antisense morpholino molecules, neural tissue failed to develop (Oelgeschlager et al. 2003). Since Chordin also blocks the binding of BMP-4 (Piccolo et al. 1996) to its receptor, it appeared that Chordin and Noggin were acting in concert to induce the nervous system. Doug Melton's group revealed that a dominant negative Activin receptor (Activin is a member of the TGF-β family) caused the entire animal pole ectoderm to become neural tissue (Hemmati-Brivaniou and Melton 1994). They showed that Follistatin, which binds and blocks Activin and BMP-4 function (Fainsod et al. 1997), was expressed and secreted by Spemann's organizer and notochord (Hemmati-Brivaniou et al. 1994). Activin appears to be able to induce dorsal mesoderm and hence indirectly leads to neural tissue induction (Smith et al. 1990;

Thomsen et al. 1990; van den Eijnden-Van Raaij et al. 1990; Dohrmann et al. 1993). However, the expression of Activin occurs too late during embryogenesis to act as an endogenous mesoderm inducer (Dale et al. 1992). Rather, it appeared that BMP-4 (which is expressed by the mesoderm) is able to induce mesoderm with a ventral identity (Dale et al. 1992). Dorsal mesoderm only formed when BMP-4 signaling was blocked by either Noggin, or Chordin or Follistatin (all of which are strongly expressed by dorsal mesoderm). Thus, by the end of the 1990s it was clear that at least three antagonists of BMP-4 strongly expressed by the dorsal mesoderm (Spemann's organizer) were probably responsible for both patterning this tissue, and for inducing the differentiation of neural tissue in the ectoderm of the animal pole.

However, as noted above, these proteins could not fully explain how the anterioposterior axis of the neural tube was patterned. Spemann in the 1930s had shown that anterior archenteron roof (dorsal mesoderm that first gastrulated together with attached endoderm) could induce anterior head structures when transplanted ectopically into the blastocoel. Moreover, progressively more posterior archenteron induced progressively more posterior regions of the embryos. Thus, the key to anterioposterior patterning seemed to reside in the patterning of the mesoderm along the ventrodorsal axis (note that the dorsoventral mesoderm axis is transposed into the anterioposterior axis during gastrulation so that the early dorsal mesoderm forms the prechordal plate which finally resides in the anterior head regions). During mesoderm induction and patterning a gradient of the growth factor Wnt8 develops (because Wnt8 expression is positively regulated by BMP-4 expression; Hoppler and Moon 1998), so that it is high ventrally and low dorsally. This gradient is converted into an anterioposterior gradient (low/anterior and high/posterior) as the mesoderm migrates through the blastopore and under the ectoderm during gastrulation. The high Wnt8 in the posterior mesoderm then contributes via vertical signaling to posteriorizing the neuroectoderm. A role for this factor in patterning was confirmed by the loss of anterior forebrain markers in embryos overexpressing Wnt8 (Christian and Moon 1993).

As for BMP-4, the action of Wnt8 was inhibited by molecules secreted by the prechordal plate (*i.e.* the dorsal mesoderm that migrated into the head region during gastrulation). Cerberus, Dickkopf and Frzb are secreted by the prechordal plate and prevent Wnt8 signaling through the Frizzled receptor present in the head (Kawano and Kypta 2003). Dickkopf (Dkk) means "big head" and refers to the phenotype observed when this protein is overexpressed (Glinka et al. 1998). Dkk-1 antagonizes the activity of Wnt8 by binding to the Wnt co-receptor LRP6 and inhibiting the formation of the Frizzled-LRP6 receptor complex necessary for signaling (Semenov et al. 2001). Thus the vertical signaling between the prechordal plate and the anterior neural plate is dependent on the loss of Wnt8 activity by Dkk-1 (Shinya et al. 2000). When Dkk-1 is knocked down a headless phenotype is observed, and when Dkk-1 is overexpressed there is an enlarged head region in frog, fish and mice (Glinka et al. 1998; Hasimoto et al. 2000; Mukhopadhyay et al. 2001). Thus, inhibiting the activity of Wnt8 is essential for establishing the anterior neural tube. In

summary, loss of BMP-4 and Wnt8 signaling is necessary for formation of the head and the anterior brain while Wnt8 activity induces the spinal cord.

Wnt8 did not act alone in posteriorizing the neuroectoderm. Basic fibroblast growth factor (bFGF or FGF-2) was shown in explant cultures to induce mesoderm with ventral/posterior character that could, in turn, induce ectoderm to form spinal cord (Ruiz I Altaba and Melton 1989). Okamoto subsequently showed that FGF-2 directly induced *Xenopus* ectodermal cells to generate neurons (Kengaku and Okamoto 1993). Moreover, high levels of FGF-2 caused animal pole ectodermal cells to express posterior neural tube markers while progressively lower levels of this growth factor led to expression of more anterior markers (Kengaku and Okamoto 1995). It appeared that FGF-2 was expressed by the organizer and diffused through the plane of the ectoderm, setting up a concentration gradient along the anterioposterior axis prior to gastrulation. While the necessity of FGF-2 in neural induction has been questioned (Wills et al. 2010), it appears that this factor has a dual role in both inducing the neuroectoderm and in anterioposterior patterning (Guillemot and Zimmer 2011). Most likely, the antagonism of BMP-4 alone is not sufficient for neuroectoderm induction. FGF signaling in the animal pole ectoderm is required for BMP-4 antagonism to produce neural fates (Delaune et al. 2005). Thus, BMP-4 inhibition is necessary, but not sufficient, for neural induction.

8.3.4 Organizing Centers in the Neural Tube

The anterior end of the neural tube is progressively partitioned into discrete compartments during embryonic development. Initially the brain is morphologically defined by the forebrain (telencephalon and diencephalon), midbrain (mesencephalon) and hindbrain (rhombencephalon) (Fig. 8.4). The diencephalon and hindbrain are further subdivided into anterioposterior segments called prosomeres (Fig. 8.4) and rhombomeres, respectively. The segmented nature of the rhombomeres are demonstrably clear in the chick hindbrain, where vertical stripes of neuroepithelium are identified by gross anatomical bulges as well as by restricted patterns of gene expression (*e.g.* Hox genes) (Lumsden 2004; Kiecker and Lumsden 2005; Alexander et al. 2009). However, in the classical sense, true segmentation is experimentally verified: first, by polarity defects (gene mutations that alter the number or complement of segments); second, by homeotic transformation (*i.e.* conversion of the morphological phenotype of one segment into another) by misexpression or mutation of

Fig. 8.4 (continued) differentiated from the ventral telencephalon by expression of Shh, several Dlx genes and Nkx2.1 during early telencephalic development in both mouse and chick (Eisenstat et al. 1999; Stoykova et al. 1996; Bardet et al. 2010). Cell lineage tracing in mouse embryos (Inoue et al. 2000) and fate mapping of the chick and Xenopus neural plates (Eagleson et al. 1995; Pombero and Martinez 2009) also support distinct origins of the lamina terminalis and ventral telencephalon. In fact, the anterior midline ANR (that gives rise to the lamina terminalis of the diencephalon) inhibits the formation of the telencephalon (Eagleson and Dempewolf 2002). Scale bar = 0.3 mm

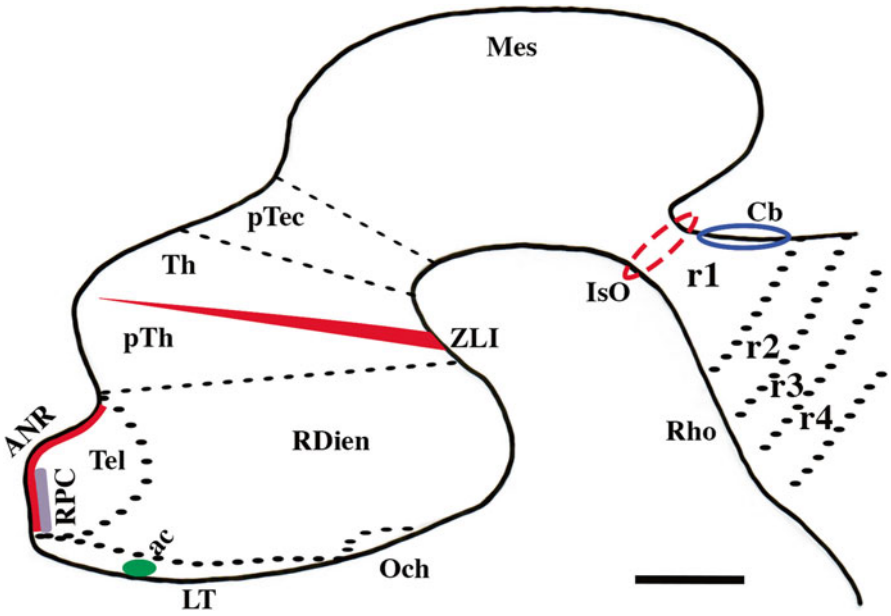

Fig. 8.4 Location of secondary organizers in chick brain. Outline of the brain of a Hamburger and Hamilton stage 22 chick traced from an embryo presented in Desmond and Levitan (2002). This drawing is from a sagittal midline section and does not include the lateral placed telencephalic vesicles. It should be noted that this schematic is used as a template for identifying features that may appear either before, or during or after this stage. The brain consists of the rhombencephalon (Rho), mesencephalon (Mes) and the prosencephalon. The prosencephalon consists of the diencephalon and a small anteriodorsal telencephalon (Tel) at stage 22. The diencephalon is partitioned into the pretectum (pTec; also called prosomere 1), thalamus (Th; prosomere 2), prethalamus (pTh; prosomere 3) and the anterior or rostral diencephalon (RDien; or presumptive hypothalamus; Puelles and Rubenstein 2003). The anterior diencephalon contains the lamina terminalis (LT) that is bounded ventrally by the optic chiasma (Och). The anterior commissure (ac; in *green*) will emerge later from telencephalic neurons in the subpallium and course within the lamina terminalis. The anterior diencephalon and telencephalon are together referred to as the secondary prosencephalon (Puelles and Rubenstein 2003). The secondary organizers are highlighted in *red* and *light purple*. The anterior neural ridge (ANR) forms the ventral lips of the anterior neuropore. As the neuropore closes this tissue gives rise to a patch of neuroepithelium called the rostral patterning center (RPC) that occupies the anterior half of the roof plate of the telencephalon (Crossley et al. 2001; Fukuchi-Shimogori and Grove 2001; Hoch et al. 2015). The zona limitans intrathalamica (ZLI) is a knife-like wedge of neuroepithelium separating the prethalamus (pTH; selectively expresses Six3) from the thalamus (Th; selectively expresses Irx3) in the diencephalon. The isthmus organizer (IsO) demarcates the boundary between the mesencephalon and rhombencephalon. The rhombencephalon is partitioned into 8 segments called rhombomeres (Lumsden 1990; r1-4 are represented). Each of the rhombomeres expresses a unique suite of genes but Hoxb1 is a selective marker of r4, and Krox 20 is restricted to r3 and r5 (Wilson and Chambers 2015). The boundaries of brain regions represented are similar to those proposed by Puelles and Rubenstein (2015). However, the preoptic area is considered here to arise from the hypothalamus (including the lamina terminalis) in the anterior diencephalon, rather than from the telencephalon as alternatively proposed (Moreno and González 2011; Puelles and Rubenstein 2015). The developmental evidence that the lamina terminalis and preoptic area form the anterior and lateral walls of the 3rd ventricle is consistent with the diencephalic origins of this tissue. Moreover, the preoptic area can be readily

specific transcription factor genes; and third, by lineage restriction (cells born in one segment remain localized to that segment) (Gehring 1987; Lumsden 2004). On this basis, rhombomeres were first revealed to be segments through lineage restriction analysis in the chick hindbrain (Lumsden 1990). The progeny of single stem cell-like neuroepithelial cells in a specific rhomobomere was shown to remain confined to that rhombomere. Subsequent loss-of-function analysis revealed that mutation of Hoxa1 resulted in segment loss in mouse hindbrain (Mark et al. 1993) whereas Hoxb1 misexpression led to conversion of rhombomere 2 into rhombomere 4 in chick (Bell et al. 1999). Thus, the evidence for hindbrain segments is very strong. It should be noted that the Hox gene expression is, by itself, probably not sufficient to create segment identity in the hindbrain. Rather, these genes most likely regulate the ability of the segment to respond to morphogen signals.

The prosomeres were initially characterized as gene expression domains (Puelles et al. 2013). Cell lineage tracing in mouse embryos has revealed forebrain and midbrain regions do not mix at a time when they appear morphologically distinct (Inoue et al. 2000) and loss-of-function of the Pax-6 forebrain marker perturbs forebrain-midbrain boundary formation (Mastick et al. 1997). These results are consistent with forebrain and midbrain behaving as large segments (although segment polarity and homeotic transformations have not been described for these compartments). Further evidence for the segmental identity came from transplantation and gene misexpression studies demonstrating that midbrain could be converted into hindbrain identity in chick (see Sect. 8.3.4.1 below). Finally, the differential identity of prosomeres 2 and 3 in the diencephalon was shown to be dependent on the Irx3-mediated responsiveness of prosomere 2 to sonic hedgehog (Shh) (Kiecker and Lumsden 2004; see Sect. 8.3.4.2 below). Despite the above evidence, the characterization of prosomeres as true segments has been debated since the expression patterns of supposed segment markers are highly dynamic during development and lineage restriction has not adequately described for each of the diencephalic prosomeres (Kiecker and Lumsden 2005). The dynamic nature of the expression patterns of forebrain marker genes was clearly demonstrated, particularly in respect to axon tract formation, in the embryonic forebrain of zebrafish (Hjorth and Key 2001, 2002). Moreover, prosomeres lack the repeated morphological similarly typical of metameric segments and there is absence of evidence for segmental polarity (Hannibal and Patel 2013). Nonetheless, the idea that the forebrain is compartmentalized has enabled the experimental interrogation of new hypotheses that explain the molecular and cellular development of this region of the neural tube.

8.3.4.1 The Isthmus Organiser

While induction of rhombomeres is associated with signals emanating from the mesoderm (most likely Wnt signals that set up an anterioposterior gradient within the neural plate; Kiecker and Niehrs 2001), the division between the hindbrain and

midbrain is dependent on signals endogenous to the neural tube Marin and Puelles 1994; Alvarado-Mallart 2005). The region between these two major subdivisions of the brain is recognised morphologically by an isthmus, which is a narrowing of the neural canal (Fig. 8.4). When the isthmus region from a quail is transplanted into the presumptive forebrain of a chick, an ectopic midbrain and cerebellum develops from the host tissue (Nakamura et al. 2008). These results revealed that the isthmus has organizer activity that induces tissue of the midbrain-hindbrain border, in what appears reminiscent of the inductive capabilities of the Spemann-Mangold organizer transplanted into the ventral embryo in amphibia. Hence, the isthmus region was referred to as the isthmus organizer (IsO). One of the defining characteristics of this tissue is its expression of FGF-8.

Analysis of transcription factor expression in the developing brain of chick revealed that Otx2 was expressed by prosencephalon and mesencephalon while Gbx2 was expressed by the rhombencephalon (Garda et al. 2001). Both genes play an important role in the development of the respective brain regions in which they are expressed (Wassarman et al. 1997; Rhinn et al. 1998). At the junction of the mesencephalon and rhombencephalon, the expression patterns of these two genes gradually overlap (Garda et al. 2001). It is in this region of overlap that the IsO emerges and begins to selectively express FGF-8. Experiments in both chick and mice revealed that FGF-8 causes the upregulation of Gbx2, that then downregulates Otx2 and consequently leads to the formation of a discrete boundary between the Gbx2-expressing IsO and the Otx2-expressing mesencephalon (Katahira et al. 2000; Garda et al. 2001; Joyner et al. 2000). If the level of Gbx2 is decreased genetically in transgenic mice then the expression of Otx2 spreads posteriorly and the IsO fails to develop. If the posterior border of expression of Otx2 is moved anteriorly via genetic manipulation, then the position of the IsO also moves anteriorly. Thus, the induction of the IsO is dependent on the overlapping expression of Oxt2 and Gbx2. This was confirmed by transplanting inert beads soaked in FGF-8 into the Otx2 expressing forebrains of chicks. Each bead became surrounded by a zone of tissue co-expressing both Gbx2 and Otx2. Otx2 was subsequently downregulated so that the bead was surrounded by an ectopic zone of Gbx2 expression, which was then surrounded by tissue expressing Otx2. Next, the Gbx2 zone expressed FGF-8, which was similar to the temporal pattern of expression in the endogenous IsO. In summary, the overlapping expression of two transcription factors is responsible for the selective expression of FGF-8 (but not for its the initial expression; Li and Joyner 2001; Martinez-Barbera et al. 2001). This overlap in expression subsequently leads to the differentiation of the cerebellum and midbrain at the anterior end of the hindbrain. How a sharp boundary between two tissue compartments is determined by the expression of two transcription factors remains to be determined. However, it is likely to involve reciprocal feedback mechanisms controlling the level of expression of these and possibly other factors.

8.3.4.2 Zona Limitans Intrathalamica

The IsO at the midbrain-hindbrain boundary is not the only organising center in the vertebrate brain. Andrew Lumsden from the MRC Center for Developmental Neurobiology in London identified a second such organizer in the zona limitans intrathalamica (ZLI) of chick. The ZLI is a narrow and knife-like wedge of cells in the diencephalon that separates the prethalamus and the thalamus (Fig. 8.4) and is recognised by its lineage restriction as well as by expression of the soluble growth factor Shh (Larsen et al. 2001; Kiecker and Lumsden 2004). Shh was shown to regulate the patterning of the diencephalic brain regions (the prethalamus and thalamus) on either side of the ZLI. The expression of Shh appears to be induced at the boundary between domains of cells expressing the Hox genes Six3 (prethalamus) and Irx3 (thalamus) in chick (Kobayashi et al. 2002). The regional identity of thalamus is dependent on Shh and this dependency is mediated by the expression of Irx3 (Kiecker and Lumsden 2004). Six3 (together with FGF8; see below) induces expression of the anterior prosencephalic marker, Bf1 (Kobayashi et al. 2002). Interestingly, Six3 null mutant mice lack a telencephalon (Lagutin et al. 2003) and humans with Six3 mutations exhibit holoprosencephaly (Labawan et al. 2009). Holoprosencephaly, which involves failure of midline separation of telencephalic vesicles, is the most common human brain defect, with an incidence of up to 50 per 10,000 pregnancies when aborted embryos are included (Orioli and Castilla 2010). Six3 represses Wnt1 expression directly in the forebrain and thereby limits its anterior expression to the midbrain (Lagutin et al. 2003). In the absence of Six3 there is anterior expansion of midbrain Wnt1 expression and concomitant loss of the telencephalon. Thus Six3 complements the early interactions occurring during gastrulation that restrict the vertical inductive roles of Wnt to the posterior neural plate (refer to Sect. 8.3.3).

In vivo gain-of-function experiments have revealed that Six3 and Irx3 mutually repress each other (Koybayashi et al. 2002). Given that both genes are initially expressed in an overlapping stripe of neuroepithelium (presumptive ZLI) at the boundary of the prethalamus and thalamus, this mutual repression should lead to sharp boundary formation and may be responsible for the formation of the ZLI. However, the ZLI does initially form in Six3 null mutant mice but is lost as the anterior brain becomes progressively posteriorized (Lavado et al. 2008). It is now realised that in mouse there is a large network of regulatory genes involved in patterning the diencephalon and that functional redundancy between these genes makes it difficult to interrogate this pathway via a gene knockout strategy (Chatterjee and Li 2012). Nonetheless, the transformation of the anterior forebrain regions into more posterior neuroepithelium is consistent with the partitioning of the brain by the interplay of transcription factors and morphogens.

The ZLI (and its expression of Shh) has a role in patterning of the diencephalon across diverse vertebrate species, including: zebrafish (Scholpp et al. 2007), *Xenopus* (Juraver-Geslin et al. 2014), chick (Larsen et al. 2001) and mice (Szabo et al. 2009). Extending on earlier work in chick (Kiecker and Lumsden 2004), Kiecker has recently demonstrated in mouse that ectopic expression of Irx3 in the telencephalon

induces expression of thalamic markers in the presence of Shh (Robertshaw et al. 2013). Thus, thalamic identity is determined through the preserved actions of Irx3 and Shh across considerable phylogenetic distance in vertebrates. The sharp anterior border of expression of Shh expression in the ZLI is shaped by a wedge of Pax6 expression in the apposing prethalamus in mouse (Caballero et al. 2014). Pax6 was shown to bind directly to the Shh promoter and repress its expression within the prethalamus. While the intricate gene regulatory network that controls the patterning associated with the ZLI remains an area of intense interest (Caballero et al. 2014; Juraver-Geslin et al. 2014), it is clear that a number of genes acting both cell and non-cell autonomously contribute to this process.

8.3.4.3 Anterior Neural Ridge

The anterior end of the neural plate in the early vertebrate embryo consists of a terminal ridge of neuroectoderm (Puelles et al. 1987), referred to as the "anterior neural ridge" (ANR). The portion of the ridge closest to the midline in both chick and mice will form the ventral lip of the "anterior neuropore" as the neural tube closes (Schoenwolf 1979; Puelles et al. 1987; Nonomura et al. 2013). The closure of the anterior neuropore occurs in a dorsal to ventral direction as the free margins of the dorsal diencephalon and telencephalon zip together. Some authors indicate that the anterior neuropore closes in a posterior to anterior direction (which is in relation to the curved longitudinal axis of the embryo), rather than in a dorsal to ventral direction (which is in relation to the axis of the spinal cord). The ANR disappears after closure of the anterior neuropore. It is replaced by a wider, and shorter, patch of neuroepithelium referred to as the "rostral patterning center" (RPC) (see Sect. 8.3.4.3). The site of final closure of the anterior neuropore marks the dorsal limit of the lamina terminalis (Puelles et al. 1987; Fig. 8.4). During subsequent development, differential growth of the lamina terminalis pushes the position of the RPC dorsally/posteriorly (Puelles et al. 1987).

Discrepancies in the description of the location of the anterior neuropore and surrounding anatomical landmarks within the old literature (Puelles et al. 1987; Shimamura et al. 1995) has led to continuing confusion, even amongst current researchers, regarding embryological relationships and developmental mechanisms in the anterior telencephalon. Most notably there is conflation of the terms ANR, RPC, roof plate, lamina terminalis and commissural plate (Rakic and Yakovlev 1968; Silver et al. 1982; Müller and O'Rahilly 1984; Hamasaki et al. 2004; O'Leary et al. 2007; Cholfin and Rubenstein 2008; Moldrich et al. 2010; Kiecker and Lumsden 2012; Robertshaw and Kiecker 2012; Suárez et al. 2014). As noted above, the ANR and RPC are morphologically distinct tissues present at different times in development. After closure of the anterior neuropore, the most dorsal midline strip of prosencephalic neuroepithelium that has zipped together is best referred to as the roof plate (Bailey 1916; Shimamura et al. 1995). The roof plate is not a homogenous structure since GDF7, BMPs and noggin are differentially expressed within the diencephalic and telencephalic regions of this structure (Furuta et al. 1997; Theil

et al. 1999; Monuki et al. 2001). The lamina terminalis is the midline neuroepithelium lying ventral to the anterior neuropore and dorsal to the optic chiasma/commissure, which is consistent with classical descriptions in human embryology (Bailey 1916). The lamina terminalis forms the anterior wall of the 3rd ventricle and will later give rise to the subfornical organ, median preoptic nuclei and the organ vasculosum in mammals (McKinley et al. 2015; Prager-Khoutorsky and Bourque 2015). The anterior commissure will develop in the dorsal portion of the lamina terminalis. The commissure plate was considered to be a contiguous sheet of neuroepithelium that contained the corpus callosum, hippocampal commissure and anterior commissure (the optic and post-optic commissures have also been included in this array). However, each of these commissures initially develop in spatiotemporally distinct regions in the midline telencephalic neuroepithelium (Silver et al. 1982; Rash and Richards 2001). With subsequent development, the callosal and hippocampal commissures grow so large that they become closely apposed. The callosal tract first develops at embryonic day 17 in mouse (Koester and O'Leary 1994), 6 days after the initial development of the post-optic and anterior commissures (Shimamura et al. 1995; Mastick and Easter 1996) and 1 day after the axons pioneer the hippocampal commissure (Silver et al. 1982). During this time, there are considerable changes in the gross morphology of the anterior forebrain, such as the development of the very large septal nuclei and the preoptic nuclei. The commissural plate is such a nebulous term that it is probably not helpful to continue to use this nomenclature.

Cell fate mapping studies in *Xenopus* revealed that the ANR contributes to the telencephalon and ventral diencephalon after the neural plate rolls into a tube (Eagleson et al. 1995). Fate mapping in chick also revealed that descendants of the midline ANR and adjacent neural neuroectoderm form the lamina terminalis (Cobos et al. 2001). In addition to the ANR giving rise to regions of the later developing forebrain, the ANR also influences the patterning of surrounding anterior neural plate. In mice, co-culture experiments revealed that the ANR most likely regulated the telencephalic expression of Bf1 (Shimamura and Rubenstein 1997). A likely candidate morphogen for this patterning event is FGF8 as it is selectively expressed by the ANR (Crossley and Martin 1995; Shimamura and Rubenstein 1997). To test this hypothesis, beads soaked in FGF8 were placed on isolated early neural plate lacking the ANR (Shimamura and Rubenstein 1997). Following 24 h of culture only neural plates exposed to FGF8 expressed Bf1, an early telencephalic marker and a critical transcription factor needed for the normal development of this brain region (Xuan et al. 1995). Similar results have been reported when these experiments were replicated in Xenopus (Eagleson and Dempewolf 2002). Thus, the ANR and its expression of FGF8 are critically important for anterior neural plate patterning throughout vertebrate evolution. Interestingly, when the FGF8 beads were positioned slightly more posterior in the mouse neural plate isolates, the transcription factor En2 was induced, rather than Bf1 (Shimamura and Rubenstein 1997). Thus, the early neural plate appears to have different competencies along its anterior-posterior axis with respect to its ability to respond to FGF8. Given that En2 is a marker of midbrain (Davidson et al. 1988) it seems that FGF8 emanating from the ANR is capable of specifying patterning of the forebrain and midbrain territories in the

early neural plate. As discussed above (Sect. 8.3.4.1), this patterning is aided later in development by FGF8 expressed in the IsO (Liu and Joyner 2001).

While the mechanisms regulating expression of FGF8 in the ANR are not completely understood, it does appear to involve a combination of Chordin and Noggin dependent inhibition of BMP signaling (Anderson et al. 2002), forebrain expression of Otx2 (Tian et al. 2002), interactions with neural crest cells in the head (Creuzet et al. 2006) and direct transcriptional regulation by Wnt/β-catenin signaling (Wang et al. 2011). As discussed above (Sect. 8.3.3), Chordin and Noggin inhibit BMP signaling during both vertical and planar induction to specify anterior neuroepithelium. Chordin and Noggin loss-of-function mice exhibit severely abnormal brain formation that seems to be a result of the reduced expression of FGF8 by the ANR in these animals (Anderson et al. 2002). BMP-2, which is expressed around the ANR, acts to decrease FGF8 expression but its effects are normally ameliorated by the actions of Chordin and Noggin. Otx2 is normally expressed in the anterior mouse brain and its loss-of-function prevents forebrain and midbrain development (Acampora et al. 1995). These morphogenetic abnormalities arise both because of the combined loss of FGF8 and Bf1 in the ANR and forebrain neuroepithelium, respectively (Tian et al. 2002). Taken together, BMP-2 decreases FGF8 expression while Otx2 acts to increase FGF8 expression in the ANR.

Deletion of the neural crest that contributes to the facial skeleton in chicks leads to failed neural tube closure and exencephaly. These morphogenetic abnormalities are accompanied by loss of FGF8 expression in the ANR (Creuzet et al. 2006). Since these defects were partially rescued by transplantation of FGF8-soaked beads, it appears that neural crest interactions with the ANR regulate FGF8 expression. Mice mutant for the zinc finger transcription factor SP8 exhibit a "faceless phenotype" due to both increased apoptosis and decreased proliferation of cranial neural crest cells (Kasberg et al. 2013). These neural crest defects occur with a concomitant reduction in the expression of FGF8 by the ANR. Together, these neural crest defects and loss of FGF8 probably account for the exencephaly that is also observed in these SP8 mutant mice.

As discussed above (Sect. 8.3.3), Wnt8 signaling posteriorizes the neural plate during gastrulation. However, Wnt signaling is subsequently needed locally to enhance expression of FGF8 by the ANR (Wang et al. 2011). Loss and gain of function of Wnt signaling in mouse embryos caused dramatic down- and up-regulation, respectively, of FGF8 expression by the ANR. Wnt signaling was found to directly alter FGF8 transcription (Wang et al. 2011).

8.3.4.4 Rostral Patterning Center

Soon after the anterior neuropore closes, the dorsal portion of the mammalian prosencephalon begins to evaginate and form telencephalic vesicles. In mice, the anterior neuropore closes at approximately embryonic day 9.5 (Crossley and Martin 1995). The formation of cerebral vesicles occurs within less than a day, so that by embryonic day 10.5 most embryos have bilateral rudimentary telencephalic

swellings emerging from the dorsolateral surfaces of the anterior brain (Sahir et al. 2000). In chick, the anterior neuropore closes at stage 12 (approximately 45–49 h incubation; Hamburger and Hamilton 1951) and telencephalic vesicles begin to bulge dorsally within 5 h (Pombero and Martinez 2009). In humans, the cerebral vesicles begin to form at stage 15 (approximately 5 weeks; Yamada et al. 2010), about 1 week after the anterior neuropore closes (stage 11; O'Rahilly and Müller 2008). The relatively enlarged cerebral cortex of mammals, in comparison to other amniotes, occurs because of tangential expansion of the telencephalic neuroepithelium due to an increased mitotic rate, a delayed exit of progenitors from the cell cycle and a longer neurogenic period (Chenn and Walsh 2002; Nomura et al. 2013).

Cell proliferation and specification along the anterioposterior axis of the developing mammalian cerebral vesicles is partly controlled by morphogens secreted from signaling centers and their downstream effects on transcription factors. After formation of the neural tube, the ANR fuses at the anterior midline as a result of neuropore closure. This midline region of the neuroepithelium is referred to as the "rostral patterning center" (RPC) and selectively expresses four FGF genes (FGF8, FGF15, FGF17 and FGF18) (Cholfin and Rubenstein 2008; Hoch et al. 2009). It should be noted that each of these genes are expressed in overlapping but slightly different patches of anterior neuroepithelium in the margin between the emerging cerebral vesicles. By embryonic day 12.5 in mice, FGF8, FGF17 and FGF18 expression has extended ventrally into the enlarging septal nuclei in the ventromedial walls of the telencephalon, while FGF15 expression has spread further ventrally into the ganglionic eminences. By this time, only FGF8 and FGF17 expression persists in the roof plate neuroepithelium of the telencephalon at the level of the foramen of Monro (passageway between the third and lateral ventricles) (Cholfin and Rubenstein 2008). Genetic labeling strategies were recently used to identify the progeny of RPC cells expressing either FGF8 or FGF17 (Hoch et al. 2015). This fate mapping has revealed that the RPC has an important role not only in patterning the cortex but this tissue gives rise to cells which widely populate both cortical and subcortical regions by late embryogenesis. Interestingly, FGF8 and FGF17 cell populations in the RPC were found to contribute to both overlapping and distinct territories in the telencephalon.

Emx2 is expressed in an anterior-to-posterior increasing gradient in the cortical neuroepithelium and thereby confers regional identity to cortical cells (Bishop et al. 2000). FGF8 secreted from the RPC acts on anterior cortical neuroepithelium to decrease Emx2 expression and hence creates the anterioposterior identity in the cortical neuroepithelium in both chick and mice (Crossley et al. 2001; Fukuchi-Shimogori and Grove 2001; Toyoda et al. 2010; Assimacopoulos et al. 2012). The Emx2 gradient is further strengthened by Wnt signals emanating from the posterioventral telencephalic vesicles (cortical hem; see below) as well as by BMPs expressed by the roof plate of the telencephalon (Theil et al. 1999). Both BMP and Wnt signaling directly increases expression of Emx2 (Theil et al. 2002). Given that FGF8 expression is absent from the cerebral hemispheres at embryonic day 12.5 in mice (when Emx2 is already expressed in an anterior to posterior increasing gradi-

ent in this tissue) it would appear that the RPC is only functionally active during a narrow window of development between embryonic days 10 and 13 in mice. This is consistent with loss of anterior identity of cortical neuroepithelium when FGF signaling was reduced at embryonic day 11.5 in mice (Fukuchi-Shimogori and Grove 2001).

Control of the size of an organizing center has been considered essential for normal cortical development (Shimogori et al. 2004). Recently it was revealed that the size of the RPC is normally regulated by apoptosis during brain development in mice (Nonomura et al. 2013; note that these authors refer to the RPC as the ANR). When apoptosis was blocked in the early stages of neural tube formation, FGF8 expression expanded ventrally after neural tube closure. This expanded expression led to abnormal FGF8 signaling and patterning (*e.g.* reduced Shh expression) in the ventral telencephalon and highlighted the importance of tight spatial regulation of FGF8 expression for normal development of the forebrain (Nonomura et al. 2013).

The discussion of organizing centers has been restricted to those that principally have a role in establishing the anterioposterior axis in the vertebrate brain. However, there are regions that also contribute to demarcating the ventrodorsal axis, both in the neural tube and later, during enlargement of the cerebral vesicles in mammals [*e.g.* the cortical hem (Caronia-Brown et al. 2014) and dorsal roof plate (Monuki et al. 2001)]. The cortical hem is a longitudinal strip of neuroepithelium lying between the presumptive hippocampus and the emerging choroid plexus in the lateral ventricle (Grove et al. 1998). This transient structure is recognized by expression of Wnt genes (2b, 3a and 5a in mice) between embryonic days 10.5 and 12.5. During subsequent development Wnt5a expands in the medial wall of the cerebral hemispheres while expression of both Wnt2b and Wnt3a progressively reduce in size until they are lost at birth (Grove et al. 1998). Wnt signaling was subsequently shown to be essential for hippocampal development (Lee et al. 2000).

The construction of the vertebrate brain relies on the induction of neural tissue and subsequent specification by specialised organizing centers that secrete morphogens as well antagonists. Together, these molecules establish gradients of activity that have enabled increased morphological complexity of the tissue. This complexity has led to the evolution of unique cognitive abilities, particularly in the social world of humans (Herrman et al. 2007). The trade-off that occurs with increased complexity of any tissue is that regeneration becomes more challenging to achieve and in some organs, such as the mammalian brain, regenerative capacity appears to have been completely lost (Bonfanti 2011). In the next section I will discuss some evidence in vertebrate species where the regenerative capacity of brain regions has persisted.

8.3.5 Regeneration of Brain Regions

When I refer to regeneration I will restrict my discussion to portions of the brain that are able to regenerate following injury in adults. I will not interrogate the extensive literature on either the persistence of stem cells in select adult brain regions, or the

differentiation of new neurons and their subsequent incorporation into adult circuitry. Furthermore, I do not consider either the plasticity of synaptic connections and the associated retraction and elaboration of axon and dendritic branches or the regeneration of axons following physical or pathological lesions. I will assess examples in vertebrates where the brain is capable of mounting a functionally-appropriate regenerative response to a gross injury. This targeted approach will hopefully illuminate the "best case" scenario for comparing and contrasting with outcomes from potential therapeutic strategies in human clinical trials. Using the above criteria, regeneration of brain regions in vertebrates following injury occurs only in non-mammalian species, and in particular, in fish, amphibians and reptiles (Font et al. 2001; Kaslin et al. 2008). While birds exhibit some replacement of specific subpopulations of neurons following selective cell type ablation, these compensatory mechanisms lack the power to regenerate specific brain regions following gross lesions (Kaslin et al. 2008). Most of the discussion here will be restricted to a few teleost species where comprehensive data is available on regeneration; reference to amphibians and reptiles is limited to where experimental results provide novel insights above and beyond that provided by teleost animal models. It should be noted that while adult amphibians have been reported to mount regenerative responses in the brain following injury, compensatory responses in these animals vary greatly in different species. For instance, regeneration in *Xenopus* is typically limited to larval stages of development before neural cell proliferation wanes in post-metamorphic animals (Kaslin et al. 2008; Bernardini et al. 2010). In contrast, regeneration occurs readily in the brain of adult newts (Minelli et al. 1987, 1990).

8.3.5.1 Telencephalon

The regeneration of ablated brain regions in vertebrates is a relatively rare event. There are some examples where reconstitution of lesioned brain does occur, but this regeneration is limited to certain species and only occurs in some brain regions. In a series of experiments examining the behavioural consequences of telencephalon lesions in adult stickleback fish it became apparent that the brain was exhibiting signs of regeneration (Segaar 1961). When fish were histologically examined after unilateral lesions that removed the lateral half of the pallium (dorsal telencephalon), there was evidence at 6 weeks survival of a new sheet of neuroblasts at the surface of the lesion, and by 12 weeks nerve cells were arranged in layers, as in the control side of the brain. Despite the return of the cytoarchitecture, the regenerated side of the brain was smaller than the unoperated control side. It was noted during these experiments that the olfactory bulbs did not regenerate after the anterior telencephalon (containing the olfactory bulbs) was severed by a transverse section. However, subsequent experiments in goldfish revealed that while transversely-lesioned telencephalons showed progressive signs of neural regeneration, whole unilateral or bilateral ablation of the telencephalic hemispheres failed to elicit any regeneration (Bernstein 1967).

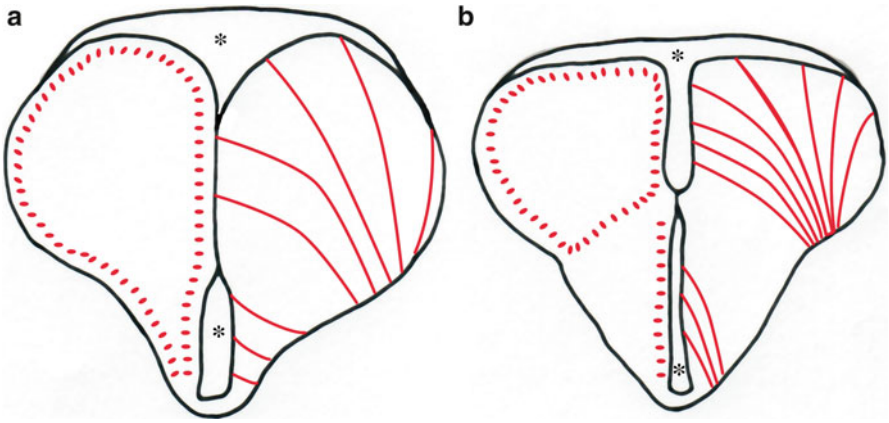

Fig. 8.5 Localization of neural stem cells in adult teleost forebrain. Schematic representations of coronal sections of an adult teleost at (**a**) anterior and (**b**) posterior levels. The *asterisks* denote ventricles. In teleosts, the lateral ventricles are dorsal and on the external surface of the brain due to eversion of the pallium. The location of the perikarya of the neural stem cells are represented by *red dots* on the left-hand sides of the brains. These cells line the lateral margins of the forebrain (which is equivalent to the subventricular zone of invaginated palliums of tetrapods). The *red lines* on the right sides of the brains depict the radial glial arrangement of the cell processes of these stem cells as they span the width of the brain wall (Lam et al. 2009)

Needlestick injuries that graze the dorsolateral surface of the anterior telencephalon in adult zebrafish are able to regenerate within 35 days (Kishimoto et al. 2012). While these injuries are less severe than those reported by the earlier studies of Segaar (1961) and Bernstein (1967), they do suggest that the regenerative capacity of the fish brain may depend on the technical delivery and size of the lesion. The needlestick injuries inflicted by Kishimoto et al. (2012) led to a preferential increase in cell proliferation around the circumference of the pallium on the injured, rather than on the uninjured side of the brain. Interestingly, there were bilateral increases in cell proliferation in the ventral ventricular proliferative zone of the subpallium. There was also considerable migration of newly born cells from the ipsilateral subpallial ventricular zone to the injury site where they differentiated into neurons. Thus, preservation of cell proliferative zones and migration routes are most likely essential to a successful regenerative response in teleosts. It should be noted that adult teleosts, unlike mammals, typically exhibit proliferative zones with abundant cycling cells at various locations along the anterioposterior axis of the brain (Adolf et al. 2006; Grandel et al. 2006; Zupanc and Sirbulescu 2011; Olivera-Pasilio et al. 2014) (Fig. 8.5). These cells were shown to have neural stem cell-like properties whose progeny could migrate into adjacent parenchyma and differentiate into neurons (Adolf et al. 2006; Grandel et al. 2006).

Deep stab injuries to the telencephalon in zebrafish are also rapidly repaired and this regeneration has been partly attributed to the absence of a permanent glia scar at the injury site (März et al. 2011). Such injuries also led to ipsilateral increases in cell proliferation at the ventricular surface. A relatively large stab wounds (~75 μm

diameter) in the adult zebrafish telencephalon (which is ~400 μm wide) is repaired quickly and with minimal gliotic scarring (Kroehne et al. 2011). While there is a strong inflammatory response, it is temporally confined to several days after the injury. This stab injury results in unilateral increases in cell proliferation in the ventricular zone. In particular, radial glia progenitor proliferation increases and these cells give rise to neuroblasts that migrate into the parenchyma where they replenish neuronal cells at the lesion site. When a neurotoxin is injected into the telencephalon at the same time as the stab wound, there is a massive two-fold increase in radial glial stem cell proliferation in comparison to needlestick injury alone (Skaggs et al. 2014). Moreover, the neurotoxin induced cell death also leads to more effective repair of the lesion site. In contrast to mammals, there is little *de novo* reactive gliosis, accumulation of inflammatory cells and deposition of extracellular matrix at the stab wound site (Kroehne et al. 2011). In zebrafish, astrocytes restrict their proliferative activity to the vicinity of the ventricular margins (which in teleosts are towards the outside surface of the telencephalon; Fig. 8.5). The normal cytoarchitecture of the telencephalon is restored within about 30 days of the stab injury (Kroehne et al. 2011). It should be noted that the ability to regenerate damaged neural tissue in teleosts is also linked to the rapid apoptotic death of injured neurons and the lack of necrotic and pathological (*e.g.* cavitation, scarring) damage at the injury site, which typically occurs in the injured mammalian brain (Zupanc 1999).

The inflammatory response after injury highlighted by Kroehne et al. (2011) was subsequently revealed to be essential to regeneration in the zebrafish brain (Kyritis et al. 2012). When the inflammatory response (increase in infiltrating leukocytes and endogenous microglia as well as elevated levels of proinflammatory cytokines IL-8, IL-1b and TNF-a) was induced experimentally, in the absence of a brain lesion, there was an increase in neural progenitor (radial glial) cell proliferation (Kyritis et al. 2012). Importantly, this response was shown to be independent of the normal constitutive activity of progenitor cells in the zebrafish brain. The critical question is how the acute inflammatory response in zebrafish (which leads to regeneration) is prevented from becoming a chronic response that inhibits repair, as it does in mammals (Kyritsis et al. 2014).

As noted above, both reptilian and amphibian brains exhibit regenerative properties following injury. This regenerative capacity has also been linked to the continual neurogenesis in the telencephalon that occurs throughout life in lizards (Lopez-Garcia et al. 1988; Garcia-Verdugo et al. 2002). The dorsal telencephalon in lizards is referred to as cortex with three principal layers, consisting of a cell layer containing projection neurons sandwiched between two plexiform layers. Early studies revealed that removal of portions of the dorsoposterior telencephalon led to regenerative responses that were ongoing at 260 days post-surgery (Minelli et al. 1977). While the thickness of the cortex was restored during this time, the cellular layer was not appropriately repaired. In contrast, when neurons in the cellular layer of the medial cortex were selectively ablated using neurotoxins, there was a concomitant increase in cell proliferation in the ventricular zone that resulted in full neuronal reconstitution of the cortex within 6 weeks (Font et al. 1991).

The axolotl telencephalon, like that of the lizard, also possesses a remarkable ability to repair itself and this was clearly illustrated by transplantation experiments. When the anterior one-third of the telencephalon was extirpated and replaced by equivalent tissue from a donor, the explanted tissue became fully incorporated into the host brain (Kirscher 1983). Within 60 days of surgery the donor anterior telencephalon had developed a separate ventricular space that was encapsulated by a dense band of neural cells (as was observed in the posterior host telencephalon). Approximately 1 year after surgery the transplanted tissue was fully contiguous with host telencephalon and a single ventricle was present. Moreover, when the middle third of the telencephalon was removed in a juvenile axolot (these animals remain in the larval state and do not metamorphose), there was complete regeneration of the tissue by 15 weeks after surgery (Maden et al. 2013). This regeneration was accompanied by increased neural cell proliferation in the ventricular zone of the surrounding unlesioned ipsilateral telencephalon. While repair proceeded for up to 15 weeks, neural cell proliferation in the ventricular zone returns to control levels by 6 weeks post-surgery. In contrast, local cell proliferation continued to persist at the margins of the lesion site within the parenchyma of the telencephalon (Maden et al. 2013). When the anterior one-third of the telencephalon was removed (including the olfactory bulb and its innervation by the olfactory nerve), repair of the telencephalon was noticeably delayed until the olfactory nerve had regenerated and reformed contact with the anterior end of the brain (Maden et al. 2013). Further lesions studies confirmed that olfactory nerve innervation of the anterior telencephalon was needed to achieve full telencephalic repair. The nature of the influence of the olfactory innervation on the regenerative processes remains to be better understood at both the cell and molecular levels.

Newts, like axolotls, also exhibit regenerative responses in the telencephalon. The newt telencephalon possesses highly active areas or hotspots of cell proliferation in the ventricular zone that lines the ventricle as well as in areas with typically low levels of proliferative activity (Kirkham et al. 2014). There appears to be two types of proliferating cells in the ventricular zone: there are those with stem cell-like properties (*i.e.* slow turnover) and cells with transient amplifying properties. During normal homeostasis, non-hotspot regions lack the latter transient amplifying population. Injection of a neurotoxin that selectively ablated cholinergic neurons led to massive loss of these neurons in the ventral telencephalon (Kirkham et al. 2014). However, these neurons were fully reconstituted within 25 days of application of the neurotoxin. In non-hotspot regions, regeneration involved *de novo* generation of neurogenic niches of transient amplifying cells in the ventricular zone. Understanding how ablation selectively up-regulated the generation of these transient amplifying cells has important implications for modulating regenerative responses in adult brain regions lacking these cells. Nonetheless, these transient amplifying cells arise from the neural stem cell population and the maintenance of this population into adult life is essential for regenerative responses (Barbosa et al. 2015).

Not all amphibians have similar brain regenerative capabilities. When the anterior one-third of the telencephalon is ablated in *Xenopus* larvae there is rapid regeneration that restores normal-like gross morphology within 30 days of surgery (Yoshino

and Tochinai 2004). However, after metamorphosis (referred to as adult) the telencephalon is unable to regenerate. Part of the reason for this difference is attributed to the inability of adult neural cells to seal the telencephalic ventricles after injury. Surprisingly, injection of a suspension of dispersed adult telencephalic cells into the adult lesion site facilitates regeneration of an otherwise unresponsive telencephalon. This regeneration appears to be associated with the sealing of the lesioned ventricle by the transplanted cells (Yoshino and Tochinai 2004). Consequently, it has been proposed that failed telencephalic regeneration in adult *Xenopus* results from an inability of cells lining the ventricles to migrate and seal these cavities after injury, rather than from a loss of neurogenic capability with age in this species (Endo et al. 2007). While it is often reported that the brain cannot regenerate in adult *Xenopus* (Ferretti and Prasongchean 2015), it should be recognized that regeneration has only been examined after severe physical insults that remove large portions of the telencephalon. It is quite possible that more modest injuries associated with stab wounds may lead to regenerative responses.

8.3.5.2 Mesencephalon

Reconstitution of the midbrain tectal cytoarchitecture occurred within 100 days after lateral lesions of the optic tectum in Crucian carps (as discussed in Zupanc and Sirbulescu 2013). This regeneration required that the proliferative zone of neuroepithelium at the posterior dorsomedial region of the tectum remained intact following the lesion. However, complete removal of the tectum did not lead to regeneration. Numerous studies have also failed to report regeneration following half-tectal ablations in goldfish (Yoon 1971; Scott 1977; Hayes and Meyer 1988). No regeneration was observed within 5 months of the ablation of either the posterior half of the tectum, or the caudomedial quarter of the tectum in goldfish (fish were 8–11 cm in body length; Yoon 1971). The lack of tectal regeneration following removal of the posterior half of the tectum in adult goldfish has been attributed to the absence of a massive increase in cell proliferation in the remaining anterior half-tectum (Stevenson and Yoon 1980). Similar findings were also reported following removal of the posterior half of the tectum in adult frogs (Udin 1977). In general, there has not been sufficient interrogation of the size and site of the tectal ablations particularly with respect to whether the ablations have perturbed or preserved resident neural stem cells in the periventricular gray zone of the tectum (Raymond and Easter 1983; Ito et al. 2010). However, the tectum can regenerate in carp provided that the proliferative mantle zone is left intact during the ablation (Kirsche et al. 1983). These regenerated tecta became functional. These results are consistent with implant studies in goldfish. One-quarter of the dorsal tectum in goldfish can be excised, rotated and reimplanted. These implants both survive and become fully functional within 6 months of the surgery (Yoon 1973).

As noted above for telencephalic lesions, there is extensive regeneration leading to reconstitution of the laminar cytoarchitecture following tectal ablations in early *Xenopus* larvae (Endo et al. 2007; Bernardini et al. 2010). However, this ability is

progressively lost as animals undergo metamorphosis (Bernardini et al. 2010; Tao et al. 2015). The mechanisms underlying this failed regeneration of the tectum in adult *Xenopus* remain unknown. In adult newts, regeneration of tectal tissue occurs when only a small plug of tissue is removed (rather than half tectal ablations) (del Grande et al. 1982, 1984; Minelli et al. 1987, 1990; Endo et al. 2007). Moreover, in adult newts, midbrain tegmental neurons regenerate within 30 days of neurotoxic ablation (Parish et al. 2007). This remarkable response is associated with an upregulation of radial glia-like progenitor cells in the ventricular zone of the midbrain (Parish et al. 2007). Given that ventricular zone cells are quiescent in the midbrain of adult newts (as in frogs; Almli and Wilczynski 2007) it would appear that regeneration is not dependent on constitutive neural cell proliferation in the adult brain. The regeneration of dopaminergic neurons in newts was instead found to be dependent on induction of *de novo* neurogenesis in this otherwise quiescent region (Parish et al. 2007). Understanding the mechanism of this regulation has important implications for therapeutic approaches aimed at stimulating neurogenesis in quiescent zones in the mammalian brain.

Sonic hedgehog, a potent developmental morphogen, was subsequently shown to play a role in dopaminergic differentiation of newly generated neurons in the newt midbrain following neurotoxic lesioning (Berg et al. 2010). These observations suggest a connection between developmental and regenerative programs in these animals. It is a link that could provide new opportunities for dissecting the molecular genetics of regeneration in the vertebrate brain. Most recently it was revealed that the expression of dopamine by midbrain neurons acts as a negative feedback to inhibit cell proliferation in the ventricular zone (Berg et al. 2011). Treating unlesioned animals with dopamine receptor antagonists was sufficient to induce significant levels of neurogenesis in the midbrain. Taken together, successful regeneration in the newt midbrain depends first on the re-entry of quiescent cells into the cell cycle and secondly, on terminating neurogenesis once sufficient dopaminergic neurons have been generated. Understanding the interplay of these two processes will hopefully provide plausible targets of therapeutic value, particularly in conditions like Parkinson's disease where the homeostasis of dopaminergic neurons is perturbed.

8.3.5.3 Cerebellum

Regeneration has also been examined in the cerebellum of teleosts (Clint and Zupanc 2001; Zupanc 2013). A scalpel blade wound to the cerebellum quickly heals within 2 weeks. Within 5 days of lesion there is massive upregulation of cell proliferation in normal proliferative zones in the cerebellum of uninjured brains as well as in regions near the wound. Many of these cells migrate to the wound, guided by radial glial cells, where they participate in neural repair (Zupanc 2001). When the entire cerebellum was ablated in zebrafish larvae at 36 h post-fertilization, it was found that these fish exhibited normal body posture, balance and locomotion at 6 days (Köster and Fraser 2006). Histological examination revealed that the

cerebellum (including Purkinje cells and associated axon tracts) had regenerated in these animals. The cerebellum normally develops in the anterior hindbrain from a dorsal rim of tissue called the rhombic lip (Fig. 8.4). Its differentiation is dependent on FGF8 signaling associated with the isthmus organiser in zebrafish (Reifers et al. 2000). Although the rhombic lip is totally removed during cerebellum ablation, new tissue expressing rhombic lip markers appears in the anterior hindbrain and becomes the precursor for regenerated cerebellum (Köster and Fraser 2006). Knockdown experiments revealed that FGF8 signaling at the midbrain-hindbrain boundary was responsible for induction of the regenerating cerebellum from remaining anterior hindbrain. Thus, regeneration was dependent on normal development signaling cascades that persisted in the isthmus organizer. The length of the temporal window during which the cerebellum is able to regenerate following ablation remains unknown. Furthermore, the role FGF8 signaling plays in adult cerebellum regeneration is also unknown.

8.3.6 Concluding Statement

There have been extraordinary advances over the last 100 years in our understanding of how a simple sheet of epithelial cells is majestically converted into the vertebrate brain. This knowledge is currently being transferred across to experimental and clinical studies aimed at the repair and regeneration of injured human brain. What is known of the genetic circuitry required to generate neural cell types specific for defined brain regions is being used to fine-tune the differentiation of embryonic stem cells *in vitro* and to modify the survival and incorporation of human fetal brain tissue transplants *in vivo*. The adoption of the novel technique of therapeutic genome editing for clinical approaches in human neuropathologies and brain injury is expected. Progress in these pursuits will be empowered by the continual interrogation of the molecular and cellular mechanisms underlying brain development and regeneration. The most significant advances will lie ahead of us, as the next generation begins to search for, and then dissect the genetic interactions lying at the interface of development and regeneration in the vertebrate brain.

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Chapter 9
Role of Innate Immune Signaling in Nuclear Reprogramming

Shu Meng, Palas Chanda, and John P. Cooke

Abstract In 2012 Shinya Yamanaka received the Nobel Prize for his discovery of four transcriptional factors that could induce pluripotency when overexpressed in somatic cells. Recently our lab discovered that innate immune signaling is also critical for this process (Lee et al., Cell 151:547–558, 2012). Specifically, we found that activation of the TLR3-NFκB pathway is required for efficient reprogramming by modulating the expression of epigenetic modifiers to favor an open chromatin configuration. Our unpublished data also suggest that activation of other pattern recognition receptors such as TLR4 or RIG-1 may facilitate reprogramming. Transdifferentiation of one somatic cell to another lineage is another form of nuclear reprogramming. We have shown that transdifferentiation of human fibroblasts to endothelial cells, another form of nuclear reprogramming, also requires innate immune signaling (Sayed et al., Circulation 131:300–309, 2015). Thus innate immune signaling plays a key role in nuclear reprogramming by regulating epigenetic plasticity (Fig. 9.1).

Keywords Immune signaling • Transcriptional factor • Nuclear reprogramming • Chromatin configuration • Transdifferentiation • Epigenetic plasticity • Induced pluripotent stem cells • Fibroblast-derived induced endothelial cells

9.1 Introduction

Nuclear reprogramming describes a global change in the epigenetic landscape and transcriptional profile of one cell type so that it resembles that of another cell type (Gurdon and Melton 2008). Nuclear reprogramming to generate induced pluripotent stem cells (iPSCs) is a breakthrough discovery that has many applications in

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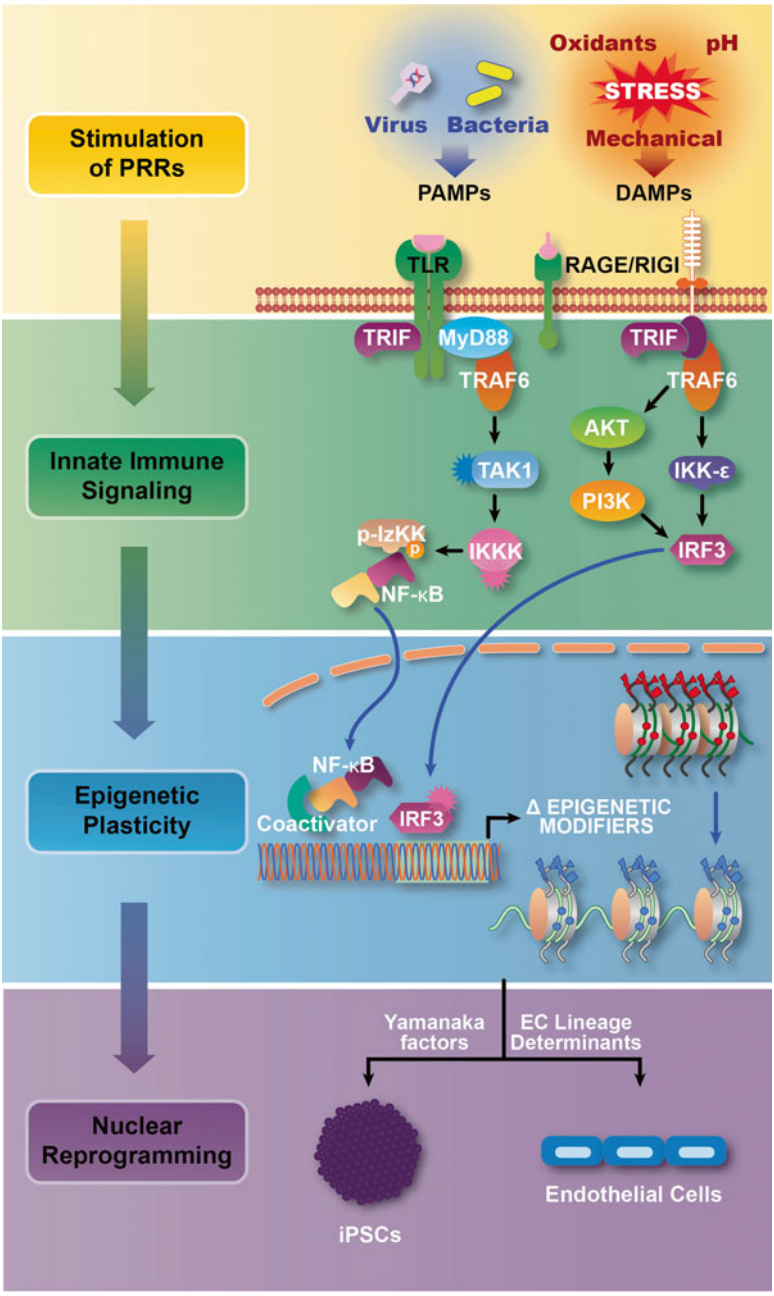

Fig. 9.1 Role of innate immune signaling in nuclear reprogramming: Pattern-recognition receptors (PRRs) on mammalian cells are stimulated by various pathogen-associated molecular patterns (PAMPs) displayed by bacteria/viruses or damage-associated molecular patterns (DAMPs) released from stressed cells. These stimuli activate innate immune signaling to activate transcriptional factors like NFκB or IRF3 leading to changes in the expression of epigenetic modifiers.

biotechnology and medicine. Because the iPSCs are capable of differentiating into cells derived from the 3 major germ layers (endoderm, ectoderm and mesoderm), the iPSCs may be useful for generating somatic cells for understanding the pathobiology of a disease; for generating high throughput cellular assays for screening of molecules that therapeutically modulate the pathobiology; or for use in cell therapy or tissue regeneration.

Nuclear reprogramming also occurs during the process of transdifferentiation, when one somatic cell undergoes a metamorphosis to a somatic cell of another lineage. The process of transdifferentiation may occur in pathobiological states, such as the transformation of squamous esophageal epithelial cells to a cell type that resembles the columnar epithelium of the intestine. This so-called Barrett's esophagus occurs in the setting of chronic inflammation induced by gastroesophageal reflux, and is a pre-malignant change (Stairs et al. 2008). However, the process of transdifferentiation may also be wielded with therapeutic intent. We have recently found that transdifferentiation of human fibroblasts to endothelial cells also requires activation of innate immunity. Therapeutic modulation of this process may be useful in diseases characterized by loss of the microvasculature associated with fibrosis.

9.2 Nuclear Reprogramming: Applications to Regenerative Medicine

9.2.1 The Transformative Effect of iPSCs in Regenerative Medicine

Nuclear reprogramming of a somatic to a pluripotent cell was first induced by somatic cell nuclear transfer, in which the nucleus of a somatic cell is placed into an enucleated egg cell (Gurdon and Melton 2008). But it was the induction of pluripotency by ectopic gene expression of a defined set of transcriptional factors that was transformative for the field of regenerative medicine (Takahashi and Yamanaka 2006). Yamanaka and colleagues revealed that the overexpression of four transcriptional factors (Oct4, Sox2, Klf4 and c-Myc) could induce pluripotency. These iPSCs are highly similar to embryonic stem cells (ESC), maintaining a capacity for indefinite self-renewal, as well as the ability to be differentiated to all three germ layers

Fig. 9.1 (continued) Consequently, a state of epigenetic plasticity is established where chromatin is in an open-state configuration. In this state of epigenetic plasticity, cellular gene expression is responsive to transcriptional factors that favor pluripotency (eg. Oct4, Sox2, Klf4 and c-Myc), leading to the generation of induced pluripotent stem cells. Alternatively, in this state of epigenetic plasticity, cells may transdifferentiate to another cell type in the presence of specific cell lineage determinants. The activation of epigenetic plasticity by inflammatory signaling is a process that we term "transflammation". Transflammation is required for cellular response to the challenge of pathogens or tissue damage

and theoretically any somatic cell type. Thus human iPSCs can be used as a surrogate for human ESCs which avoids the ethical issues surrounding the latter cells. The iPSCs can be generated and manipulated with relative ease, facilitating studies of pluripotency and differentiation.

Patient-specific iPSCs may be generated from somatic cells that are easily obtained (eg. skin fibroblasts), and then differentiated into cells that are less easily obtained (eg. neurons) so as to understand the pathobiology of disease (eg. a neurological disease that is not well characterized). To the extent that the patient's disorder has a genetic basis, the generation of patient-specific cells is likely to replicate the genetic substrate for the pathobiology. Once the pathobiology is understood, such iPSC-derived cells may be used to develop cellular assays for high throughput screening for therapeutic molecules (Chamberlain et al. 2010; Volz et al. 2012; Ebert et al. 2009; Hiura et al. 2013; Israel et al. 2012; Martins-Taylor et al. 2014; Nguyen et al. 2011; Nishino et al. 2011; Reinhardt et al. 2013; Yang et al. 2010).

Patient-specific iPSC-derived cells may have application in regenerative medicine. One could potentially use such cells to replace or regenerate tissues or organs to restore or reestablish normal cell function (Eguizabal et al. 2013). The iPSCs have indefinite replicative capacity, and thus sufficient iPSC-derived cells could be generated for human therapy. These autologous cells would not confront the immune barrier that would need to be surmounted by human ESC-derived cells. However, because of the logistical issues, iPSC-derived cells for therapy are more likely to be derived from banks of allogeneic iPSCs that are chosen (or engineered) to express the major histocompatibility (MHC) antigens most frequently represented in the population (Kaneko and Yamanaka 2013; Okita et al. 2011). Patients requiring a cell therapy would undergo testing to identify their MHC profile, and receive an iPSC-derived cell therapy using matched cells. Such therapy might be administered as the cells alone; cells bioengineered into a matrix or device; or a cell-derived product.

9.2.2 Nuclear Reprogramming and Transdifferentiation

The ability to generate iPSCs from somatic cells highlighted the fact that somatic cell phenotype is fluid and cell fate can be manipulated by the overexpression of exogenous transcription factors. So-called "direct reprogramming" or transdifferentiation is manifested by a change in the epigenetic and transcriptional profile of a somatic cell nucleus as it undergoes a metamorphosis toward a different lineage (Yamanaka and Blau 2010). This form of nuclear reprogramming was first accomplished by cell fusion. For example, fusion of a liver and muscle cell caused the nucleus of the liver cell to express muscle proteins (Blau et al. 1985). The overexpression of lineage determination genes may have the same effect. For example, the overexpression of MyoD in the liver cells recapitulates many of the same effects as cell fusion with a skeletal muscle cell (Yamanaka and Blau 2010). Transdifferentiation via the overexpression of specific lineage determination factors has now been

broadly applied to induce many cell lineages, eg. fibroblasts have been transdifferentiated into endothelial cells (Li et al. 2013), cardiac muscle cells (Nam et al. 2013), and neurons (Son et al. 2011).

In some applications, transdifferentiation might be a simpler approach to generating therapeutic cells. The direct reprogramming avoids the steps required for induction of pluripotency, the differentiation of the iPSCs, and the selection of therapeutic cells from a heterogeneous mixture. Also, iPSCs must be generated ex vivo and differentiated to the therapeutic cell type prior to administration (to avoid the adverse event of teratoma formation). The administration of the iPSC-derived cells is also problematic. Among the hurdles to iPSC-derived cell therapy is how to deliver the cells where they are needed, in the number that is necessary (too many cells could invite ischemia-induced apoptosis), and in a fashion that recreates the normal tissue architecture. It seems far better to infuse small molecules into the tissue or organ that may promote transdifferentiation of resident cells (eg. fibroblasts) to a therapeutic cell type (eg. endothelial cells). For example, in the setting of a myocardial infarction, some of the resident fibroblasts that proliferate and migrate to the site of injury could be induced to form endothelial cells (inducible ECs, iECs) by administration of a small molecule cocktail for therapeutic transdifferentiation. These iECs would be expected to self-assemble into capillaries that would provide the nutrition and the niche for true cardiac repair rather than scar formation.

However, until recently transdifferentiation has largely been accomplished using viral vectors that encode lineage determination factors including transcriptional factors and small RNAs. A more tractable approach for clinical use would be a small molecule strategy that could avoid the concerns of using viral vectors. Our recent discovery that innate immune signaling promotes epigenetic plasticity has provided a new approach for nuclear reprogramming that can be modulated by small molecule (Sayed et al. 2015). Thus an understanding of innate immune signaling is necessary for regenerative medicine applications.

9.3 Innate Immunity Signaling and Regeneration

Innate immune response is the first line defense of the host to invading pathogens. Pathogens are recognized by cells because they display pathogen-associated molecular patterns (PAMPs) (Akira et al. 2001; Janeway and Medzhitov 2002) which trigger an inflammatory response on mammalian cells (Medzhitov and Janeway 2000; Mogensen 2009). PAMPs are a group of highly conserved structures present in large groups of microorganisms (Medzhitov and Janeway 2000).

Pattern-recognition receptors (PRRs) on mammalian cells bind to these PAMPs to initiate innate immune signaling. The PRRs are comprised mainly of Toll-like receptors (TLRs) (Akira et al. 2006; Medzhitov and Janeway 1997; Medzhitov et al. 1997) as well as cytosolic PRRs which include the retinoid acid-inducible gene I (RIG-1)-like receptors (RLRs) (Yoneyama et al. 2004) and the nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (Kanneganti et al. 2007).

TLRs recognize microbes on the cell surface and in endosomes, whereas RLRs and NLRs detect microbial components in the cytosol. In addition, endogenous components derived from dying host cells may display damage-associated molecular patterns (DAMPs) that can also bind to and activate PRRs (Desmet and Ishii 2012).

The toll receptors were first described in Drosophila as sensors of pathogens (Lemaitre et al. 1997), and homologues (the TLRs) were subsequently identified in mammals. So far 13 TLR members are known. These receptors may be classified into two large groups: those that are expressed on the cell surface [TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10] and those that are located in intracellular compartments such as the endosome [TLR3, TLR7, TLR8, TLR9, TLR11, TLR12, and TLR13] (Kawai and Akira 2010). Cell surface TLRs generally recognize microbial membrane components such as lipids and lipoproteins. For example, TLR4 recognizes bacterial lipopolysaccharide (LPS). By contrast, the intracellular TLRs recognize pathogenic RNA or DNA. For example, TLR3 recognizes viral double-stranded RNA (Alexopoulou et al. 2001). TLR7 and TLR8 recognize ssRNA (Mancuso et al. 2009; Guiducci et al. 2013). TLR9 recognizes unmethylated CpG-DNA (Coban et al. 2010).

TLRs are type I integral membrane glycoproteins. They are comprised of an exodomain containing leucine-rich-repeat motifs, a membrane-spanning segment and a signaling domain homologous to that of the IL1 receptor (TIR domain). The activation of TLRs by their PAMP ligand triggers monomers of the TLRs to form active homodimers or heterodimers. Subsequently, adaptor proteins are recruited, triggering the downstream signaling cascades. Several adaptor proteins have been shown to bind to the TIR domain so as to mediate the downstream signaling. These include myeloid differentiation primary response gene 88 (MyD88) and TIR-domain- containing adapter-inducing interferon-β (TRIF) (Kato et al. 2006; Kawai and Akira 2006). MyD88 is the adaptor for all TLRs except for TLR3, which signals through TRIF. Assembly and localization of receptor signaling complexes (Gay et al. 2014) MyD88 and TRIF activate proinflammatory signaling pathways including NFκB, AP1, ERK and p38, and anti-viral signaling pathways through IRFs.

TLR3 is responsible for viral recognition (Alexopoulou et al. 2001) and signals through the TRIF adaptor following recognition of double stranded RNA (dsRNA). This TLR3–TRIF pathway can also be stimulated by a synthetic analog of dsRNA, polyinosinic-polycytidylic acid (Poly I:C) (Gitlin et al. 2006; Kato et al. 2006). NOD1 and NOD2 binds to cytosolic peptidoglycan fragments meso-DAP and muramyl dipeptide, respectively. A different set of NLRs induces caspase-1 activation through inflammasomes (Kanneganti et al. 2007).

These pathways ultimately induce the generation and release of inflammatory cytokines and chemokines that represent the first volley in the inflammatory response to a pathogen. These pathways represent the initial inflammatory response and have been well characterized. Recently we have discovered another process that is sparked by innate immune signaling and which may be important for the response to a pathogen or damage. This process, which we call ‘transflammation’, results in an epigenetic plasticity that facilitates the phenotypic fluidity needed to respond to a cellular challenge.

9.4 Innate Immune Signaling and Nuclear Reprogramming to Pluripotency

One of the favorite aphorisms in our Center is "There is no such thing as a failed experiment, so long as the work was carried out well". If the experiment was performed meticulously and thoughtfully, but the results are not what you expected (or hoped for), you have learned something valuable. Perhaps you should use an alternate approach, different reagents or different conditions. Or perhaps your hypothesis is wrong, and you need to revise it. Or perhaps it is time to go in a different direction. Or maybe, if you are insightful enough to appreciate your luck, you have stumbled upon something wonderful. This serendipity has occurred a few times in our group because we value rigorous work, negative data, honesty and humility, just as much as we value the validation of a hypothesis.

This philosophy led us to the surprising observation that the Yamanaka approach to generate iPSCs requires more than the master regulators (Oct4, Sox2, Klf4 and c-Myc), but also relies heavily upon activation of innate immunity (Lee et al. 2012). Our discovery began with an attempt to develop a non-integrating approach to generating iPSCs, shortly after Yamanaka published his papers showing the feasibility of generating human iPSCs. We are a translational group interested to generate fundamental insights that transform cardiovascular care. Accordingly we intended to generate human iPSCs without using a viral vector that would complicate the regulatory roadmap to iPSC-derived cell therapy. We embarked upon the generation of cell permeant peptides of the Yamanaka factors, using the technologies of Paul Wender and James Swartz at Stanford University. Each of the Yamanaka factors was generated as a fusion peptide, containing three domains: the Yamanaka factor; a short bridging sequence; and a highly basic cell penetrating peptide. We documented that the fusion proteins could bind to their consensus sequence, and could enter cells and localize to the nucleus. We also showed that they could rescue iPSCs where one of the Yamanaka factors was knocked down by siRNA technique. We then used different doses and timing of administration of the fusion peptides encoding the Yamanaka factors. However, despite many attempts, we initially failed to generate iPSCs using cell permeant peptides.

Jieun Lee in the lab persisted to understand what might be the cause of this failure. She examined the expression of the core pluripotency genes downstream of the Yamanaka factors. She found that within 24 h of exposure to a retroviral vector encoding Sox2, downstream genes such as Nanog were dramatically upregulated. By contrast, with the fusion peptide Sox2 there was no activation of the downstream genes in the first 24 h. A similar pattern was observed with retroviral Oct4 and fusion peptide Oct4. It then occurred to one of us (JPC) that perhaps the retroviral vector itself had a role in the process. Years before, working with Ed Mocarski at Stanford, we had found that even empty viral vectors could have a dramatic effect on the phenotype and function of endothelial cells. Thus, it seemed possible that the retroviral vector (carrying the Yamanaka factors) could somehow play a role in nuclear reprogramming.

On the basis of this insight, we decided to assess the effect of an irrelevant retroviral vector (encoding GFP) on the reprogramming process. When retroviral GFP was combined with the fusion peptide Yamanaka factors, everything worked! The combination of the cell-permeant peptides encoding the Yamanaka factors, together with the irrelevant viral vector, induced the expression of the core pluripotency network.

Subsequent studies revealed that the TLR3 receptor was involved in this process. When we knocked down elements of the TLR3 signaling pathway, reprogramming to pluripotency using the Yamanaka approach was dramatically suppressed (in unpublished studies, we have found that RIG-1 is also involved. Both of these receptors sense viral double-stranded RNA). We documented the role of innate immune activation using other reprogramming approaches as well. We showed that reprogramming using mmRNA encoding the Yamanaka factors was also dependent upon activation of innate immunity. We also examined the modulation of innate immune activation on reprogramming using murine embryonic fibroblasts carrying a doxycycline-inducible cassette encoding the Yamanaka factors. In this system, addition of the TLR3 agonist polyI:C enhanced, whereas the NFκB decoy oligonucleotide suppressed, reprogramming to pluripotency.

Subsequent studies revealed that activation of innate immune signaling caused global changes in epigenetic modifiers that favored an open chromatin configuration. We observed dramatic downregulation of epigenetic modifiers known to enforce suppressive epigenetic markers, such as Dot1L and members of the histone deacetylase family (HDAC 1, 5, 8, 9 and 10). We also observed upregulation of epigenetic modifiers known to enhance gene activation, including members of the histone acetyltransferase family. These effects of TLR3 activation were associated with increased H3K4 trimethylation and decreased H3K27 trimethylation at the Oct4 and Sox2 promoters.

9.5 Innate Immune Signaling and Transdifferentiation

A number of groups have forced transdifferentiation of one cell type into another cell lineage by using viral vectors to overexpress lineage determination factors. Is it possible that the viral vectors themselves (by activating innate immunity) might play a role in this forced transdifferentiation? Our discovery of the role of innate immune signaling in nuclear reprogramming led us to ask if this process may be involved in transdifferentiation. Accordingly, we posed this hypothesis: Human fibroblasts may be transdifferentiated into endothelial cells simply by activating innate immunity (to increase epigenetic plasticity) together with external cues (eg. vascular endothelial growth factor and other endothelial growth factors) to generate endothelial cells.

Recently, we have published our confirmation of the hypothesis (Sayed et al. 2015). Priming cells with polyI:C, together with endothelial growth factors, could directly convert a small percentage of the treated fibroblasts into endothelial cells.

These fibroblast-derived induced endothelial cells (iECs) expressed all of the expected immunohistochemical markers; manifested endothelial functions not present in fibroblasts (eg. generation of nitric oxide, formation of tubular networks in matrigel; uptake of acetylated LDL); and had a transcriptional profile by RNA seq that was highly similar to genuine endothelial cells. Expected histone modifications were observed in the iECs (such as increased H3K4me3 and decreased H3K27me3 in endothelial specific gene promoters such as CD31).

This transdifferentiation was absolutely dependent upon innate immune activation, as in the absence of polyI:C priming, the endothelial growth factors had no effect. Furthermore, when we reduced innate immune activation by knocking down TLR3, these fibroblasts had reduced transdifferentiation efficiency. More notably, iECs derived from these KD fibroblasts had markedly defective endothelial cell function. The RNA sequencing of these KD iECs revealed that they were incompletely reprogrammed. Our unpublished data reveal that NFκB inhibitors also block the transdifferentiation. The data in aggregate confirmed that innate immune signaling can be modulated to enhance transdifferentiation of human somatic cells.

9.6 What Is the Role of Innate Immune Signaling in Regenerative Processes?

Lower vertebrates are well known for their ability to regenerate. Urodele amphibians can regenerate limbs, tails and other body parts after injury (Brockes and Kumar 2005). Zebrafish can regenerate the apex of the heart after its resection (Zhang et al. 2013). By contrast, mammals have limited ability to regenerate certain tissues. Adult humans can partially regenerate liver, skeletal muscle and the peripheral nervous system, but have very limited regenerative capacity in some organs, such as the heart (Stoick-Cooper et al. 2007). Furthermore, any regenerative capacity of humans decreases substantially with age.

Based on our studies of reprogramming and transdifferentiation, we suspect that activation of innate immunity plays a significant role in regeneration and repair *in vivo*. Such regeneration and repair must involve a facilitation of epigenetic plasticity. To respond effectively to an injury, cells in the vicinity of the damage must change their basal phenotype from a quiescent to an activated state. Resident cells of all lineages must proliferate, migrate and integrate into the newly forming tissue. Indeed, in the salamander, the blastema that forms after a limb amputation consists of skin, muscle, nerve and other tissue cells that have “de-differentiated”, and have substantially increased their rate of proliferation and migration (Stoick-Cooper et al. 2007). As the limb bud grows, the cells that are behind the proliferating front begin to differentiate back into their original lineage, presumably guided by an epigenetic memory of their previous state. This process must necessarily involve an epigenetic plasticity that facilitates the dynamic changes in the transcriptional profile that facilitates the metamorphosis.

It has been shown that inhibition of inflammation can substantially disrupt the repair process in many organ systems in the early stage of injury such as in ischemia/reperfusion injury (Boros and Bromberg 2006), lung, kidney, muscle (Tidball and Villalta 2010), intestine (Scheeren et al. 2014) and liver injury (Sun and Gao 2004). Conversely, acute inflammation initiates the regenerative response in the adult zebrafish brain (Kyritsis et al. 2012). Furthermore, muscle cell proliferation is enhanced by activation of the innate immune effector NFκB.

Tlr3 knockout mice manifest a delayed skin wound closure as characterized by impaired re-epithelialization, granulation formation, and neovascularization together with decreased proinflammatory cytokine expression and less recruitment of myeloid cells (Lin et al. 2011). These findings suggest that TLR3 and its downstream signaling facilitates early wound healing in tissue repair.

TLR4 is also required for wound healing. Tlr4 deficient mice have delayed wound healing, reduced neutrophil infiltration and reduced chemokine levels (Chen et al. 2013). The Tlr4 deficient mice also exhibit an impaired response to cardiovascular stress. Specifically, a reduced cardiac hypertrophy is observed following pressure overload (due to aortic banding) compared with WT mice (Ha et al. 2005). This phenotype is also associated with a decreased NFκB binding activity.

Myd88 KO mice also exhibit impaired wound healing (Stoick-Cooper et al. 2007). The impairment in repair and regeneration extends to other organs. For example, Myd88(−/−) and Tlr4(−/−)Tlr2(−/−) mice have impaired lung repair after injury (Jiang et al. 2005). Myd88 knockout mice also demonstrate an attenuation of liver regeneration after hepatectomy (Seki et al. 2005) in association with reduced levels of proinflammatory cytokines such as IL6 and TNFα. Consistent with this observation, there is defective hepatocyte regeneration in IL6 deficient mice (Cressman et al. 1996). By contrast, knockout of a single TLR such as TLR2, TLR4 or TLR9 did not cause a defect in liver regeneration (Campbell et al. 2006).

Deletion of MyD88 or TLR2 in the intestinal epithelium markedly reduces dextran sodium sulphate (DSS)-induced colitis regeneration. Limiting dilution transplantations of breast epithelial cells devoid of TLR2 or MYD88 manifest a reduction in the number of mammary repopulating units compared with the control (Scheeren et al. 2014).

To conclude, many of the components of innate immune signaling are required for tissue regeneration in mouse models (Table 9.1). That being said, there are some contradictory data from the literature. For example, deficiency of INFγ (a downstream effector of innate immunity) enhances skin wound healing. This observation suggests a negative role for IFNγ in skin regeneration (Ishida et al. 2004). IFNγ and the class II transactivator (CIITA) also inhibit muscle development (Londhe and Davie 2013). Similarly, Tlr3−/− mice manifest early liver regeneration (Zorde-Khvalevsky et al. 2009) whereas injection of the TLR3 agonist polyI:C inhibits liver regeneration (Sun and Gao 2004). Furthermore, LPS injection suppresses liver regeneration (Akita et al. 2002), suggesting that hyper activation of TLR may inhibit the regeneration process. On the other hand, TNFα deficient mice did not have impaired liver regeneration (Hayashi et al. 2005).

Table 9.1 Regenerative phenotype of mice deficient of key innate immune signaling molecules

Mice	Regenerative phenotype
Tlr3–/–	Delayed skin wound healing (Lin et al. 2011)
	Promoted liver regeneration (Zorde-Khvalevsky et al. 2009)
Tlr4–/–	Reduced cardiac hypertrophy following pressure overload (Ha et al. 2005)
	Delayed skin wound healing (Chen et al. 2013)
MyD88–/–	Delayed skin wound healing (Stoick-Cooper et al. 2007)
	Impaired lung repair after acute lung injury (Jiang et al. 2005)
	Delayed liver regeneration (Seki et al. 2005)
	Delayed intestine regeneration (Scheeren et al. 2014)
Tlr4–/– Tlr2–/–	Impaired lung repair after acute lung injury (Jiang et al. 2005)
Tlr2–/–	Delayed intestine regeneration (Scheeren et al. 2014)
INFγ–/–	Promoted skin wound healing (Ishida et al. 2004)
IL6–/–	Defective liver regeneration (Cressman et al. 1996)

9.7 The Goldilocks Zone for Innate Immunity in Nuclear Reprogramming

These apparently contradictory data for the role of innate immune activation in regenerative processes *in vivo* might be explained by our discovery of a 'Goldilock's zone' for innate immune activation in nuclear reprogramming. At levels of innate immune activation that are less or greater than within the 'zone', reprogramming is minimal or absent. We discovered the zone when we examined the dose-dependency of TLR3 stimulation on generation of iPSCs using murine embryonic fibroblasts containing a doxycycline-inducible cassette encoding the Yamanaka factors. In this model, when doxycycline is added to the cells, iPSC colonies are observed within 3 weeks. We transfected the dox-inducible MEFs with an NFκB promoter luciferase construct to assess the level of innate immune activation. At baseline, the MEFs manifested a modest activation of NFκB, and colony formation after 3 weeks (unpublished data). The addition of polyI:C from 3 to 100 ng/ml, increased NFκB activity as well as colony generation. At higher doses of polyI:C, we saw a further increase in NFκB activity, but a reduction in colony formation. These studies revealed an optimal activation of innate immunity to increase iPSC generation.

In parallel, we examined the effect of an oligonucleotide decoy on iPSC generation in this model. The decoy scavenges NFκB, and its addition was associated with a decline in NFκB activity from baseline. The decoy also caused a dose-dependent decline in colony generation. Indeed, colony generation could be virtually suppressed when the dose of decoy was sufficient to abrogate NFκB signaling.

9.8 Perspectives and Significance

Our studies confirm that innate immune activation is necessary for reprogramming. Furthermore, the studies reveal an optimal zone of innate immune activation for efficient reprogramming. We propose that there is a similar zone *in vivo* (Fig. 9.2). For example, the pre-clinical studies suggest that some amount of innate immune activation is necessary for reprogramming. However, with excessive activation of innate immune signaling, repair and regeneration may be attenuated. There are clinical correlates of our pre-clinical data. For example, excessive activation of innate immune signaling (such as that which occurs in the "fixed inflammation" circumscribing a diabetic foot ulcer) may impair wound healing. On the other hand, insufficient activation of inflammatory cascades (as in patients treated with high dose corticosteroids) might be responsible for the impaired wound healing observed in these patients. We anticipate that a more comprehensive elucidation of the molecular determinants of this Goldilocks zone will provide insights and potential therapeutic targets to improve regeneration.

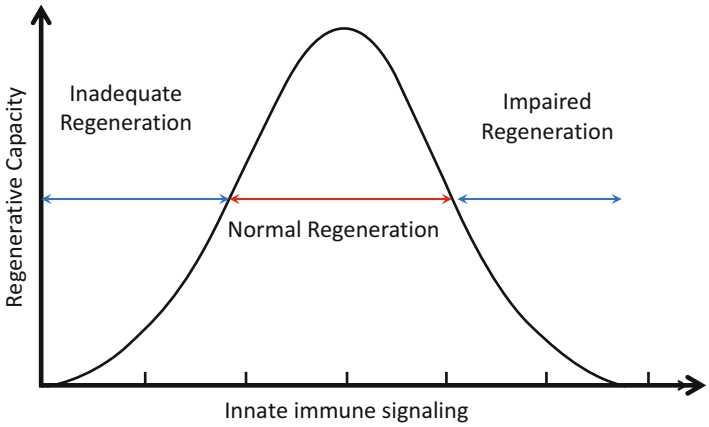

Fig. 9.2 Innate immune signaling, nuclear reprogramming, and regeneration: Our experimental data reveal that nuclear reprogramming of somatic cells to pluripotent cells requires innate immune activation. Furthermore, transdifferentiation of one somatic cell to another also requires activation of innate immune signaling. Furthermore, we find that innate immune signaling causes global changes in epigenetic modifiers that increase epigenetic plasticity, so as to facilitate phenotypic fluidity. We hypothesize that these mechanisms underlying epigenetic plasticity and phenotypic fluidity are required for regeneration and repair in vivo. Notably, our unpublished data indicate that the reprogramming response to innate immune activation is parabolic, in that there is an optimal level of activation. Too little activation leads to inadequate induction of nuclear reprogramming, whereas too much activation may activate processes that oppose reprogramming. We hypothesize that this is a general phenomenon for cellular response and regeneration. That is, there is an optimal activation range of innate immune signaling required *in vivo* for cellular plasticity and regeneration

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Chapter 10
Cardiac Regeneration in Zebrafish

Chi-Chung Wu and Gilbert Weidinger

Abstract Heart failure caused by cardiomyocyte loss after ischemic tissue damage is a leading cause of death worldwide, since adult mammals cannot regenerate heart injuries. While some new cardiomyocytes are produced in adult mammals during normal ageing and after infarction, this occurs at insufficient rates for effective cardiac regeneration. Zebrafish, on the contrary, are able to regenerate multiple organs including the heart. Injuries induce complex cellular and molecular responses in endocardium, epicardium and myocardium, which robustly regenerate in a coordinated manner, resulting in full morphological and functional recovery. In particular, differentiated cardiomyocytes re-enter the cell cycle and proliferate to regenerate the myocardium. Thus, the zebrafish has emerged as an important model to study mechanisms of naturally occurring cardiac regeneration. Here, we describe zebrafish heart injury techniques and review current data on mammalian cardiomyocyte turnover and production in response to injury as well as our current knowledge of the cellular and molecular mechanisms of zebrafish heart regeneration.

Keywords Zebrafish • Heart regeneration • Cardiomyocytes • Cell proliferation • Epicardium • Endocardium

10.1 Introduction

10.1.1 Cardiomyocytes Are Insufficiently Replaced After Injury of the Adult Mammalian Heart

Myocardial infarction (MI) is a leading cause of death worldwide. Compared to other organs in mammals (e.g. lung, liver, bone, skin), the adult heart displays very little regenerative capacity in response to injury. In particular, lost adult mammalian cardiomyocytes (CMs) are largely not replaced after MI or other disorders resulting

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in CM death, leading to muscle loss, irreversible scarring and eventually heart failure (Laflamme and Murry 2011). In contrast, adult zebrafish can completely regenerate their heart via CM replacement, resulting in scar-free recovery (Poss et al. 2002; Schnabel et al. 2011; Gonzalez-Rosa et al. 2011; Chablais et al. 2011; Wang et al. 2011). Intriguingly, neonatal mice can regenerate the myocardium after partial surgical resection as well, but this ability is lost by 7 days after birth (Porrello et al. 2011). While complete functional heart regeneration requires not only restoration of lost muscle tissue, but also coordinated replacement of the other tissues of the heart, in particular of endocardium, epicardium and coronary vasculature, a central question in heart regeneration research has been how CMs can be restored. Genetic lineage-tracing experiments have indicated that during neonatal mouse and adult zebrafish heart regeneration CMs are replaced via proliferation of pre-existing CMs, and not from stem or progenitor cells (Kikuchi et al. 2010; Jopling et al. 2010; Porrello et al. 2011). In adult mammals, CMs have long been believed to be terminally differentiated and thus unable to proliferate (Zebrowski and Engel 2013). Instead, much of the growth of the postnatal mammalian heart occurs via enlargement of pre-existing CMs, i.e. hypertrophy (Li et al. 1996; Yoshizumi et al. 1995). Thus, one explanation for the differential ability of the adult zebrafish and mammalian heart to regenerate would be the idea that CMs retain the ability to re-enter the cell cycle and proliferate in the zebrafish, while such capacity is lost during ontogeny in mammals.

However, there is now good evidence that new CMs do form during adult mammalian life, including humans. Taking advantage of the integration of atmospheric ^{14}C, which was generated by nuclear bomb tests during the Cold War, into cells born during that time, Frisén and colleagues estimated the age of CMs in humans. They showed that human CMs are being renewed during adult life, but at a very low rate of 1 % per year at the age of 25 and 0.45 % at the age of 75 (Bergmann et al. 2009). Thus, approximately 45 % of all CMs are exchanged during a human lifetime while 55 % remain from neonatal growth (Bergmann et al. 2009).

While it is not possible to identify the cellular source of newly forming CMs in the adult human heart, several elegant studies in mice have addressed whether CM renewal during aging and the low-rate CM replacement after cardiac injury are due to proliferation of existing CMs or due to CM differentiation from progenitor cells (Ali et al. 2014; Ellison et al. 2013; Hsieh et al. 2007; Malliaras et al. 2013; Senyo et al. 2013, 2014; van Berlo et al. 2014). Unfortunately, the conclusions drawn by several studies based on genetic lineage-tracing of CMs or cardiac progenitor cells differ considerably. While most reports using different means to track the fate of differentiated CMs found that renewal of CMs during homeostasis is due to proliferation of existing CMs (Senyo et al. 2014), one study concluded that progenitor cells contribute to CM formation after cardiac injury, since the progeny of differentiated CMs got diluted with other cells in injured hearts (Hsieh et al. 2007). In contrast, other studies using lineage-tracing of differentiated CMs and multi-isotope imaging mass spectrometry concluded that the limited CM replacement after injury is due to CM proliferation (Senyo et al. 2013; Ali et al. 2014). However, c-kit positive cardiac progenitor cells have been reported to contribute significantly to CM

production after cardiac injury as well (Ellison et al. 2013). In contrast, another study found that c-kit positive cells form negligible numbers of CMs both during homeostasis and after injury (van Berlo et al. 2014).

Thus, while further studies are needed to clarify how CMs are formed in the adult mammalian heart, there is a growing consensus that the rate of CM formation under homeostatic conditions and in response to heart injury is low, too low for effective myocardial regeneration after heart injury (Senyo et al. 2014).

10.1.2 Mammalian Cardiomyocyte Proliferation Can Be Experimentally Induced

Despite the uncertainties about the cellular basis of CM turnover in adult mammals, the above mentioned studies suggest that adult mammalian CMs are able to proliferate and could potentially be augmented therapeutically. In support of this idea, injection of the growth factor Neuregulin 1 (Nrg1) has been shown to induce proliferation of CMs in both healthy and injured adult mouse hearts, to reduce scar size and to improve functional recovery after MI (Bersell et al. 2009). Likewise, induction of constitutively active ERBB2, a tyrosine kinase receptor of Nrg1, in neonatal, juvenile or adult mouse CMs was found to lead to cardiomegaly caused by CM hypertrophy and proliferation, while ERBB2 induction after MI improved functional recovery (D'Uva et al. 2015).

Interestingly, several studies indicated that proliferation might be actively inhibited in adult mammalian CMs. First, overexpression of several miRNAs was found to stimulate both DNA synthesis and cytokinesis in neonatal mouse and rat CMs in culture and in adult animals (Eulalio et al. 2012). Second, deletion of components of the Hippo signaling pathway, which controls organ size and suppresses cell proliferation, has been shown to induce adult CM renewal via cell cycle re-entry and mitosis (Heallen et al. 2013). In addition, removal of Hippo components in P7 mice, a time at which natural heart regeneration is compromised (Porrello et al. 2011), followed by apical resection at P8 resulted in significant muscle regeneration from spared CMs and improved functional recovery (Heallen et al. 2013). Together, these studies suggest the potential to augment cardiac regeneration by stimulating adult mammalian CM proliferation *in vivo* through supplying pro-proliferation factors and/or removing natural inhibitors.

10.2 Zebrafish Heart Regeneration – Injury Models

A promising way to uncover factors that are able to stimulate CM proliferation after heart injury would be to elucidate the molecular basis underlying naturally occurring adult heart regeneration in non-mammalian vertebrates. Cardiac regeneration

after injury has predominantly been studied in zebrafish, but also in salamanders (Oberpriller and Oberpriller 1974; Piatkowski et al. 2013). Interestingly however, it has been reported that heart regeneration does not occur in medaka, a teleost fish model organism that otherwise shares many features with zebrafish (Ito et al. 2014). Thus, it is unclear whether heart regeneration is a common feature amongst teleost fish and other lower vertebrates, and more data will be needed to address the intriguing question of how heart regeneration evolved or whether cardiac regenerative capacity was lost during evolution in certain lineages.

Since the discovery of heart regeneration in zebrafish, this model has developed rapidly over the past years, owing to its robust regenerative capacity and its propensity for genetic manipulation (Gemberling et al. 2013). In this chapter, we summarize the current knowledge about the cellular basis and molecular regulation of zebrafish heart regeneration.

10.2.1 Ventricular Resection

Zebrafish hearts consist of one atrium, one ventricle and a prominent non-muscular extension of the aorta, termed the bulbus arteriosus. The ability of zebrafish to regenerate the heart was first reported by Keating and colleagues (Poss et al. 2002). After surgical removal of around 20 % of the apex of the ventricle, the injury was found to be rapidly sealed by a fibrin clot. This fibrinous wound tissue increased in size till 7–9 days post injury (dpi). Evidence for CM cell cycle activity was obtained, and a contiguous wall of muscle was restored by 30 dpi (see Sect. 10.3 below). By 60 dpi, the fibrin clot disappeared and the size and shape of the ventricle appeared to be normal, with little or no collagen deposition, indicating that, in contrast to adult mammals, permanent scarring does not occur (Fig. 10.1a) (Poss et al. 2002). To date, ventricular resection remains a frequently used injury model, but several studies have developed additional ways to damage the heart, which better mimic some aspects of mammalian cardiac injury.

10.2.2 Cryoinjury

Acute loss of myocardial tissue after MI is typically caused by ischemia and subsequent CM death throughout the affected area. After clearance of dead cells and matrix debris from the infarct zone, damaged tissue is replaced by scar tissue characterized by collagen deposition. While zebrafish effectively regenerate the resected heart, the correlation between ventricular resection in zebrafish and MI in mammals is limited. For instance, ventricular resection does not cause massive cell death and thus no cell debris removal before muscle replacement is required. In addition, scar tissue formed after resection mainly comprises fibrin with little or no collagen deposition. Therefore, the value of ventricular resection as a translationally relevant

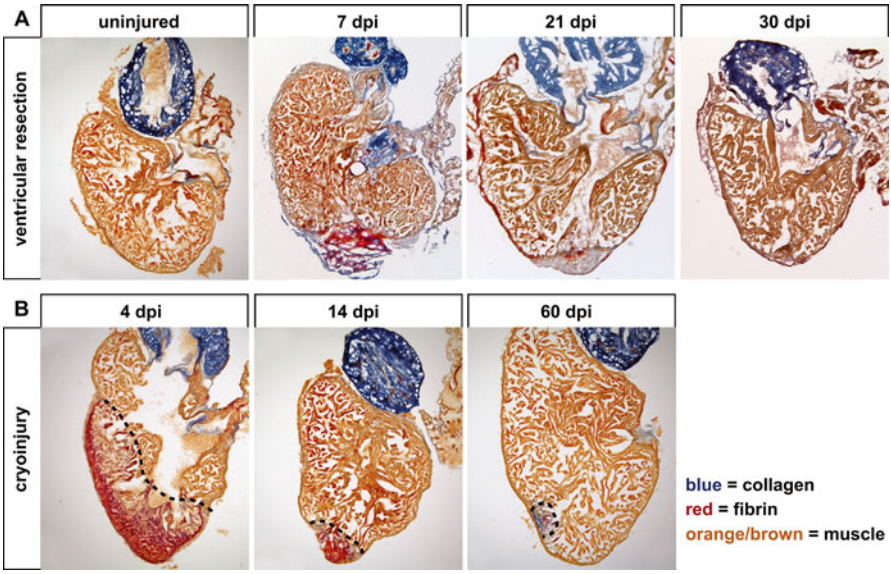

Fig. 10.1 Regeneration of ventricular myocardium in the resected and cryoinjured zebrafish heart (**a**) Acid fuchsin orange G (AFOG) staining on longitudinal sections of uninjured and resected hearts at different times after injury. At 7 days post injury (dpi), the injured area is sealed by a fibrin clot (*red*). The wound tissue progressively reduces in size from 7 to 21 dpi. At 30 dpi, the resected apex is filled with muscle (*orange/brown*) and no obvious wound tissue and collagen deposition (*blue*) is observed, indicative of complete morphological regeneration.
(**b**) Acid fuchsin orange G (AFOG) staining on longitudinal sections of cryoinjured hearts at different time points after injury. At 4 dpi, the injured area is characterized by fibrin deposition (*red*). The wound tissue progressively reduces in size from 4 to 60 dpi where little or no collagen deposition is observed. *Dashed lines*, wound boundary

cardiac injury model has been questioned and cryoinjury has been employed in zebrafish as an alternative to better model certain aspects of MI (Chablais et al. 2011; Gonzalez-Rosa et al. 2011; Schnabel et al. 2011).

In these models, either a small piece of dry ice (Schnabel et al. 2011) or a thin metal filament cooled by liquid nitrogen is applied to the surface of the ventricle (Chablais et al. 2011; Gonzalez-Rosa et al. 2011). This treatment was found to cause extensive cell death and to result in an injury affecting approximately 25 % of the ventricle size. Infiltration of immune cells was observed as early as 1 dpi (Schnabel et al. 2011), while morphological regeneration (muscle restoration and wound tissue resolution) took longer than that after ventricular resection, around 60–130 days. Cellular responses including elevated CM proliferation and reactivation of developmental gene programs in the epicardium and endocardium were found to be largely similar to those seen in the resection model (see Sect. 10.3 below; Gonzalez-Rosa et al. 2011; Schnabel et al. 2011). In contrast to ventricular resection, scar formation with extensive collagen deposition was observed as early as 3 days after cryoinjury (Gonzalez-Rosa et al. 2011). Intriguingly however, the

collagen-scar was progressively resolved and scar-free regeneration occurred (Fig. 10.1b) (Gonzalez-Rosa et al. 2011; Schnabel et al. 2011; Chablais et al. 2011). Transient scar formation has been suggested to be functionally important for regeneration in zebrafish, implying that scarring and regeneration are not necessarily mutually exclusive (Chablais and Jazwinska 2012). However, whether the molecular composition of the transient scar in zebrafish differs from that of permanent mammalian scars is unknown, and the molecular basis underlying scar resolution remains to be elucidated.

10.2.3 Genetic Ablation

10.2.3.1 Cardiomyocyte Ablation

In addition to mechanical and cryoinjury, in which several cardiac cell types are affected, myocardial-specific injury has also been achieved. Poss and colleagues generated an ablation system which induces expression of the diphtheria toxin A chain (DTA) in CreERT2-expressing CMs upon 4-hydroxytamoxifen (4-HT)-mediated recombination (Wang et al. 2011). This system caused massive cell death and depleted > 60 % of the ventricular CMs, resulting in lethargy, severe stress hypersensitivity, gasping phenotypes and reduced swimming performance, which are classical signs of heart failure and are not seen in other zebrafish heart injury models. Surprisingly, complete muscle regeneration occurred rapidly within 30 days and full functional recovery was observed at 45 dpi. Such rapid recovery could be explained by the fact that proliferation levels of spared CMs were found to be two to four times higher than those seen in other injury models. Intriguingly, robust cellular responses in the epicardium and the endocardium similar to other injury models were also observed, suggesting that CM cell death is sufficient to initiate an organ-wide regenerative program (Wang et al. 2011).

10.2.3.2 Epicardial Ablation

Poss and colleagues also employed an inducible cell ablation system using *tcf21*-driven expression of bacterial nitroreductase, which converts the drug metronidazole into a cytotoxin, to inducibly kill epicardial cells in the adult zebrafish heart (Wang et al. 2015). While this technique revealed that the epicardium is required for myocardial regeneration (see Sect. 10.4.2), it also demonstrated that the epicardium itself can rapidly and efficiently regenerate from spared epicardial cells. Epicardial regeneration was found to involve directed migration of proliferating epicardial cells from the base of the ventricle towards its apex (Wang et al. 2015). The bulbus arteriosus, which is located at the base of the ventricle, appears to provide signals that direct this migration by repelling epicardial cells, as shown in a series of elegant tissue recombination experiments in cultures of explanted hearts (Wang et al. 2015).

Sonic hedgehog (Shh) is a strong candidate for such a bulbus arteriosus-derived signal, since Hedgehog signaling was shown to be required for epicardial regeneration and Shh-soaked beads could restore epicardial migration in hearts whose bulbus arteriosus had been removed (Wang et al. 2015). It will be interesting to test to which extent Shh-mediated directed cell migration is also involved in epicardial development.

10.3 Functional Recovery

Assays for assessing heart function, in particular quantification of cardiac hemodynamics using ultrasound, are difficult to perform in zebrafish due to the small size of the adult heart (Huang et al. 2015; Kang et al. 2015). Nevertheless, there is increasing evidence for functional recovery during zebrafish heart regeneration. Myocardium at the apex of the ventricle, which presumably has formed anew during regeneration in response to ventricular resection, was found to be electrically coupled to the rest of the ventricle, indicating functional recovery (Kikuchi et al. 2010). Likewise, coupling measured by optical mapping and injury-induced lengthening of action potential duration was found to be restored to pre-injury levels 45 days post genetic ablation of CMs (Wang et al. 2011). Electrocardiogram recordings showed prolonged QT intervals after cryoinjury, which recovered by 30 dpi (Chablais et al. 2011). However, another report found that regenerated hearts retained a prolonged QT interval after ventricular resection (Yu et al. 2010). Side-by-side comparison of different injury models as well as measurements of the electrophysiological properties of individual CMs (Nemtsas et al. 2010) will be instrumental in clarifying whether zebrafish can completely recover the electrophysiological properties of ventricular myocardium. Measurements of cardiac performance based on echocardiography indicated that heart function does recover; yet this might take longer than morphological regeneration. One study reported that heart function after cryoinjury recovered only within 180 days (Hein et al. 2015), while another showed that pumping function based on relative fractional volume shortening recovered within 60 days in cryoinjured hearts, while ventricular wall motion remained altered even after 140 days (Gonzalez-Rosa et al. 2014). Overall, these data indicate that zebrafish cannot only regenerate the architecture of the heart but also its function, albeit it remains unclear to what extent.

10.4 Regeneration of Non-myocardial Tissues

Heart injury in zebrafish triggers regenerative responses in all three primary layers of the heart, namely endocardium, epicardium and myocardium, plus regeneration of coronary vasculature. In this section, we summarize the current knowledge of cellular responses and their molecular regulation in the non-myocardial layers.

10.4.1 Endocardium

Endocardial cells were found to be the first to respond to heart injury after ventricular resection (Kikuchi et al. 2011b). In the uninjured ventricle, endocardial cells typically possess elongated nuclei and thin cell bodies that tightly adhere to myofibers, as revealed by transmission electron microscopy (TEM). As early as 1 h post ventricular resection (hpi), endocardial cells both near and distant from the amputation plane appear to round up and to detach from the underlying myofibers (Kikuchi et al. 2011b). Endocardial cells close to the amputation plane were reported to retain a disorganized appearance until 7 dpi, coinciding with rapid CM proliferation. There is also evidence that the endocardium is a source of retinoic acid, which promotes CM proliferation. As early as 3 and 6 hpi, expression of the retinoic acid-synthesizing enzyme *aldh1a2* (*raldh2*) was found to be upregulated in the entire endocardium, representing an intriguing organ-wide response. From 1 dpi onwards, *aldh1a2* expression became localized to endocardial cells at the injury site, where it persisted throughout the early stages of regeneration (until 14 dpi). Intriguingly, endocardial upregulation of *aldh1a2* occurred also in response to spontaneous infarcts observed in explanted adult zebrafish hearts and upon lipopolysaccharide (LPS) injection, which triggers a systemic inflammatory response. These data suggest that endocardial upregulation of *aldh1a2* is a fundamental response to cardiac injury and inflammation (Fig. 10.2) (Kikuchi et al. 2011b). To test the role of RA signaling in heart regeneration, Poss and colleagues inhibited the signal by systemic overexpression of dominant-negative retinoid acid receptors or the RA-degrading Cyp26 enzyme. Both treatments inhibited CM proliferation at 7 dpi, while RA injection was not sufficient to induce CM proliferation. These results suggest that

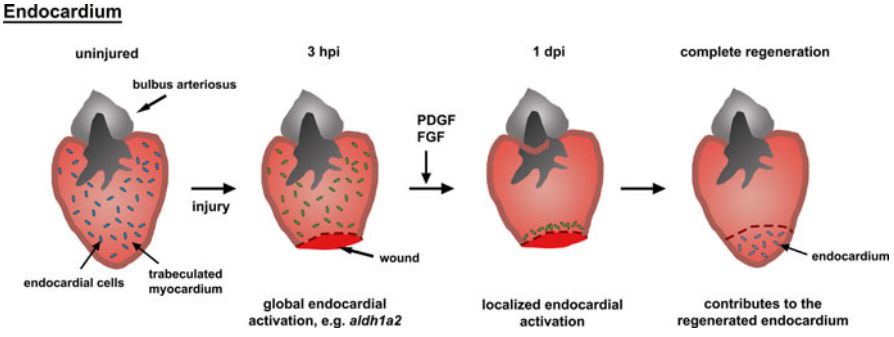

Fig. 10.2 Regeneration of the endocardium following injury
In the uninjured heart, endocardial cells tightly adhere to myofibers of the trabeculated myocardium. Starting very early after injury, endocardial cells show signs of morphological and transcriptional alterations. In particular, at 3 h post injury (hpi), the retinoic acid-synthesizing enzyme *aldh1a2* is upregulated in endocardial cells in the entire heart, while expression is later restricted to endocardium at the wound border (1 dpi). PDGF and FGF signaling might be required for endocardial regeneration. Lineage tracing indicates that pre-existing endocardial cells contribute to the regenerated endocardium upon complete regeneration.

RA is a permissive but not instructive signal for CM proliferation and regeneration (Kikuchi et al. 2011b). Since the epicardium was also found to upregulate *aldh1a2* expression in response to heart injury (albeit later than the endocardium) (Kikuchi et al. 2011b), tissue-specific interference with RA production will be required to clarify whether endocardium or epicardium-derived RA regulates CM proliferation. However, injured mammalian hearts were found to display epicardial, but not endocardial *aldh1a2* expression, suggesting that endocardial RA production supports myocardial regeneration (Kikuchi et al. 2011b).Platelet derived growth factor (PDGF) and Fibroblast growth factor (FGF) signaling appear to be required for revascularization of the wound, and thus likely also for restoration of the endocardial layer.

10.4.2 Coronary Vasculature

Vascular supply of the myocardium varies widely among teleost fish, but the compact wall of the zebrafish ventricle is supported by coronary vessels which are located in the subepicardial space (Hu et al. 2001). No molecular marker that is expressed only in the coronary endothelium but not in endocardial cells has been described. Nevertheless, several studies have argued that in addition to endocardial cells, which express a transgenic endothelial marker, *fli*:EGFP, at low levels, a population of cells displaying stronger *fli*:EGFP could be identified in the wound tissue during heart regeneration, and these cells have been addressed as regenerating coronary vessels (Lepilina et al. 2006; Kim et al. 2010; Zhao et al. 2014). Genetic lineage-tracing experiments indicated that the endothelial cells of this presumptive regenerating coronary vasculature are derived from pre-existing endothelial cells, while perivascular cells are derived from the epicardium (Fig. 10.3) (Zhao et al.

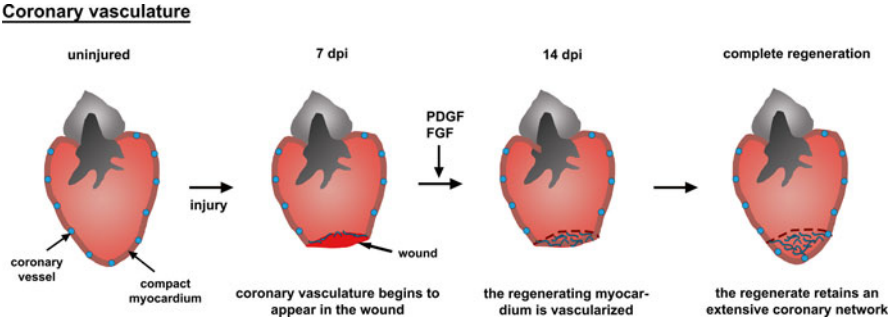

Fig. 10.3 Regeneration of coronary vasculature
In the uninjured heart, coronary vasculature is observed in the compact layer myocardium. At 7 dpi, coronary vasculature beings to appear in the wound. At 14 dpi, the regenerating myocardium is heavily vascularized, which persists after complete regeneration. Regeneration of the coronary vasculature appears to be regulated by PDGF and FGF signaling.

2014; Kikuchi et al. 2011a). Systemic inhibition of FGF signaling via overexpression of a dominant-negative FGF receptor or pharmacological inhibition of PDGF signaling interfered with re-vascularization of the wound tissue (Lepilina et al. 2006; Kim et al. 2010). While FGF inhibition also resulted in defects in myocardial regeneration (Lepilina et al. 2006), neither FGF nor PDGF signaling are thought to regulate CM proliferation (Lepilina et al. 2006; Kim et al. 2010), indicating that re-vascularization is important for myocardial regeneration.

10.4.3 Epicardium

The epicardium, a mesothelial cell layer covering the outer surface of the ventricle, has been found to respond to heart injury by re-expression of genes associated with epicardial development, including *tbx18*, *wt1b* and *aldh1a2* and also by becoming proliferative at 3 dpi (Gonzalez-Rosa et al. 2011; Kikuchi et al. 2011b; Lepilina et al. 2006; Schnabel et al. 2011). By 7 dpi, epicardial cells were reported to enclose the wound and to integrate into the fibrous wound tissue. For instance, a large number of cells retaining the expression of *tbx18* was found in the wound by 14 dpi and in the regenerate at 30 dpi (Lepilina et al. 2006). During embryonic heart development epicardial cells are thought to undergo epithelial-to-mesenchymal transition (EMT) and to invade the subepicardial space and the myocardium to form smooth muscle cells of the coronary vasculature and myocardial fibroblasts (Dettman et al. 1998). In addition, some lineage tracing studies have found evidence for formation of CMs from epicardial cells during development and after heart injury, although the specificity of the Cre lines used for some of these studies has been questioned (Zhou et al. 2008; Cai et al. 2008; Smart et al. 2011; Rudat and Kispert 2012). To address the potential of epicardial cells during zebrafish heart regeneration, Poss and colleagues used inducible Cre lines driven by *tcf21* regulatory sequences and found that epicardial cells give rise to perivascular cells but not CMs after injury (Kikuchi et al. 2011a). This finding was substantiated by Mercader and colleagues using transplantation experiments, which in addition indicated that myofibroblasts are derived from epicardial cells during heart regeneration as well (Gonzalez-Rosa et al. 2012). Thus, during naturally occurring heart regeneration, the epicardium does not adopt CM fate (Fig. 10.4).

During embryonic development, the epicardium is thought to provide signals regulating myocardial development, including CM proliferation (Lavine and Ornitz 2008; Riley 2012). To test the requirement of the epicardium for zebrafish myocardial regeneration, Poss and colleagues ablated epicardial cells using *tc21*-driven expression of nitroreducatase (Wang et al. 2015). Epicardial ablation induced early after ventricular resection (from 2 to 5 dpi) reduced CM proliferation at 7 dpi and resulted in defects in myocardial restoration, as evidenced by presence of scar tissue by 30 dpi. Thus, the epicardium is essential for myocardial regeneration in zebrafish. The molecular signals mediating its effects on CMs during regeneration are however unknown.

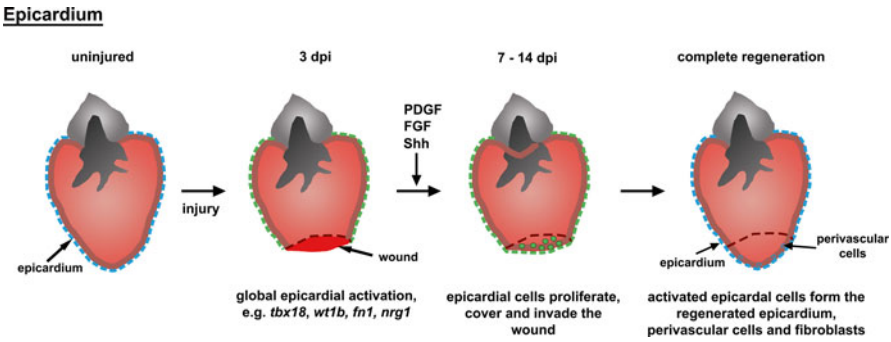

Fig. 10.4 Regeneration of the epicardium
In the uninjured heart, the epicardium consists of a single layer of cells at the outline of the compact myocardium. At 3 dpi, epicardial activation, which is characterized by upregulation of *tbx18*, *wt1b*, *fn1* and *nrg1*, is observed in the entire heart. From 7 to 14 dpi, epicardial cells cover and invade the wound tissue. Lineage-tracing experiments demonstrate that epicardial cells give rise to the regenerated epicardium, perivascular cells of the coronary vasculature and myofibroblasts. Regeneration of the epicardium appears to be regulated by PDGF, FGF and Shh signaling.

Epicardial EMT during heart regeneration appears to be controlled by FGF and PDGF signaling (Lepilina et al. 2006; Kim et al. 2010). Inhibition of FGF signaling by systemic overexpression of a dominant-negative FGF receptor compromised integration of *tbx18*-positive cells into the regenerating myocardium in addition to its effects on re-vascularization (see Sect. 10.4.2) (Lepilina et al. 2006). Using an adult zebrafish epicardium explant culture, Lien and colleagues showed that PDGF signaling triggers epicardial cells to detach and form mesenchymal-like cell types. PDGF signaling was also required for epicardial cell proliferation both *in vitro* and *in vivo*, and treatment with a PDGF receptor inhibitor reduced the expression of the EMT marker *snail2* in regenerating hearts (Kim et al. 2010).

The epicardium also appears to be an important source of extracellular matrix (ECM), which influences the cellular behavior of regenerating CMs. Poss and colleagues showed that expression of fibronectin, a major ECM component, is strongly induced upon heart injury and mainly deposited by injury-activated epicardial cells (Wang et al. 2013). Myocardial regeneration was significantly inhibited in fish overexpressing a dominant-negative human fibronectin fragment as well as in fish homozygous for a *fibronectin1* mutation, resulting in severe fibrosis. Interestingly, such myocardial regeneration defects were not caused by defective CM proliferation. Instead, at 30 dpi, a larger number of CMs was present in the area flanking the injury in homozygous *fibronectin1* mutants, suggesting that fibronectin might be required for CM mobilization and integration into the injury site (Wang et al. 2013). Altogether, these data suggest that the epicardium regulates zebrafish heart regeneration in a number of ways.

10.5 Regeneration of the Myocardium

10.5.1 Cellular Sources of Regenerating Cardiomyocytes

Central to the ability of zebrafish to recover from heart injuries is the fact that they can replace lost CMs. So far, research into the cellular and molecular mechanisms of heart regeneration thus has largely focused on CM restoration. This is usually studied in the adult heart, where homeostatic myocardial cell cycle activity is very low, which allows for the identification of injury-induced regenerative mechanisms of CM production. Nevertheless, injuries to the rapidly developing embryonic zebrafish heart at stages where CMs are normally produced have also been proposed to represent a useful model of regeneration (Zhang et al. 2013). Using a nitroreductase-based CM-specific ablation system, Chi and colleagues reported that lost embryonic ventricular CMs are rapidly replaced (Zhang et al. 2013). Interestingly, they suggested that this is not only due to elevated proliferation of spared CMs, but also due to transdifferentiation of atrial into ventricular CMs as revealed by genetic lineage-tracing experiments. This process is at least partly mediated by Notch signaling since treatment of ablated hearts with the γ-secretase inhibitor DAPT reduced the appearance of atrially-derived CMs in the injured ventricle. Though intriguing, such atrial contribution to the regenerating ventricle was found to be minimal in adult CM-ablated hearts, suggesting that adult zebrafish rely on other sources of CMs for ventricular regeneration (Zhang et al. 2013).

For a period of time, it remained unclear whether the newly forming CMs in adult regenerating hearts are derived from existing differentiated CMs or other sources like stem cells. Initial findings from Poss and colleagues suggested that CMs regenerate from progenitors, but not from differentiated spared CMs (Lepilina et al. 2006). However, subsequent genetic lineage-tracing experiments from two independent studies provided strong evidence for the latter mechanism (Jopling et al. 2010; Kikuchi et al. 2010), indicating that the earlier data which were based on differential maturation and stability of fluorescent proteins were artefacts (Kikuchi et al. 2010). Using an inducible CM-specific Cre line (*myl7*:CreERT2), Poss and colleagues demonstrated that differentiated CMs labeled 5 days before ventricular resection in adult fish contribute to the vast majority of the regenerated myocardium (Fig. 10.5) (Kikuchi et al. 2010). Izpisua-Belmonte and colleagues genetically lineage-traced embryonic CMs and similarly concluded that adult regenerating CMs are derived from cells that once had CM character (Jopling et al. 2010). Thus, the current consensus in the field is that zebrafish myocardial regeneration is achieved via cell cycle re-entry and proliferation of spared differentiated CMs (Figs. 10.6 and 10.7a). Similarly, neonatal mouse heart regeneration is thought to occur via spared CM proliferation as well (Porrello et al. 2011). Whether all CMs can equally contribute to regeneration or whether a particular subset of potentially less differentiated cells has higher regenerative potential is currently unknown. It is also worth noting that quantitative data showing that heart regeneration involves the

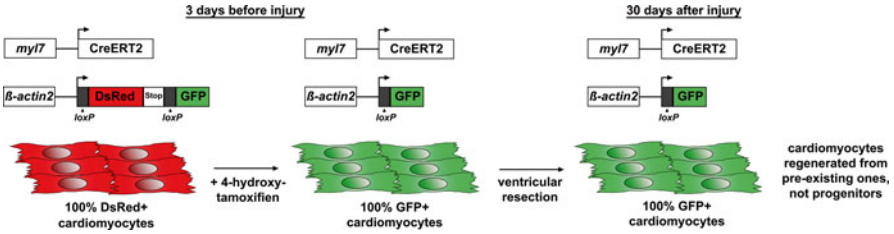

Fig. 10.5 Cardiomyocyte lineage tracing experiments
To test whether differentiated cardiomyocytes are the precursors of regenerating cardiomyocytes, a dual transgenic system has been used. Tamoxifen-inducible CreERT2 recombinase is expressed specifically in differentiated cardiomyocytes under control of the *myl7* promoter. Three days before injury, Cre activity is induced by treating the fish with 4-hydroxytamoxifen. Consequently, CreERT2 removes a floxed DsRed-STOP cassette from a second transgene expressed under control of a *β−actin2* promoter, which is active in cardiomyocytes in the adult heart. Removal of the STOP cassette allows transcription of GFP, thus cardiomyocytes are genetically labeled with GFP. At 30 dpi, all of the regenerated cardiomyocytes are GFP positive, indicating that cardiomyocytes regenerate from pre-existing cardiomyocytes but not from stem or progenitor cells, which are GFP negative.

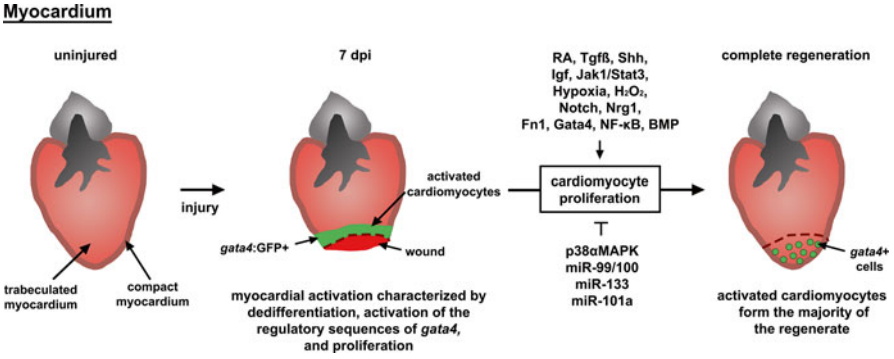

Fig. 10.6 Regeneration of the myocardium
At 7 dpi, cardiomyocytes close to the wound are activated, as indicated by dedifferentiation, activation of the regulatory sequences of *gata4* and proliferation. Cardiomyocyte proliferation is regulated positively by RA, Tgfß, Shh, Igf, Jak1/Stat3, Hypoxia, H_2O_2, Notch, Nrg1, Fn1, Gata4, NF-κB and BMP and negatively by p38αMAPK, miR-99/100, miR-133 and miR-101a. Lineage tracing indicates that regenerated cardiomyocytes are derived from spared, "activated" CMs at the wound border.

full restoration of CM numbers is missing. In addition, whether CM hypertrophy, which is prominent in injured mammalian hearts (Zebrowski and Engel 2013), also occurs in the zebrafish and if so, to which extent injury-induced CM cell cycle activity could be due to CM polyploidization has not been explored.

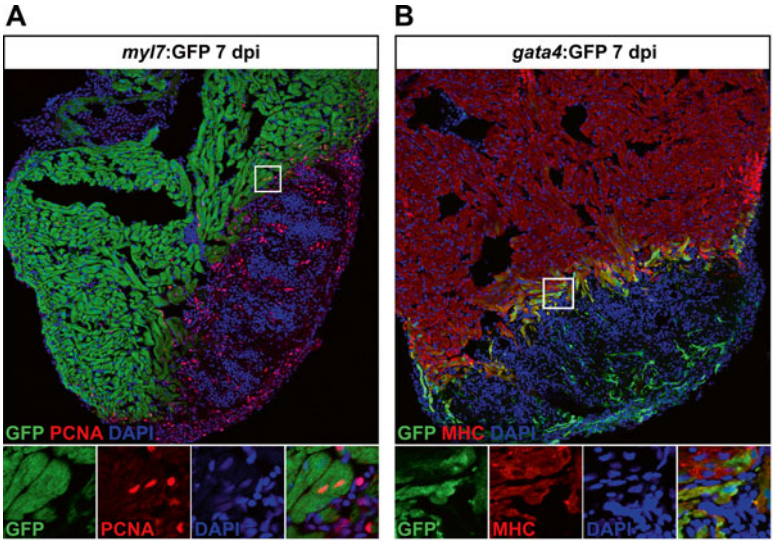

Fig. 10.7 Cellular responses of cardiomyocytes to injury
(**a**) At 7 dpi, cardiomyocytes (marked by *myl7*:GFP) at the wound border re-enter the cell cycle and express the cell-cycle marker PCNA.
(**b**) At 7 dpi, cardiomyocytes (marked by myosin heavy chain (MHC) expression, *red*) at the wound border activate the regulatory sequence of *gata4* in *gata4*:GFP transgenic fish.

10.5.2 Cardiomyocyte Dedifferentiation

Cardiomyocytes do not only re-enter the cell cycle, but also appear to dedifferentiate during regeneration, which is both suggested by reported loss of characteristics of the differentiated state and by upregulation of embryonic genes. After injury, CMs close to the wound were found to be characterized by reduced expression of GFP driven by the *myl7* promoter, which is active in differentiated CMs, by partial loss of sarcomeric structures revealed by transmission electron microscopy and by alpha-actinin disorganization (Jopling et al. 2010; Kikuchi et al. 2010; Sallin et al. 2015; Schnabel et al. 2011; Wang et al. 2011; Wu et al. 2016). These changes, which are suggestive of CM dedifferentiation in response to injury, were found to be accompanied by reactivated expression of genes that are highly expressed in embryonic hearts, including *nppa, nppb*, embryonic myosin chains, and likely also cardiogenic transcription factors like *gata4* (Kikuchi et al. 2010; Sallin et al. 2015; Wang et al. 2013; Wu et al. 2016). Poss and colleagues found that a transgenic line driving GFP from regulatory sequences of *gata4*, a cardiac transcription factor expressed during embryonic heart development, is re-expressed in a subpopulation of CMs in the compact layer myocardium near the wound from three to seven dpi in injured hearts (Fig. 10.7b). By 14 dpi, GFP-positive cells were found in many cells around and within the injury site. A sub-set of these *gata4*:GFP-expressing CMs expressed markers of cell cycle activity, and their descendants, genetically marked by

inducible *gata4*:CreERT2 from five to seven dpi, contributed significantly to the regenerated myocardium (Kikuchi et al. 2010). While strong evidence for the re-expression of endogenous *gata4* or other cardiogenic factors in CMs in injured hearts is still missing, overexpression of a dominant-negative form of Gata4 reduced CM proliferation in the compact layer myocardium, compromised myocardial regeneration at 30 dpi and resulted in extensive scarring (Gupta et al. 2013). Thus, reactivation of *gata4* in the compact layer myocardium appears to be crucial for successful heart regeneration (Gupta et al. 2013).

Izpisua-Belmonte and colleagues also suggested that dedifferentiation is of functional importance for CMs to proliferate since no mitotic CMs displayed highly organized sarcomeric structures (Jopling et al. 2010). However, mammalian CMs also reactivate expression of embryonic genes and show partial loss of sarcomeric structure after heart injury (D'Uva et al. 2015; Kubin et al. 2011). Therefore, CM dedifferentiation could be a shared injury response between the non-regenerating mammalian and the regenerating zebrafish heart (Szibor et al. 2014). Dedifferentiation thus might represent a prerequisite for proliferation, but likely is not sufficient or causal for CM proliferation, considering that mammalian CMs do not proliferate in response to heart injury. More detailed systematic analyses of molecular changes in CMs induced by injury in both models and the identification of experimental interventions that specifically block dedifferentiation will be essential to understand the functional role of CM dedifferentiation during heart regeneration.

Migration of CMs into the injured area, regulated by the Cxcl12-Cxcr4 system, has also been suggested to be an important event during regeneration (Itou et al. 2012). Pharmacological inhibition of Cxcr4 reduced the number of CMs in the injury site at 14 dpi and compromised myocardial regeneration at 60 dpi without affecting CM proliferation. While these data have been interpreted as indicative of the importance of CM migration during regeneration, more direct evidence for active CM migration is lacking.

10.5.3 Molecular Regulation of Cardiomyocyte Regeneration

Several studies have identified factors and signaling pathways regulating CM proliferation during zebrafish heart regeneration. Obvious candidates for such factors are those that also regulate embryonic CM proliferation during heart development. To screen for molecular regulators of embryonic CM proliferation, Poss and colleagues employed the fluorescent ubiquitination-based cell cycle indicator (FUCCI) system, which comprises two fusion proteins, mCherry-zCdt1 and Venus-hGeminin, which are expressed cyclically in the G1 and S/G2/M phases of the cell cycle, respectively (Choi et al. 2013). By combining transgenic expression of these proteins specifically in CMs (*myl7*:FUCCI) with a small-scale chemical screen targeting common developmental signaling pathways, Hedgehog (Hh), Insulin-like growth factor (Igf), and Transforming growth factor ß (Tgfß) signaling were identified as positive regulators of CM proliferation during development (Choi et al. 2013).

10.5.3.1 Hedgehog Signaling

Hh signaling has been implicated in driving cardiac specification in the zebrafish embryo and reduction of Hh signaling causes cardiac morphogenesis defects in mice (Washington Smoak et al. 2005). Treatment of *myl7*:FUCCI embryos with the Smoothened agonist SAG, which activates Hh signaling, increased CM proliferation by 60 % as well as CM numbers by 10 % (Choi et al. 2013). On the other hand, treatment with cyclopamine (a Smoothened antagonist) reduced CM proliferation and numbers by 27 % and 19 %, respectively. These data suggest that Hh signaling is required for embryonic CM proliferation.

Interestingly, Hh signaling was also activated in CMs in the adult zebrafish heart 7 days after ventricular resection, as indicated by the expression of the Hh target gene *ptch2* in a transgenic reporter line (Choi et al. 2013). Treatment with cyclopamine 6 days after ventricular resection or genetic CM ablation reduced expression of the cell cycle maker PCNA in CMs. SAG treatment, on the other hand, increased CM cell cycle activity remarkably by 65 % after ventricular resection while it had no effect on adult uninjured zebrafish hearts. Together, these data suggest that zebrafish CM proliferation requires Hh signaling, which on its own is not sufficient to trigger regenerative proliferation (Choi et al. 2013). Hh ligands might be provided by the epicardium, which was reported to express a transgenic sonic hedgehog reporter line (Choi et al. 2013). Whether Hh signaling acts directly on CMs and is indeed activated by epicardium-derived ligands will have to be clarified using tissue-specific experimental manipulations.

Hh signaling was also found to be activated in the mouse myocardium after myocardial infarction (Kusano et al. 2005). Injection of Sonic Hedgehog (Shh) plasmid into infarcted hearts was sufficient to promote neovascularization, to protect CMs from apoptosis, to reduce post-MI remodeling and to improve functional recovery (Kusano et al. 2005). Therefore, although the beneficiary effects of Hh signaling on cardiac repair are conserved between zebrafish and mammals, the mode of actions seems to be different. It would be interesting to characterize the interaction partners and downstream targets of Hh signaling in both models to understand the molecular basis behind these differential responses to the activation of the same signaling pathway.

10.5.3.2 Insulin-Like Growth Factor Signaling

Igf signaling has been described to have positive effects on CM proliferation during mouse development (Li et al. 2011). In *myl7*:FUCCI zebrafish embryos, treatment with the Igf receptor antagonist NVP AEW541 reduced CM proliferation and number by 33 % and 14 %, respectively (Choi et al. 2013). This proliferation defect was verified by Lien and colleagues in an independent study (Huang et al. 2013). On the other hand, treatment with an Igf signaling agonist increased embryonic CM proliferation and number by 41 % and 17 %, respectively (Choi et al. 2013). These data suggest that Igf signaling is required for CM proliferation in the developing

mammalian and zebrafish heart. Components of the Igf signaling pathway were also found to be upregulated in the adult zebrafish heart in response to injury. RT-PCR analysis showed that expression of the ligand *igf2b* is upregulated by three dpi and peaks at seven dpi, in a temporal profile similar to that of CM proliferation, which peaks between seven and 14 dpi. *igf2b* expression appeared to be restricted to endocardial cells in the injury site as detected by *in situ* hybridization while Igf receptor 1 was expressed in CMs close to the amputation plane (Choi et al. 2013; Huang et al. 2013). Systemic Igf signaling inhibition in adult fish either by treatment with the NVP AEW541 antagonist or by overexpression of a dominant-negative form of Igf receptor 1 (*hspl70*:dnigf1ra) reduced PCNA expression in CMs after injury. Lien and colleagues found that activation of *gata4*:EGFP in a subset of CMs at the wound border was not affected by Igf receptor antagonist treatment, while Igf signaling inhibition blocked proliferation of this population and appeared to reduce their accumulation in the injured area by 14 dpi (Huang et al. 2013). Prolonged treatment with antagonist until 30 dpi inhibited regeneration, resulting in excessive fibrin and collagen deposition (Huang et al. 2013). On the other hand, overactivation of Igf signaling was sufficient to increase CM proliferation by 65 % and 36 % after ventricular resection and genetic CM ablation, respectively (Choi et al. 2013; Huang et al. 2013). Together, these data suggest that endocardium-derived Igf ligands regulate regenerative CM proliferation and successful regeneration. Again, whether proliferation is cell-autonomously regulated by Igf signaling activation in CMs and whether it is indeed activated by endocardially produced ligands will have to be clarified using tissue-specific manipulations.

10.5.3.3 Transforming Growth Factor ß Signaling

Treatment of *myl7*:FUCCI embryos with the Tgfß receptor inhibitor SB431542 reduced CM proliferation and number by 30 %, suggesting that Tgfß signaling promotes CM proliferation during development (Choi et al. 2013). The role of Tgfß signaling during zebrafish adult heart regeneration was further studied by Chablais and Jaźwińska (Chablais and Jazwinska 2012). Following cryoinjury, expression of three Tgfß isoforms (*tgfß1, tgfß2 and tgfß3*) was upregulated in the wound at four and 14 dpi as detected by *in situ* hybridization. These Tgfß isoforms appeared to be expressed in several cell types including fibroblasts, epithelial cells of the wound, as well as some CMs at the wound border. Tgfß receptors, on the other hand, appeared to be expressed in the wound (both *alk4* and *alk5a*) and in the entire heart possibly in Vimentin-positive fibroblasts (*alk5a*), suggesting that both CMs and non-CMs can be responsive to Tgfß ligands. This idea was verified by immunostaining against phosphorylated Smad3, a readout for active Tgfß signaling, which was expressed in a large number of cells in the wound and in a sub-set of CMs at the wound border (Chablais and Jazwinska 2012). To understand the functional role of Tgfß signaling, cryoinjured fish were treated with the Tgfß receptor antagonist SB431542 and analyzed by histological staining at different time points. At four dpi, in both control and treated fish, fibrin deposition was evident in the wound. Interestingly, at 14 dpi,

while scar tissue containing fibrin and collagen was evident in control hearts (Chablais et al. 2011; Gonzalez-Rosa et al. 2011), Tgfß signaling-inhibited fish failed to deposit collagen in the wound (Chablais and Jazwinska 2012). At 30 dpi, while little scar tissue could be detected in control hearts, implying that the fibrin and collagen depositions present at 14 dpi had been resolved and replaced by new muscle, Tgfß inhibitor-treated fish still contained fibrin-rich wound tissue, which showed signs of mechanical deformation (Chablais and Jazwinska 2012). These data suggest an unexpected positive correlation between transient collagen deposition and successful heart regeneration in zebrafish. Based on drug-shift experiments, Chablais and Jaźwińska suggested that Tgfß signaling-mediated transient collagen-rich scar formation is required to stabilize the injured myocardium (Chablais and Jazwinska 2012). In addition to collagen deposition, Tgfß also appears to be required for the production of other ECM proteins including the tissue remodeling protein Tenascin C and Fibronectin, which is crucial for zebrafish heart regeneration (Chablais and Jazwinska 2012; Wang et al. 2013). Lastly, consistent with its role during embryonic development, Tgfß signaling inhibition reduced CM proliferation detected by either PCNA expression or BrdU incorporation (Chablais and Jazwinska 2012; Choi et al. 2013). Altogether, these studies suggest that Tgfß signaling is required for both CM proliferation and collagen deposition in the wound after injury. Chablais and Jaźwińska's study is the first to provide evidence for the functional importance of transient scarring during zebrafish heart regeneration (Chablais et al. 2011; Gonzalez-Rosa et al. 2011). This observation challenges the prevalent concept in the heart regeneration field that scarring and regeneration is mutually exclusive, and also opens up a new area of research into the molecular mechanisms of transient scar resolution. Similar to the pathways discussed above, future studies using cell-type specific manipulations will be necessary to clarify whether the effects of Tgfß signaling on CM proliferation are direct.

10.5.3.4 Jak1/Stat3 Signaling

Several studies have performed transcriptional profiling of regenerating zebrafish hearts using whole ventricle samples (Lien et al. 2006; Sleep et al. 2010). Poss and colleagues performed profiling of RNAs that are specifically translated in zebrafish CMs using translating ribosome affinity purification (TRAP) technology (Fang et al. 2013). To do this, an EGFP reporter gene was fused to the N-terminus of the ribosomal protein L10a, whose expression was driven specifically in CMs under the regulatory sequence of *myl7* (*myl7*:TRAP). Translating mRNAs were isolated by immunoprecipitation with an antibody against EGFP, which were then processed for microarray analysis. Among the differentially regulated mRNAs, several members of the Janus kinases1/Signal transducer and activator of transcription 3 (Jak1/Stat3) pathway were found to be upregulated in 1 dpi samples, including *interleukin 6 signal transducer* (*il6st*), *jak1*, *stat3* and the Jak1/Stat3 target gene *suppressor of cytokine signaling 3b* (*socs3b*) (Fang et al. 2013). *In situ* hybridization showed that these Jak1/Stat3 pathway genes are induced in an organ-wide manner at 1 dpi but

later expression appeared to localize to CMs at the amputation plane. To investigate the role of Jak1/Stat3 signaling during heart regeneration, Poss and colleagues used a Cre/lox-based system for inducible overexpression of a dominant-negative Stat3 (dnStat3) in CMs upon 4-hydroxytamoxifen induction, which was able to reduce expression of the target gene *socs3b* by 80 % following injury. Continuous dnStat3 expression from prior to heart injury compromised myocardial regeneration by 30 dpi and resulted in extensive scarring compared to control fish. In line with this, CM proliferation was also strongly reduced (~80 %) in dnStat3-expressing fish (Fang et al. 2013). Expression of *relaxin 3a* (*rln3a*), which codes for a peptide hormone, was found to be directly regulated by Stat3 in the regenerating heart. Daily retro-orbital injection of Rln3a protein in dnStat3-expressing fish partially rescued CM proliferation defects at seven dpi, suggesting that the effect of Jak1/Stat3 signaling on heart regeneration is at least partly mediated by Rln3a (Fang et al. 2013).

In contrast to the signaling pathways described above, the requirement of Jak1/Stat3 signaling for CM proliferation appears to be regeneration-specific. Larvae subjected to dnStat3 overexpression from 4 days post fertilization survived to adulthood, suggesting that Jak1/Stat3 signaling is not required for heart development (Fang et al. 2013). Furthermore, CM proliferation in juvenile and young adult zebrafish stimulated by low density growing conditions (Wills et al. 2008) was not affected by dnStat3 expression (Fang et al. 2013). Altogether, these data identify Jak1/Stat3 signaling as an injury-induced pathway that directly regulates regenerative CM proliferation.

10.5.3.5 Notch Signaling

Treatment of *myl7*:FUCCI embryos with a Notch signaling inhibitor (γ-secretase inhibitor) had no effect on CM proliferation, suggesting that Notch signaling is dispensable for CM proliferation in the embryonic heart (Choi et al. 2013). In contrast, Burns and colleagues found that Notch signaling is required for adult CM regeneration (Zhao et al. 2014). In adult zebrafish, three out of four Notch receptors (*notch1a*, *notch1b* and *notch2*) were upregulated in both endocardial and epicardial cells but not in CMs 7 days after ventricular resection as revealed by *in situ* hybridization (Zhao et al. 2014). To understand the role of Notch signaling during regeneration, Burns and colleagues inhibited Notch signaling by ubiquitously overexpressing a dominant negative isoform of the murine mastermind-like protein (dnMAML), which cannot recruit essential cofactors to the Notch transcriptional complex, rendering the complex inert. Continuous systemic Notch inhibition compromised myocardial regeneration in the resected ventricular apex by 30 dpi and resulted in extensive scarring with collagen deposition, suggesting that Notch signaling is required for heart regeneration (Zhao et al. 2014). Although Notch receptors were predominantly upregulated in endocardial and epicardial cells after injury, Notch signaling inhibition did not interfere with the early injury responses reported previously in both cell types (Kikuchi et al. 2011b; Lepilina et al. 2006), namely the formation of epicardium-derived cells and activation of *aldh1a2* expression in both

epicardium and endocardium at seven dpi (Zhao et al. 2014). Interestingly, while myocardial regeneration was compromised in Notch-inhibited hearts at 30 dpi, coronary endothelium regeneration as detected by *kdrl*:mCherry transgenics was not inhibited, indicating that Notch signaling is dispensable for neovascularization after injury. Cardiomyocyte proliferation, on the other hand, was found to be regulated by Notch signaling since continuous overexpression of dnMAML from one to seven dpi significantly reduced PCNA expression in CMs by 53 %. Intriguingly, Notch hyperactivation by systemic overexpression of the intracellular domain of the Notch receptor (NICD) also significantly reduced CM proliferation at seven dpi as well as myocardial regeneration at 30 dpi, suggesting that the right balance of Notch signaling activity is essential for successful heart regeneration (Zhao et al. 2014).

10.5.3.6 Neuregulin1 Signaling

As discussed above, while a number of pathways/factors have been identified to regulate CM regeneration, none of them are able to stimulate CM hyperplasia in the absence of injury. In contrast, a recent study by Poss and colleagues showed that Neuregulin1 (Nrg1) is a potent injury-induced mitogen for zebrafish heart regeneration and that its activation in the healthy adult heart is sufficient to induce rapid regenerative programs, resulting in excessive myocardial hyperplasia and cardiomegaly (Gemberling et al. 2015). Neuregulin1 is an extracellular factor of the EGF family that might act as a transmembrane protein, but can also give rise to secreted peptides due to ectodomain shedding (Mei and Nave 2014).

After genetic ablation of CMs, *nrg1* expression was rapidly induced in the heart at three and seven dpi, coinciding with induction of CM proliferation. *nrg1* was predominantly expressed in perivascular regions in the ventricular wall after genetic ablation and in the region surrounding the injury site after ventricular resection, as revealed by *in situ* hybridization. Co-localization studies with transgenic reporter lines marking different cardiac cell types showed that the dominant source of *nrg1* in the ventricular wall is *tcf21*-expressing epicardial cells. After ventricular resection, treatment with a small molecule inhibitor of Erbb receptors, which are required for Nrg1 signaling, reduced CM proliferation at seven dpi by 54 %. On the other hand, inducible overexpression of Nrg1 specifically in CMs using the Cre-Lox system increased proliferation at seven dpi by 84 %. These data suggest that Nrg1 is a potent mitogenic factor for CMs after injury (Gemberling et al. 2015).

Using the same CM-specific overexpression system, Poss and colleagues tested the effect of Nrg1 on the uninjured adult heart. Nrg1 overexpression for 7 days significantly induced CM proliferation, resulting in a remarkable thickening of the ventricular wall by 460 % after 30 days of overexpression (Gemberling et al. 2015). These changes are likely the result of CM hyperplasia instead of hypertrophy since CM cell size was not affected by continuous Nrg1 overexpression. However, prolonged Nrg1-induced hyperplasia compromised cardiac function as revealed by echocardiography and a swimming test which is an assay for cardiac function that is sensitive for heart injury and failure (Wang et al. 2011).

Nrg1 overexpression in the uninjured heart was sufficient to elicit responses similar to those seen during heart regeneration. During heart development, cortical muscle in the ventricular wall was reported to be typically formed from a small number of CMs, which expand laterally on the ventricular surface to form large clones, as revealed by CM clonal analysis (Gupta and Poss 2012). In contrast, after heart injury, CM regeneration appears to occur through proliferation near the injury site, which results in a number of small CM clones in the regenerated ventricular wall (Gupta et al. 2013). Overexpression of Nrg1 in juvenile fish 5 weeks post fertilization (wpf), a time at which cortical muscle first appears, resulted in ectopic ventricular wall thickening by 10 wpf and clonal analysis showed obvious clone mixing, which is more reminiscent of injury-induced regeneration (Gemberling et al. 2015). Furthermore, Nrg1 overexpression also activated the regulatory sequence of *gata4*, as well as the expression of *tgfß2*, *aldh1a2* and *fibronectin 1* which have all been functionally implicated in zebrafish heart regeneration (Gupta et al. 2013; Kikuchi et al. 2010, 2011b; Chablais and Jazwinska 2012; Wang et al. 2013). Finally, CM dedifferentiation, epicardial activation and myocardial vascularization were also rapidly induced by Nrg1 overexpression in uninjured hearts (Gemberling et al. 2015). Together, these data suggest that Nrg1 signaling is a potent inducer of regenerative programs that involve different cardiac cell types. Such a mitogenic and pro-regenerative role of Nrg1 on CMs appears to be conserved between zebrafish and mammals since both injection of Nrg1 or expression of a constitutively active Erbb2 has been reported to induce CM proliferation both *in vitro* and *in vivo* and to improve functional recovery after MI (Bersell et al. 2009; D'Uva et al. 2015).

10.5.3.7 Bone Morphogenetic Protein signaling

To identify regulators of zebrafish CM regeneration, Weidinger, Bakkers and colleagues employed Tomo-Seq, a recently developed method providing genome-wide transcriptional expression data with spatial resolution (Wu et al. 2016). This resulted in the identification of several hundred genes whose expression is enriched at the wound border, where CMs re-enter the cell cycle. Ligands, receptors and target genes of Bone Morphogenetic Protein (BMP) signaling were found to be expressed at the wound border, and nuclear accumulation of phosphorylated Smad1/5/8, a readout of active BMP signaling, was detected in dedifferentiating CMs at the wound border. Systemic interference with BMP signaling showed that the pathway is required for CM proliferation and muscle regeneration, while overexpression of the ligand Bmp2b was sufficient to augment CM proliferation and to speed up muscle regeneration (Wu et al. 2016). Weidinger, Bakkers and colleagues found that BMP signaling is not required for physiological CM proliferation in juvenile fish, indicating that it represents an injury-specific regulator of CM regeneration. In part, this might be due to BMP signaling being required for CM dedifferentiation (Wu et al. 2016). Interestingly, BMP signaling has also been reported to be upregulated in injured mammalian hearts, but there it appears to

play the opposite role than in zebrafish. Interference with BMP signaling protects mammalian CMs from apoptosis and improves the outcome after MI (Pachori et al. 2010). These results indicate that the differential ability of zebrafish and mammalian hearts to regenerate depends in part on opposing responses of CMs to BMP signaling.

10.5.3.8 NF-κB signaling

NF-κB signaling has also been identified as important regulator of zebrafish heart regeneration by Poss and colleagues (Karra et al. 2015). Using a transgenic reporter line, NF-κB activity was detected in CMs adjacent to the amputation plane throughout the first two weeks after injury. Inhibition of NF-κB activity specifically in CMs inhibited CM proliferation and myocardial regeneration at 7 and 30 dpi, respectively. In addition, NF-κB transcriptional complexes also appear to contribute to CM dedifferentiation by directly activating transcription of gata4. Interestingly, myocardial inhibition of NF-κB activity also resulted in defects in infiltration of epicardial cells into the wound at 14 dpi, suggesting a potential cross-talk of CMs and epicardial cells during regeneration (Karra et al. 2015).

10.5.3.9 p38α MAPK

The activity of p38α MAPK is inversely correlated with CM proliferation in mammals; inhibition of p38α MAPK induces proliferation of cultured neonatal and adult CMs, which are normally non-proliferative (Engel et al. 2005). Moreover, co-treatment of adult rats with FGF1 and p38α MAPK inhibitor after MI reduced scarring and markedly improved functional recovery (Engel et al. 2006). A study from Izpisua-Belmonte and colleagues revealed a similar role of p38α MAPK on CM proliferation in zebrafish (Jopling et al. 2012b). To manipulate p38α MAPK activity, Izpisua-Belmonte and colleagues targeted expression of either a dominant negative form of p38α MAPK (dnp38α MAPK) or a constitutive active (ca) MKK6, an upstream activator of p38α MAPK, to CreERT2-expressing CMs upon 4-hydroxytamoxifen (4-HT)-mediated induction (Jopling et al. 2012b). While p38α MAPK inhibition had no effect on myocardial regeneration after ventricular resection, caMKK6 overexpression significantly inhibited myocardial regeneration at 30 dpi. Moreover, mosaic expression of caMKK6 using a partially silenced CM-specific Cre line showed that caMKK6-expressing CMs were unable to incorporate BrdU, suggesting that p38α MAPK activity inhibits CM proliferation and regeneration (Jopling et al. 2012b). These results suggest that p38α MAPK activity in adult zebrafish CMs must be low for heart regeneration to occur.

10.5.3.10 Hypoxia

Hypoxia-inducible factors (HIFs) are the direct effectors of the hypoxic response. In mammals, it has been reported that HIF1α overexpression in the myocardium reduces infarct size and improves functional recovery after MI (Kido et al. 2005). Izpisua-Belmonte and colleagues reported that at 7 days post ventricular resection in zebrafish, the wound tissue and a subset of CMs close to the amputation plane showed hypoxia induction as evidenced by Hypoxyprobe staining (Jopling et al. 2012a). Cardiomyocyte BrdU incorporation and regeneration in adult zebrafish preconditioned with phenylhyrazine, an anemia/hypoxia-inducing drug, was enhanced, while zebrafish overexpressing a dominant-negative form of HIF1α in CMs showed inhibition of these processes. Together, these data suggest that hypoxia is required for myocardial regeneration in zebrafish (Jopling et al. 2012a). In cultured zebrafish CMs hypoxic treatment appeared to increase the number of mitotic and dedifferentiated CMs (Jopling et al. 2012a). Whether or not these effects could also be observed *in vivo* is, however, unclear.

10.5.3.11 Hydrogen Peroxide

Reactive oxygen species (ROS), despite their toxic potential, are crucial for regeneration of the tadpole tail in *Xenopus* and of the caudal fin in zebrafish (Gauron et al. 2013; Love et al. 2013). A study by Xiong and colleagues revealed a positive role of hydrogen peroxide (H_2O_2) in zebrafish heart regeneration as well (Han et al. 2014). After ventricular resection, *duox*, a member of the NADPH-oxidase and related dual oxidase family of proteins, which is responsible for the formation of superoxide anions and subsequently H_2O_2, was upregulated in epicardial cells at the site of injury from three to 14 dpi (Han et al. 2014). In line with the temporal expression profile of *duox*, the level of H_2O_2 in CMs was also found to be elevated between three and 14 dpi, as detected by expression of Hyper, a fluorescent protein-based H_2O_2 sensor (Belousov et al. 2006), in CMs in *myl7*:Hyper transgenics. Pharmacological inhibition of Duox activity by diphenyleneiodonium (DPI) or apocynin reduced H_2O_2 production and CM proliferation at seven dpi. Prolonged treatment with DPI also compromised myocardial regeneration and resulted in scarring. These data suggest that H_2O_2 is required for CM proliferation and regeneration (Han et al. 2014).

Intriguingly, H_2O_2 was found to regulate CM proliferation and regeneration through its interaction with Erk1/2 signaling and its feedback regulator Dusp6 (Han et al. 2014). Dusp6 expression is stimulated by phosphorylated Erk1/2 (pErk1/2) but reciprocally deactivates pErk1/2 by dephosphorylation, hence forming a negative feedback loop that limits Erk1/2 activity (Nichols et al. 2000). H_2O_2, on the other hand, directly oxidizes Dusp6, which renders the protein prone for degradation, and hence leads to Erk1/2 overactivation (Chan et al. 2008). In zebrafish, both

phosphorylation of Erk1/2 and *dusp6* expression were found to be activated after ventricular resection (Han et al. 2014). DPI treatment in regenerating hearts induced Dusp6 stability and therefore reduced phosphorylation of Erk1/2. The effect of DPI on CM proliferation could be counteracted by co-treatment with BCI, a small molecule that inhibits phosphatase activity of Dusp6. In addition, systemic overexpression of Dusp6 in *hspl70*:dusp6 transgenics reduced pErk1/2 expression, and compromised CM proliferation, *gata4* activation and myocardial regeneration. These data strongly suggest that endogenous H_2O_2 regulates CM proliferation and regeneration by promoting Erk1/2 signaling through repressing Dusp6 activity (Han et al. 2014).

10.5.3.12 miRNAs

miRNAs have been implicated in various responses to MI in mammals, including cell death, proliferation, and metabolism (reviewed in Wang and Martin 2014). In zebrafish, several studies have demonstrated the importance of regulation of miRNAs for successful heart regeneration (Yin et al. 2012; Aguirre et al. 2014).

Using microarrays and real-time quantitative PCR (qPCR), Poss and colleagues found that a number of miRNAs are differentially regulated 7 days after ventricular resection, a large number of which have been shown to be modulated in injured mammalian heart (Yin et al. 2012). One of the identified miRNAs was the CM-specific miR-133, which had been found to negatively regulate zebrafish fin regeneration (Yin et al. 2008). *In situ* hybridization and qPCR analyses demonstrated that miR-133 levels decrease by seven and 14 dpi compared to uninjured hearts, and later return to baseline level at the completion of heart regeneration by 30 and 60 dpi, suggesting that downregulation of miR-133 levels could be important for heart regeneration (Yin et al. 2012). Indeed, loss- and gain-of-function experiments demonstrated that miR-133 negatively regulates CM proliferation and regeneration. Systemic inducible overexpression of the miR-133 precursor sequence increased the levels of mature miR-133 and reduced CM proliferation and regeneration at seven and 30 dpi, respectively (Yin et al. 2012). On the other hand, inducible overexpression of a miR-133 sponge RNA, which contained triplicate perfect binding sites for miR-133, reduced the levels of miR-133 and induced CM proliferation at seven and 30 dpi. Poss and colleagues further identified the gap junctional protein *connexin-43* (*cx43*) and the mitotic check point kinase *mps1* as targets of miR-133. While Mps1 mutants showed heart regeneration defects (Poss et al. 2002), treatment with carbenoxolone (CBX), an inhibitor of Cx43, reduced CM proliferation at seven dpi after ventricular resection (Yin et al. 2012). Together, these data demonstrate the importance of downregulation of miR-133 levels during heart regeneration, whose effect is mediated through, at least partly, Mps1 and Cx43 (Yin et al. 2012).

Yin and colleagues recently also described that downregulation of miR-101a after zebrafish heart injury is essential for CM proliferation (Beauchemin et al. 2015). In addition, this miR also seems to regulate transient scar removal. While its expression is downregulated shortly after injury, expression was found to actually

increase above levels in uninjured hearts between seven and 14 dpi. Depletion of miR-101a during this time using transgenic overexpression of a sponge construct resulted in increased scarring by 30 dpi (Beauchemin et al. 2015). Thus, miR-101a appears to play a dual role during heart regeneration, where its downregulation early allows CM proliferation, while increased levels later are required for scar removal.

The miRNAs miR-99/100 and Let-7a/c were also identified as important regulators of zebrafish heart regeneration and differential regulation of their expression was suggested as part of the reason why zebrafish can but adult mammals cannot regenerate their heart. Izpisua-Belmonte and colleagues showed that expression of these miRNAs is downregulated during zebrafish heart regeneration (Aguirre et al. 2014). Injection of miR-99/100 mimics into regenerating hearts reduced CM proliferation and compromised myocardial regeneration, which was suggested to be mediated through their targets beta subunit of farnesyl-transferase (Fnt) and SWI/SNF-related matrix associated actin-dependent regulator of chromatin subfamily a, member 5 (Smarca5). Interestingly, downregulation of miR-99/100 and Let-7a/c and subsequent upregulation of Fnt and Smarca5 was not observed in mouse hearts after MI (Aguirre et al. 2014). Blockage of both miRNAs in mouse after MI resulted in CM dedifferentiation and improved functional recovery (Aguirre et al. 2014). These data suggest that downregulation of miR-99/100 and Let-7a/c is an essential regenerative response, which is conserved but dormant in the mammalian heart. This study demonstrates the importance of zebrafish as a model for deciphering molecular mechanisms underlying natural heart regeneration which could be applied to heal the non-regenerating mammalian heart.

10.6 Conclusions

The zebrafish has rapidly developed over the last decade as a model for studying cardiac regeneration. Recent discoveries have greatly improved our understanding of how natural heart regeneration is regulated. After injury, developmental gene programs are activated globally in the endocardium and epicardium, which proliferate and contribute to the regenerated endocardial (including new vasculature) and epicardial cells in a lineage-restricted manner, respectively. Activation of these two layers precedes that of CMs and is likely to provide endocardial scaffold and epicardial covering of the wound to support muscle regeneration. In addition, these two layers also act as a source of pro-regenerative factors which affect CM proliferation and regeneration in a paracrine manner, likely including ligands of FGF, RA, Igf, Tgfß and Notch signaling, and Nrg1.

In response to injury, CMs have been shown to partially dedifferentiate, re-enter the cell cycle and proliferate to regenerate the lost muscle. Several signaling pathways have been identified as being required for regenerative CM proliferation, most of which appear to be also involved in regulating CM proliferation during embryonic development (RA, Hh, Igf, Tgfß), while injury-specific regulators have been identified as well (Jak1/Stat3, H_2O_2, Notch, BMP). Since most pathways have been

studied using systemic interference, the strongest evidence for a direct role on CMs exists for Jak1/Stat3 and BMP signaling; cell type-specific pathway manipulation will be required in the future to clarify cellular interactions during heart regeneration. Currently, heart regeneration studies in adult zebrafish are also limited by the lack of inducible genetic loss-of-function systems. Hopefully, in the near future, genome-editing tools like CRISPR/Cas9 will enable zebrafish researchers to induce tissue-specific genetic deletion of genes to further dissect the requirement of various factors or signaling pathway components during heart regeneration. Together with rapidly developing transcriptomic, proteomic and epigenomic tools, future studies promise to provide insights to important outstanding questions such as: (1) Why do zebrafish but not mammalian CMs retain the ability to proliferate throughout life? (2) How are regenerative responses initiated in the zebrafish heart? (3) What are the underlying mechanisms of scar removal in zebrafish? We believe the zebrafish will continue to thrive as an important model to understand natural heart regeneration, which might provide valuable insights to refine strategies for improving mammalian cardiac repair.

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Chapter 11
Genetics and Regeneration in Vertebrates

Elizabeth D. Hutchins and Kenro Kusumi

Abstract Regeneration is a common trait in vertebrates, with regrowth of entire appendages carried out by a number of groups including teleost fish, amphibians, and squamate reptiles. While humans are also vertebrates, we have very limited ability to regenerate as adults. Cellular and molecular studies in zebrafish, *Xenopus* frog, axolotl, and green anole lizard model systems have identified components of genetic programs for regeneration that include both developmental and adult repair mechanisms shared with mammals. Regeneration in vertebrates involves the genetic regulation of wound epithelium formation, modulation of the immune response, remodeling of the extracellular matrix, patterning of the regrowing appendage, and activation of Wnt/β-catenin and FGF signaling pathways. By understanding the mechanisms by which vertebrates are able to regenerate their appendages, we can translate these processes to develop clinically relevant regenerative therapies.

Keywords Vertebrate • Tetrapods • Non-tetrapod vertebrate • Teleost fish • Amphibians • Squamate reptiles • Zebrafish • *Xenopus* frog • Axolotl • *Ambystoma mexicanum* • Mammals • Inflammation • Immune response • Extracellular matrix • Regeneration

11.1 Introduction

Regeneration is a common trait in vertebrates, with regrowth of entire appendages carried out by teleost fish, amphibians, and squamate reptiles (Agata and Inoue 2012; Bely and Nyberg 2010). However, the capacity of an animal to regenerate an appendage can vary between different periods of its lifespan and between anatomical structures. All these vertebrate groups share a common ancestor and their genomes reflect this common heritage in sharing multiple homologous genetic pathways that regulate developmental patterning and differentiation (Cañestro et al. 2007). Researchers have developed resources to study development in a number of

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model systems, and these species have been used for genetic studies of regeneration. A number of the models are tetrapods, or vertebrate animals evolved for life on land, with changes in the anatomy and function of the limbs, spinal column, and cardiovascular system (Table 11.1). Non-tetrapod vertebrates include teleost fishes, and among that group, the zebrafish (*Danio rerio*) has emerged as a major developmental genetic model. The first branch of the tetrapod group is the amphibians, which include salamanders (order Caudata and also referred to as urodeles) and frogs (order Anura and also referred to as anurans; Table 11.1). Urodele amphibians and teleost fish are capable of regenerating their tails and fins following amputation (Gemberling et al. 2013; Stocum and Cameron 2011). In anuran amphibians such as the *Xenopus* frog, tail and fin regeneration is observed in tadpole stages but following metamorphosis, adults regenerate a spike-like, cartilaginous structure as a replacement limb (Endo et al. 2000).

Amniotes, or non-amphibian tetrapods, evolved approximately 325 million years ago with further adaptations to embryos, skin, and other structures to life on land (Donoghue and Benton 2007; Pyron 2010; Shedlock and Edwards 2009). There are two main groups of amniotes–the synapsids, which includes modern day mammals, and the sauropsids, which encompasses modern day reptiles (including lizards, snakes, turtles, alligators) and birds. Regeneration is a common trait in lizards (Fisher et al. 2012; Gilbert et al. 2013b; McLean and Vickaryous 2011; Ritzman et al. 2012). In alligators, tail regeneration has been reported in the field, though the structure and regenerative mechanism are unknown (Bellairs and Bryant 1985; Han et al. 2005). In contrast, birds have very limited regenerative capacity, as do mammals. Regenerative capacity in mammals is namely limited to the neonatal and juvenile stages, including the ability to regrow digit tips (Borgens 1982; Han et al. 2008; Illingworth 1974; Singer et al. 1987). Additionally, some species such as the African spiny mice (*Acomys*) can lose and regenerate large amounts of skin (Seifert et al. 2012; Table 11.1).

Next-generation sequencing technologies developed following the human genome project (Lander et al. 2001) have enabled the whole genome sequencing of many vertebrate species, including squamate reptiles, frogs, and several species of teleost fish (Tollis et al. 2015). This now permits researchers to utilize comparative genomics to search for the origins of various biological functions, including complex processes such as regeneration. Below, we will outline the commonalities and differences in regenerative capacity in key model systems, and afterwards outline what we know about the biological processes involved in regeneration and their genetic regulation.

Table 11.1 Vertebrate models used for analysis of regeneration at the cellular, molecular, and genomic levels

Amniote	Class	Order	Species	Common name	Tail regeneration	Limb/fin regeneration	Other regeneration
Yes	Mammalia	Rodentia	*Mus musculus*	Mouse			Digit tips *(neonatal stages)*
Yes	Mammalia	Rodentia	*Acomys kempi*	African spiny mouse			Skin *(regeneration of up to 60 % of dorsal skin)*
			A. percivali				
Yes	Mammalia	Primates	*Macaca mullata*	Rhesus monkey			Digit tips (*neonatal stages)*
Yes	Mammalia	Primates	*Homo sapiens*	Human			Digit tips *(neonatal stages)*
Yes	Reptilia	Squamata	*Anolis carolinensis*	Green anole	Yes		
			Anolis sagrei	Brown anole			
Yes	Reptilia	Squamata	*Eublepharis macularius*	Leopard gecko	Yes		
No	Amphibia	Caudata	*Ambystoma mexicanum*	Axolotl	Yes	Yes	Spinal cord, brain, retina, jaw, and heart
No	Amphibia	Caudata	*Notophthalmus viridescens*	Eastern newt	Yes	Yes	Spinal cord, brain, retina, jaw, heart, and lens
No	Amphibia	Anura	*Xenopus laevis*	African clawed frog western Clawed frog	Yes *(at tadpole stage)*	Yes (Regeneration at tadpole stages. At *adult stages, the regenerated appendage lacks digits and has a central cartilage skeleton)*	
			Xenopus tropicalis				
No	Actinopterygii	Cypriniformes	*Danio rerio*	Zebrafish	Yes	Yes	Spinal cord, brain, retina, heart

11.2 Comparison of Regeneration in Vertebrates

11.2.1 Teleost Fish: Zebrafish

Zebrafish fins are complex, segmented appendages containing dermal bone surrounded by vascularized, innervated mesenchymal tissue. Following amputation, the fin ray is (1) covered by an epidermis within a few hours, followed by (2) formation of a mass of proliferative cells underlying the epidermis, called a blastema, within 1–2 days, and finally (3) differentiation and regenerative outgrowth (Haas 1962; Géraudie and Singer 1992; Nabrit 1929; Santamaría and Becerra 1991). This blastema consists of proliferative, morphologically similar cells that originated from the amputation stump via disorganization of osteoblasts and fibroblasts. Cre recombinase-based lineage analysis has revealed that osteoblasts in the stump dedifferentiate, migrate distally and contribute to the proliferating blastema. They then contribute in a lineage-specific fashion to osteoblasts in the regenerated fin (Knopf et al. 2011; Singh et al. 2012; Sousa et al. 2011; Stewart and Stankunas 2012; Tu and Johnson 2011). Similarly, the endothelium, epidermis, and fibroblasts are also lineage restricted (Tu and Johnson 2011). Curiously, however, the zebrafish fin can regenerate following osteoblast ablation, suggesting that there may be an additional source of cells (Singh et al. 2012). During fin outgrowth, FGF signaling interactions between the epidermis and underlying mesenchyme maintain the proliferative cells at the distal portion of the regenerating fin and promote outgrowth (Lee et al. 2009).

In addition to regenerating amputated fins, zebrafish can regenerate spinal cord, brain, retina, and heart (Becker et al. 1997; Cameron 2000; Kizil et al. 2012; Otteson and Hitchcock 2003; Poss et al. 2002; Reimer et al. 2008). Microarray-based transcriptome studies have been carried out for zebrafish spinal cord regeneration, and proliferation and outgrowth is mediated by *sox2* expression in the neuroependymal cells (Hui et al. 2014; Ogai et al. 2014). Due to the developmental and genetic tools available for the zebrafish model, rapid progress is being made on understanding regenerative mechanisms in this species.

11.2.2 Urodele Amphibians: Axolotl and Newt

Urodele amphibians are widely studied for their highly developed regenerative abilities. Juvenile and adult animals are capable of regenerating many tissues that are nearly identical to the original, including limbs, jaw, tail, retina, spinal cord, heart, and brain (Maden et al. 2013; Table 11.1). A key urodele model species for limb regeneration studies has been the axolotl (*Ambystoma mexicanum*), and regrowth of the limb has been a major area of research.

The limb is a complex biomechanical structure, and regeneration involves formation of many tissue types including dermis, epidermis, muscle, cartilage, nerve, vasculature, and other skeletal elements. After amputation, a key regenerative

structure forms that is known as the "blastema." The blastema was originally defined in amphibians, but subsequent studies have shown that the structure differs between vertebrate groups and its definition is evolving as we learn more about progenitor cell origins. In amphibians, this mass of progenitor cells arises from either dedifferentiation of mature tissues (newt) or activated stem cells residing in these tissues (axolotl and *Xenopus* frog), is in a proliferative state, expresses developmental genes, and is induced by signaling interactions with the overlaying wound epithelium (Brockes and Kumar 2008; Han et al. 2005; Morrison et al. 2006; Satoh et al. 2008, 2012).

Based on transcriptomic and cellular studies in the axolotl model, investigators have been able to divide the process of salamander limb regeneration into three phases: (1) formation of an epithelium and scar-free wound healing, (2) activation of progenitor cells, and (3) patterning and differentiation of structures in the regrowing limb (Bryant et al. 2002; Knapp et al. 2013). During the wound healing phase, which begins immediately following amputation, epidermal cells migrate over the wound stump and form a new layer of epidermis. These epidermal cells do not contribute to the blastema (Carlson et al. 1998; Hay and Fischman 1961; Namenwirth 1974; Satoh et al. 2008). Following wound healing, progenitor cells are activated and form a blastema underneath the epidermis. Regenerative success is dependent upon signaling from the damaged nerve during this phase (Kumar and Brockes 2012; Satoh et al. 2009; Stocum and Cameron 2011).

Historically, the blastema was defined as being populated by cells generated via dedifferentiation (Butler and O'Brien 1942; Echeverri and Tanaka 2002; Hay and Fischman 1961; Namenwirth 1974; Thornton 1938; Wallace and Wallace 1973). Recent studies, however, demonstrated that both dedifferentiation of tissues adjacent to the stump and activation of tissue resident stem cells contribute to the blastemal pool of progenitor cells present in the regenerating limb (Tanaka and Reddien 2011) and that these progenitor cells retain a memory of their tissue or embryonic origin (Kragl et al. 2009; Morrison et al. 2010; Sandoval-Guzmán et al. 2014). Cells that dedifferentiate from the dermis display the greatest plasticity, contributing to regenerated tissues of the cartilage and tendons, which all derive embryonically from the lateral plate mesoderm. Some dedifferentiated cells have only a single potential. For example, Schwann cells, which form the myelin sheaths surrounding peripheral motor axons, appear to only give rise to Schwann cells, even when rescuing irradiated limbs that would not otherwise regenerate. In addition, the regenerated skeletal myofibers were found to derive from the adult skeletal muscle, without contribution from other tissues (Kragl et al. 2009).

Lineage tracing experiments of skeletal muscle cells comparing the eastern newt (*Notophthalmus viridescens)* with the axolotl have identified differences between these two urodele amphibians in terms of dedifferentiation and stem cell-mediated regeneration. In the eastern newt, muscle progenitor cells in the blastema arise from both dedifferentiated cells from local skeletal muscle fibers as well as migrating PAX7-positive satellite cells from nearby tissue. In contrast, all of the muscle progenitor cells in the blastema of the axolotl arise from PAX7-positive satellite cells, and there is no evidence of progenitor cells deriving from dedifferentiation (Morrison

et al. 2010; Sandoval-Guzmán et al. 2014). Sequencing studies in the newt pointed to a large number of proteins unique to urodele amphibians, suggesting that new genes may have evolved that are responsible for the high regenerative capacity (Looso et al. 2013). However, a complete genome sequence of the eastern newt, estimated to be 30 billion base-pairs (Mihaylova and Aboobaker 2013) is not yet available, and this may complicate assignment of orthologous genes.

The final phase of limb regeneration in salamanders has been termed "re-development" because of the recapitulation of patterning and differentiation that takes place in embryonic limb development, including the regulation by FGF, sonic hedgehog, and Hox pathways (Hutchison et al. 2007; Muneoka and Bryant 1982; Nacu and Tanaka 2011; Roy and Gardiner 2002; Roy et al. 2000; Torok et al. 1998). The wound epithelium that forms in the regenerating limb is referred to as the apical epithelial cap (AEC) and is analogous to the FGF8 expressing apical ectodermal ridge (AER) in limb bud development (Christensen and Tassava 2000; Christensen et al. 2002; Han et al. 2005). Advances in genetic studies of regeneration in the axolotl are limited by the lack of a complete genome sequence. The predicted genome sizes of urodeles are quite large, with estimates of 13–30 billion base-pairs (Mihaylova and Aboobaker 2013).

11.2.3 Anuran Amphibians: Xenopus *Frogs and Tadpoles*

Studies of regenerative capacity in anuran amphibians have primarily focused on the model species *Xenopus laevis* and *X. tropicalis*. Regeneration in *Xenopus* frogs is temporally constrained to the period prior to metamorphosis (Table 11.1). Prior to metamorphosis, *Xenopus* tadpoles use their tails for locomotion, but during metamorphosis, this tail is resorbed by adult stages. In tadpoles, amputation of the tail is followed by regeneration of a fully functional replacement with coordinated swimming by 7 weeks (Gaete et al. 2012). This regenerated tail contains spinal cord, notochord, smooth and skeletal muscle, vasculature, and skin (Love et al. 2011; Slack et al. 2007). Though the regenerated tail is functional, it lacks the segmented, chevron shaped skeletal muscle groups that derive from the embryonic process of somitogenesis, and the associated segmental structures such peripheral nerve axons and vasculature (Love et al. 2011; Slack et al. 2007).

The process of tadpole tail regeneration has been categorized into three major phases: (1) acute inflammatory response, (2) cell proliferation, and (3) regrowth of differentiating tissues, including neurons, notochord, muscle, and vasculature (Love et al. 2011). In anuran amphibians, in contrast to urodele amphibians, there is no evidence that transdifferentiation produces multipotent cells contributing to the regenerated tissue. Cell lineage tracing indicates that the tadpole spinal cord regenerates from adjacent spinal cord cells in the stump and, similarly, the notochord regenerates from adjacent notochordal cells in the stump (Gargioli and Slack 2004). Instead of arising from dedifferentiation of pre-existing myofibers, the regenerating myofibers originate from PAX7-positive satellite progenitor cells in adjacent muscle

tissue that migrate into the regenerating tail (Gargioli and Slack 2004; Ryffel 2003). During the larval stage, tadpoles regenerate their tails from stage 40 until metamorphosis with the exception of a transient refractory period around stage 46–47 (Beck et al. 2003; Bosco 1979). The genetic mechanisms regulating the shutdown of regenerative capacity during this brief refractory period remain unknown.

While tadpoles can regenerate their tails, the capacity of *Xenopus* frogs to regenerate their forelimbs declines as animals approach metamorphosis (Dent 1962). At the onset of metamorphosis, regenerative ability gradually declines from stage 55 to stage 60. *Xenopus* froglets can regrow a patterned limb with digits following amputation at stage 53. Whereas salamanders are able to regenerate forelimbs or hindlimbs as adults, *Xenopus* frogs do not demonstrate scar-free wound healing (Godwin and Rosenthal 2014). After metamorphosis, *X. laevis* froglets regenerate a spike-like, cartilaginous structure without digits or proximal-distal joints following limb amputation (Dent 1962; Endo et al. 2000; Korneluk and Liversage 1984). This regenerated appendage is radially symmetrical in shape, reflecting the lack of dorsal-ventral and left-right asymmetry found in the original forelimb (Endo et al. 2000; Ohgo et al. 2010; Satoh et al. 2005; Yakushiji et al. 2007). Additionally, the regenerated appendage spike was not reported to have any myofibers, even though there are PAX7+ positive myofibers in the adjacent stump that could contribute satellite progenitor cells (Satoh et al. 2005). Following amputation of the forelimb, (1) a wound epithelium is established, (2) proliferative mesenchymal cells accumulate at the distal tip, and (3) cells differentiate and the limb grows outward (Endo et al. 2000). In adults, the formation of proliferative cells at the tip of the regenerating limb and its regenerative outgrowth are dependent on nerve innervation (Endo et al. 2000; Korneluk and Anderson 1982; Suzuki et al. 2005). In contrast, tadpoles are capable of forming limb buds even following resection of a limb nerve (Filoni and Paglialunga 1990).

Though the adult regenerative capacity of anuran amphibians does not reach that of urodele amphibians such as the eastern newt or axolotl, *Xenopus* frogs are an informative regenerative model for two reasons (Beck et al. 2009). First, the decline in regenerative capability over developmental time has many parallels with the changes in mammals (see below). Second, *Xenopus* frogs have been a classic developmental and genetic model for over a century, and a large and interdisciplinary research community has generated a number of resources that would advance regenerative biological studies. As a biomedical model, genetic commonalities between *Xenopus* and humans have been extensively investigated.

11.2.4 Squamate Reptiles: Lizards

Lizards are the most closely related group to humans that can regenerate appendages (Fig. 11.1). Many lizard species are capable of tail autotomy and/or regeneration (Bellairs and Bryant 1985). Autotomy is a self-amputation process induced by physiological and/or mechanical stress whereby the lizard sheds its tail as a

Fig. 11.1 Image of a green anole lizard with a fully regenerated tail (*arrow at break point*) (Photograph by Joel Robertson)

mechanism of predator evasion. The caudal vertebrae of many lizards possess fracture planes in the middle of the centrum to facilitate autotomy (Simpson 1968). Following autotomy, the amputated tail continues to move, serving to distract a predator while the lizard is able to escape (Dial and Fitzpatrick 1983). Studies in the leopard gecko (*Eublepharis macularius*) suggest that tail regeneration does not depend on autotomy. Tails can regenerate regardless of proximity to the fracture plane and whether or not the tail is removed naturally via autotomy or mechanically amputated (Delorme et al. 2012).

There have been a number of studies describing tail regeneration in lizards. Research published within the past decade has focused on the green anole (*Anolis carolinensis*) (Eckalbar et al. 2013; Fisher et al. 2012; Hutchins et al. 2014; Ritzman et al. 2012) and the leopard gecko (*Eublepharis macularius*) (Delorme et al. 2012; Gilbert et al. 2013a, b; McLean and Vickaryous 2011) as model organisms. The green anole has recently been used a model for studies of development (Eckalbar et al. 2012; Sanger et al. 2008), population genetics (Tollis and Boissinot 2014; Wordley et al. 2011), reproductive physiology and behavior (Lovern and Wade 2003; Wade 2012), and functional morphology (Montuelle 2009; Ritzman et al. 2012). As such, it was the first non-avian reptile to have its genome sequenced (Alföldi et al. 2011), permitting molecular genetic studies of regeneration. An extensive database of historical studies of regeneration of the green anole (Alibardi 1995a, b, 2010a, 2014a, b; Chlebowski et al. 1973; Cox 1968; Egar et al. 1970; Kamrin and Singer 1955; Maderson and Licht 1968; Simpson 1968; Turner and Singer 1973; Zika 1969) informs molecular, cellular, and anatomical analysis published recently (Fisher et al. 2012; Hutchins et al. 2014; Ritzman et al. 2012).

While the regenerated tail of lizards serves as a biomechanical replacement, it is not anatomically identical, reflecting patterning and differentiation processes distinct from embryonic development (Fisher et al. 2012; Ritzman et al. 2012). The regenerated tail consists of a central, unsegmented cartilaginous tube. The regenerated spinal cord runs through the center of this tube, and new muscle groups are arranged in a radially organized fashion around the outside of the cartilaginous endoskeleton. The proximal region of the cartilage tube undergoes endochondral ossification at the boundary with the original tail skeleton (Lozito and Tuan 2015). The regenerated spinal cord has a different structure as well and consists of an ependymal tube that is continuous with the original spinal cord but lacks the surrounding white matter (axons) and gray matter (cell bodies) (Fisher et al. 2012; Gilbert et al. 2013a). Dorsal root ganglia for the peripheral sensory system are not regenerated, and the regenerating tail is innervated from immediately proximal ganglia within the original tail stump (Duffy et al. 1990; Egar et al. 1970). Anatomically, the original tail possesses equally spaced, interdigitated myomeres along the length of the tail, while the regenerated tail consists of longitudinal myomeres of varying size that are radially organized (Ritzman et al. 2012). Histologically, these regenerated myomeres possess unique tendinous attachments, and the distribution of connective tissue is distinct from the original tail. Additionally, there are irregularly spaced foramina in the cartilage tube that transmit vasculature but not nerves (Fisher et al. 2012). This structure is more rigid than the original tail, suggesting that the regenerated tail is less capable of controlled, fine-scale movements.

Following autotomy, where the tail vertebra breaks about halfway through at the fracture plane, there is (1) formation of a blood clot and ECM remodeling associated with contraction of tissues in the stump, (2) formation of the wound epithelium and ablation of the scab, (3) formation of proliferating cells, angiogenesis, and thickening of the wound epithelium, and (4) outgrowth and differentiation of mature tissues, including myofibers, cartilage, and the neuroependyma (Lozito and Tuan 2015; McLean and Vickaryous 2011; Nambiar et al. 2008). While blastema formation is fairly well characterized during limb and fin regeneration in amphibians and teleost fish, lizards follow a distinct mechanism of regeneration, though the source of cells is still relatively unknown. In the newt model, a blastema is traditionally characterized by dedifferentiation of tissue, the formation of a mass of pluripotent proliferating cells focused at the tip of the regenerating appendage, and the absence of a vascular bed at the distal tip (Butler and O'Brien 1942; Echeverri and Tanaka 2002; Hay and Fischman 1961; Iten and Bryant 1973; Mescher 1996; Namenwirth 1974; Peadon and Singer 1966; Singer 1974; Smith and Wolpert 1975; Thornton 1938; Wallace and Wallace 1973). Unlike the newt, but in common with the axolotl and *Xenopus* frog, there is no evidence of dedifferentiation in tail regeneration of the lizard (Cox 1969; Hughes and New 1959; Kahn and Simpson 1974; Simpson 1970). Additionally, in the leopard gecko, proliferating cells are present throughout the regenerating tail, rather than localized at the distal tip, and the distal tip is highly vascular (McLean and Vickaryous 2011). As in *Xenopus* frog tadpoles and salamanders, regeneration in lizards is nerve-dependent. Damage to the spinal cord immediately proximal to the regenerating tail inhibits regeneration (Kamrin and Singer 1955; Simpson 1964; Whimster 1978). The ependymal cells of the spinal

cord, which lie at the core of the regenerating tail, provide positional identity to the forming appendage (Wang et al. 2011).

Building on a whole genome assembly and annotation available for the green anole lizard, RNA-sequencing based analysis has identified 326 genes that are differentially expressed within the regrowing tail (Hutchins et al. 2014). This group includes genes regulating wound response, thyroid hormonal response, muscle and cartilage development, and genes in the Wnt and FGF/MAPK pathways. Genes in the Wnt-Ca2+ pathway have been identified to play a role in both modulation of the inflammatory response as well as regeneration in a number of vertebrates (Kim et al. 2010; Knapp et al. 2013; Sugiura et al. 2009). In addition, PAX7+ satellite progenitor cells were isolated from green anole skeletal muscle that could contribute to regenerating tissue (Hutchins et al. 2014). The regenerated lizard tail is an impressive example of formation of hyaline/articular cartilage, *de novo* muscle groups, skin, vasculature, and neural ependymal cells (Fisher et al. 2012; Gilbert et al. 2013a; McLean and Vickaryous 2011; Ritzman et al. 2012). Hyaline/articular cartilage does not typically regenerate in the adult humans, and degradation within synovial joints leads to clinical disorders including osteoarthritis. Continuing genetic, molecular, and cellular studies in the lizard should provide more information about the source of cells in the regenerating tail.

11.2.5 Mammals: Mouse and Human

Mammals have highly limited regenerative capacity, with regeneration highest at the neonatal period. Neonatal mice (*Mus musculus*), human children, and juvenile Rhesus monkeys (*Macaca mulatta*) can regenerate amputated digit tips (Borgens 1982; Douglas 1972; Illingworth 1974; Singer et al. 1987; Vidal and Dickson 1993). There have also been some clinical studies of digit tip regeneration in adults, with either the placement of a Hyphecan, a membrane-like cap made from chitin, over the wound (Halim et al. 1998; Lee et al. 1995) or application of a extracellular matrix to facilitate cartilage and bone regeneration (Benders et al. 2013) following wound debridement. In both mouse and human, regeneration occurs following amputation of the terminal phalanx only and is dependent on whether or not the amputation level includes the nail organ (Borgens 1982; Mohammad et al. 1999; Neufeld and Zhao 1995). Specifically, the extent of new bone in the regrown structure and therefore the length of the regenerated fingertip are dependent upon level of amputation (Han et al. 2008; Neufeld and Zhao 1995). One main difference between mammals and regeneration in other vertebrates is that in humans and mice, innervation is not necessary for digit tip regeneration (Mohammad and Neufeld 2000).

Regenerated digit tips contain multiple tissues including bone, connective tissue and tendons, the nail organ, hair follicles, and skin (Rinkevich et al. 2011; Said et al. 2004), and these structures are innervated and highly vascularized. Following amputation of the digit tip, the following stages are involved in mammalian digit tip

regeneration: (1) wound closure occurs in a few days and osteoclasts erode the bone in the stump, (2) a mass of proliferative cells, which originate from connective tissue cells migrating across the wound and marrow in the skeletal stump, is formed, and (3) cells differentiate into mature tissues in the regenerate (Fernando et al. 2011; Han et al. 2008; Lehoczky et al. 2011; Muneoka et al. 2008; Neufeld and Zhao 1995; Revardel and Chebouki 1987). As in zebrafish, *Xenopus* frogs, and green anole lizards, there is no evidence that transdifferentiation occurs to generate cells for regeneration. The stem/progenitor cells that contribute to regeneration are lineage-specific, i.e., epidermal stem cells and mature keratinocytes originate from the ectodermal tissue overlying the stump, and bone is derived from pre-amputation, mesodermal, osteoblasts (Lehoczky et al. 2011; Rinkevich et al. 2011). Given that mammals retain some regenerative capacity at neonatal stages, this points to the need to understand the genetic mechanisms required to maintain this capacity into adulthood.

11.3 Genetic Regulation of Regeneration in Vertebrates

Given that regeneration is a common trait in vertebrates, the question arises as to whether this capacity existed in the common vertebrate ancestor of whether it arose independently in multiple lineages (Bely and Nyberg 2010). The answer to this question is very relevant for regenerative medicine. If regeneration was a trait of a common ancestor to zebrafish, axolotl, frogs, lizards, and humans, then there would be expected to be a common genetic toolkit involved in the process. Evolution of vertebrate group-specific features may have arisen in each lineage, but this would give researchers a starting point for translating findings from model species for medical treatments. Even if regeneration arose multiple times in vertebrate evolution, they may share activation of common pathways that regulate key processes such as scar-free wound healing to form a wound epithelium, patterning of the regenerating appendage (dorsal-ventral, left-right, and proximal-distal axes), remodeling of ECM facilitated by matrix metalloproteinases, modulation of immune response genes, and activation of regulatory developmental signaling pathways, namely Wnt/β-catenin and FGF. In the section below, studies from comparison of regenerative model species will be presented.

11.3.1 Wound Epithelium Formation

Before regenerative outgrowth can occur, a wound epithelium forms over the damaged tissue as part of the scar-free wound healing process (Campbell and Crews 2008; Murawala et al. 2012; Takeo et al. 2015). Clinical cases of regeneration in adult digit tips involve proper debridement and placement of a substitute wound epithelium made of chitin over the wound, underlying the overall significance of the

presence of a wound epithelium (Halim et al. 1998; Lee et al. 1995). In the lizard and newt, the wound epithelium thickens to twice the size of the original epidermis (Delorme et al. 2012; Lozito and Tuan 2015; McLean and Vickaryous 2011). This structure is called the apical epithelial cap (AEC) in the newt to mimic the apical ectodermal ridge (AER) formed during limb development (Christensen et al. 2002; Christensen and Tassava 2000; Han et al. 2001). In addition to providing a covering for the wound, signaling between the epithelium and underlying mesenchyme promotes proliferation and outgrowth during regeneration in the frog, salamander, and zebrafish (Christensen et al. 2002; Ghosh et al. 2008; Han et al. 2001; Kawakami et al. 2006; Lee et al. 2009; Poss et al. 2000; Yokoyama et al. 2011).

11.3.2 Modulation of Immune Response

Modulation of inflammation and the immune response is a key first step scar-free healing required for regeneration (Fahmy and Sicard 2002; Godwin and Brockes 2006; Godwin and Rosenthal 2014; Godwin et al. 2013; Harty et al. 2003; Mescher and Neff 2006). Gene expression studies of the regenerating axolotl limb (Knapp et al. 2013; Monaghan et al. 2012; Stewart et al. 2013), regenerating tissues of newt (Mercola 2012), and tadpole tail in *X. tropicalis* (Love et al. 2011), have identified immune response genes that are activated during regeneration, as early as 6 h following amputation (Love et al. 2011). Following tail autotomy in the Italian wall lizard (*Podarcis sicula*), granulocytes and monocytes/macrophages are observed at the site of tail loss and participate in wound healing (Alibardi 2010b; Alibardi et al. 2012). Macrophages in particular are of interest, as they are a source of inflammatory and anti-inflammatory signals, regulating ECM remodeling, fibroblast formation, angiogenesis, and peripheral nerve innervation in wound repair (Barron and Wynn 2011; Lucas et al. 2010; Martini et al. 2008; Nucera et al. 2011). By controlling the inflammatory response, macrophages directly regulate repair and regeneration (Delavary et al. 2011). Cytokines may regulate many genes, including those involved in the cell cycle (Zhang et al. 2014), and they regulate proliferation of multiple cell types, namely that of fibroblasts, keratinocytes, and endothelial cells (Delavary et al. 2011). Additionally, macrophages stimulate the production of soluble effector molecules, including platelet-derived growth factors (PDGFs), insulin-like growth factors (IGFs), hepatocyte growth factors (HGFs), fibroblast growth factors (FGFs), transforming growth factors (TGFs), colony-stimulating factors (CSFs), Wnt ligands, and other molecules related to the immune system (Stefater et al. 2011). Both pro-inflammatory and anti-inflammatory signals are upregulated simultaneously during regeneration in salamanders and *Xenopus* tadpoles, suggesting that the balance of the inflammatory response is important for successful regenerative outcomes (Godwin et al. 2013; King et al. 2012). Depletion of macrophages in the axolotl prior to limb amputation results in collagen deposition and formation of a thick scar at the limb stump instead of regeneration, while depletion of macrophages during regeneration prior to regenerative outgrowth results in a delay in regeneration (Godwin et al. 2013).

In mammalian tissue repair, macrophages arrive at wound sites 24–48 h following injury and are responsible for clearing dead cells and releasing proinflammatory cytokines. Macrophages reduce inflammation and stimulate angiogenesis, fibroblast migration, and replication (Park and Barbul 2004). Macrophage depletion or transcriptional repression during muscle repair in mammals results in a fibrotic response (Ruffell et al. 2009; Tidball and Wehling-Henricks 2007). In axolotl limb regeneration, macrophage depletion in the first 24 h following limb amputation results in wound closure with regenerative failure associated with fibrosis and dysregulation of genes regulating extracellular matrix formation. When endogenous macrophages have been restored, regeneration is restored as well following reamputation, indicating particular importance of the macrophage response in regeneration (Godwin et al. 2013). In summary, regeneration in vertebrates follows a two-step model, with a permissive immunological environment as a requisite first step.

11.3.3 Remodeling of the Extracellular Matrix

Before regeneration can occur, there must be remodeling of the severed tissues. This process destabilizes the extracellular matrix (ECM) that provided scaffolding for differentiated tissues in the original appendage, allowing for new matrix to be created for differentiating cells in the regenerated appendage (Stocum and Cameron 2011; Yokoyama 2008). Additionally, this remodeling response is thought to be a component for upregulation of a scar-free wound healing in regenerative organisms as opposed to a fibrotic response (Godwin et al. 2014; Vinarsky et al. 2005). Matrix metalloproteases (MMPs) were initially discovered for their degradation of collagen during tadpole metamorphosis (Gross and Lapiere 1962) and have since been studied for the degradation of ECM components in developmental and disease systems (Galliera et al. 2015; Paiva and Granjeiro 2014). MMPs have been identified as playing a role in regeneration in a number of species, including the axolotl, *Xenopus* frog, and anole lizard. Following autotomy in the anole lizard, the tissues in the stump regress and osteoclasts degrade the distal half of the exposed vertebra (Lozito and Tuan 2015). The protease MMP9 is present in regenerating leopard gecko tail and most likely contributes to this remodeling phase (Delorme et al. 2012). In the *Xenopus* frog, *mmp7* is expressed within 6 h of tadpole tail amputation (Love et al. 2011). MMPs are highly expressed during salamander limb regeneration within hours of limb amputation, and are required for regeneration. Tissue remodeling genes continue to be expressed prior to outgrowth of the limb (Kato et al. 2003; Knapp et al. 2013; Monaghan et al. 2009; Stewart et al. 2013; Vinarsky et al. 2005; Yang and Bryant 1994; Yang et al. 1999). Microarray analysis of multiple regenerative tissues, including spinal cord, heart, tail, forelimb, and hindlimb, in the eastern newt identified multiple MMPs induced early in the regenerative response, with *mmp-13* upregulated in all regenerating newt tissues examined (Mercer et al. 2012).

11.3.4 Patterning of the Regenerated Appendage

Similarly to the development of the embryonic appendage, patterning is crucial to successful regeneration. Some positional cues in vertebrate regeneration are known, however many remain elusive. Genetic screening of non-regenerating mutants in the zebrafish identified a number of factors involved in patterning (Makino et al. 2005; Nechiporuk et al. 2003; Poss et al. 2002; Whitehead et al. 2005), and microarray analysis of spinal cord regeneration identified a number of genes responsible for dorsal-ventral patterning, including those involved in the establishment of SHH gradients, and anterior-posterior patterning, including Hox genes (Hui et al. 2014). The symmetrical shape of the forelimb spike in the *X. laevis* froglet reflects lack of a radial organization of the regenerated structure. Epigenetic modification of the limb-specific enhancer MFCS1 inhibits *shh* activation, disrupting patterning of the anterior-posterior axis (Endo et al. 2000; Satoh et al. 2006; Yakushiji et al. 2007). Inhibition of SHH signaling by cyclopamine in the axolotl produces a similarly shaped digitless phenotype (Roy and Gardiner 2002). Hox genes *hoxa11* and *hoxa13* specify the autopodial and zeudopodial regions during *Xenopus* tadpole limb bud regeneration but are misexpressed during froglet limb regeneration where they fail to separate, disrupting proximal-distal patterning (Endo et al. 2000; Ohgo et al. 2010). Hox genes also specify proximal-distal limb regions in the axolotl, though gene expression in the regenerating limb differs from expression during development (Torok et al. 1998). In the gecko lizard, positional identity of the proximal-distal axis in the regenerating tail is regulated by retinoic acid (RA) regulation of CD59 (Wang et al. 2011). Cd59 contains conserved motifs with Prod1, which determines proximal-distal positional identity in limb regeneration in the newt (Da Silva et al. 2002). Further genetic analysis of regenerating appendages in vertebrates should help to identify genes involved in patterning during vertebrate regeneration.

11.3.5 Activation of Wnt/β-catenin and FGF Signaling Pathways in Vertebrate Regeneration

Wnt/β-catenin signaling plays a central role in regulating vertebrate regeneration. Gene expression studies of axolotl limb regeneration have identified Wnt/β-catenin, BMP, and FGF signaling as playing key roles in wound healing, cell proliferation/blastema formation, and "re-development" of the limb (Kawakami et al. 2006; Knapp et al. 2013; Makanae et al. 2014; Satoh et al. 2011; Wu et al. 2013; Yokoyama et al. 2007). Wnt/β-catenin signaling is necessary for limb regeneration in the axolotl, developing limb and tail regeneration in *Xenopus* tadpoles, and fin regeneration in zebrafish (Ghosh et al. 2008; Kawakami et al. 2006; Sugiura et al. 2009; Yokoyama et al. 2007). Functional studies in zebrafish implicate Wnt/β-catenin signaling as a sort of control network in the regenerating fin that regulates proliferation and pattering through regulation of other pathways, including FGF, BMP, and

Hedgehog signaling (Wehner et al. 2014). While Wnt/β-catenin and FGF signaling promote blastemal proliferation and outgrowth, non-canonical Wnt signaling inhibits these processes (Lee et al. 2005; Stoick-Cooper et al. 2006).

Activation of Wnt/β-catenin and FGF signaling can also enhance regenerative capabilities. *Xenopus* froglets grew limbs with multiple digits after implantation of larval limb progenitor cells with activated Wnt/β-catenin signaling and the addition of sonic hedgehog, FGF10, and thymosin β4 (Lin et al. 2013). Modulation of Wnt/β-catenin signaling can induce limb regeneration of the apical ectodermal ridge (AER) and limb bud in chick embryos, which normally do not regenerate (Kawakami et al. 2006; Kostakopoulou et al. 1996; Yokoyama et al. 2011). Extracellular ligands such as Wnt5 in the axolotl and frog (Ghosh et al. 2008; Kawakami et al. 2006; Sugiura et al. 2009; Yokoyama et al. 2011) and FGFs in the salamander and zebrafish (Christensen et al. 2002; Han et al. 2001; Lee et al. 2009; Poss et al. 2000) produce a positive feedback loop between the epithelium and underlying mesenchyme to promote proliferation and regulate differentiation. During digit tip regeneration in mice, genes in the Wnt pathway are differentially expressed (Chadwick et al. 2007), and activation of Wnt signaling in the nail epithelium confers regenerative ability (Takeo et al. 2013). These studies demonstrate the importance of the Wnt/β--catenin pathway across vertebrate model systems, suggesting that there is a conserved genetic program for regeneration.

11.4 Future Directions in Vertebrate Regeneration

Genome sequencing of a growing number of vertebrate species has highlighted the degree to which homologous genes are conserved across evolution (Alföldi et al. 2011; Hellsten et al. 2010; Kusumi et al. 2011; Wallis et al. 2004). Transcriptomic analysis using RNA-Seq or microarrays in the zebrafish, axolotl, newt, *Xenopus* frog, green anole, and mouse has identified that common pathways are activated in regeneration, including genes regulating scar-free wound healing, embryonic development of component tissues, and tissue repair. The availability of whole genome sequences for many of these models permits genetic studies to advance to using ChIP-Seq and cis-regulatory analysis. For larger genomes, advances in sequencing technologies have facilitated sequencing and assembly of large genomes (20–40 Gbp) that were previously thought to be too difficult to assemble (Neale et al. 2014). There is a current effort to sequence the axolotl genome, which has yet to be sequenced, mainly due to its large size of approximately 20 billion base-pairs (Mihaylova and Aboobaker 2013).

The continuing emergence and decreasing cost of RNA-Seq-based gene expression studies in regenerative model organisms should aid in identification of conserved genetic mechanisms in vertebrates.

Functional studies are already possible in developmental models such as the zebrafish and *Xenopus* frog, where morpholino knock-down, transgenic reporter, and knockout technologies have already been tested (Hardy et al. 2012). The

development of the new generation of gene editing technologies, including TALEN and CRISPR-Cas9, facilitates more direct testing of gene targets identified in published studies. Additionally, small molecule inhibitors could be applied to functionally test the importance of specific pathways in regeneration, e.g., using agents targeting β-catenin or GSK3B to disrupt the canonical Wnt pathway. Given the rapid advances in identifying genes and cellular processes regulating the regeneration of appendages in vertebrate models, there is great promise for future application in developing regenerative medical therapies.

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