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Homeobox Genes and Homeodomain Proteins: New Insights into Cardiac Development, Degeneration and Regeneration

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Abstract

Cardiovascular diseases are the most common cause of human death in the developing world. Extensive evidence indicates that various toxic environmental factors and unhealthy lifestyle choices contribute to the risk, incidence and severity of cardiovascular diseases. Alterations in the genetic level of myocardium affects normal heart development and initiates pathological processes leading to various types of cardiac diseases. Homeobox genes are a large and highly specialized family of closely related genes that direct the formation of body structure, including cardiac development. Homeobox genes encode homeodomain proteins that function as transcription factors with characteristic structures that allow them to bind to DNA, regulate gene expression and subsequently control the proper physiological function of cells, tissues and organs. Mutations in homeobox genes are rare and usually lethal with evident alterations in cardiac function at or soon after the birth. Our understanding of homeobox gene family expression and function has expanded significantly during the recent years. However, the involvement of homeobox genes in the development of human and animal cardiac tissue requires further investigation. The phenotype of human congenital heart defects unveils only some aspects of human heart development. Therefore, mouse models are often used to gain a better understanding of human heart function, pathology and regeneration. In this review, we have focused on the role of homeobox genes in the development and pathology of human heart as potential tools for the future development of targeted regenerative strategies for various heart malfunctions.

Keywords

Cardiac development · Cardiac regeneration · Heart disease · Homeobox genes

Abbreviations

AMHC1	atrial myosin heavy chain-1
ANTP	Antennapedia
BMP	bone morphogenetic protein
Cdh2	cadherin 2
CDK	cyclin-dependent kinases
Cited2	Cbp/P300 interacting transactivator with Glu/Asp Rich Carboxy-Terminal Domain 2
CNS	central nerve system

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ESC	embryonic stem cells
FGF	fibroblast growth factor
FHF	first heart field
Flk1	fetal liver kinase 1
GJA5	gap junction protein alpha 5
GSC	goosecoid
H3K27me3	histone H3 methylation on the amino (N) terminal tail
Hcn4	hyperpolarization-activated cyclic nucleotide-gated channel 4 gene
HOXL	homeobox transcription factor Hox-like
Irx	Iroquois family of homeobox genes
ISL1	LIM-homeodomain transcription factor islet 1/insulin gene enhancer protein ISL-1
JMJD3	JmjC domain-containing protein 3
MEF2C	myocyte-specific enhancer factor 2C
MESP1	mesoderm posterior BHLH transcription factor 1
MSCs	mesenchymal stem cells
Myocd	myocardin
NKL	NK-like
Nkx2-5	homeobox protein NK-2 homolog E
Nodal	nodal growth differentiation factor
Nppa	natriuretic peptide A
OFT	outflow tract
PCBP2	poly(rC)-binding protein 2
Pitx2	paired like homeodomain 2
Pitx2c	paired-like homeodomain transcription factor 2
PROS	prospero
RA	retinoic acid
SAN	sinoatrial node
SHF	second heart field
Shox2	short stature homeobox 2
SMAD	main signal transducers for receptors of the transforming growth factor beta (TGF-β) superfamily;
TALE	three-amino-acid loop extension
Tbx5	T-box transcription factor 5
TF	transcription factors
TGF-β	transforming growth factor beta;
VCS	ventricular conduction system
ZEB2	zinc finger E-box binding homeobox 2
ZF	zinc finger
Ziro	zebrafish iroquois homeobox genes
ZO-3	tight junction protein 3

1 Introduction

Homeobox genes are a large family of genes that direct the formation of body structures along the head-tail axis in multicellular animal species (Innis 1997; Shashikant et al. 1991). It is also known that homeobox genes (Hox genes), as an ancient class of transcription factors, are important for the body patterning during embryo development (Innis 1997; Shashikant et al. 1991). Many of the homeobox genes play very important part in the spatiotemporal development of human heart (Lage et al. 2010). Likewise, some of these genes shape the human heart and control its multistep developmental process from simple crescent cells to a fully functional organ. For example, homeobox genes like homeobox protein NK-2 homolog E (Nkx2-5), LIM-homeodomain transcription factor islet 1 (Isl1), paired like homeodomain 2 (Pitx2) are widely known to be important for the proper development of human heart (Akazawa and Komuro 2005; Luo et al. 2014; Franco et al. 2017). However, there are many more homeobox genes that play substantial roles in cardiac function but thus far, there are less known and/or less investigated.

Specific inherited gene mutations cause congenital heart defects such as atrial or ventricle septal defects, abnormalities of outflow tract and etc. (Bao et al. 1999). Similarly, various pathological lifestyle factors like smoking, low physical activity, toxic and noxious agents and other environmental factors might also negatively affect cardiovascular function and promote heart failure (O'Toole et al. 2008; Nayor and Vasan

2015). Since it is impossible to exactly pinpoint how certain gene mutations influence development of human heart at the earliest stages, different mouse models have been created to better understand regulation of human heart development and its relation to various diseases (Camacho et al. 2016). Many of the genes studies in mouse models have similar vital roles in the development and function of human heart (Xu and Baldini 2007). Therefore, investigation of human disease and cues from mouse heart development models have revealed an important role of homeobox genes, including those that encode transcription factors.

Aside from already known homeobox genes, there are more homeobox genes that are essential for the formation of human and/or mouse myocardium. Some of these homeobox genes code transcription factors (TF), whereas other form a tight network regulating heart development and fate of heart progenitors. Several review articles have explored individual families of homeobox gene and their roles in embryo development. However, knowledge concerning the involvement of homeobox genes and homeodomain TF in the development of human heart referring mouse models are still lacking. Therefore, in this review we describe the role of more than 20 homeobox genes that are mainly involved in heart development and around 15 homeobox genes that are known to play minor or less investigated, but nonetheless important roles in cardiac development. Data summarized in this review will help to broaden the possible future applications of homeobox genes and their coded TF in targeted therapeutic strategies for cardiac regeneration and therapy.

2 Development of the Human Heart

Starting from the day first of fertilization, the zygote undergoes multiple cell divisions leading to the formation of third germ layer, known as the mesoderm (Moorman et al. 2003). Later mesodermal cells migrate towards anterior part of embryo to form a distinct crescent-shaped epithelium, named the cardiac crescent (Buckingham et al. 2005). Cells situated in the distinct anterior-lateral territory within the cardiac crescent contribute to the formation of first heart field (FHF), distinguished by the expression of hyperpolarization-activated cyclic nucleotide-gated channel 4 gene (Hcn4) (Liang et al. 2013). Cardiac progenitor cells also develop into second heart field (SHF), which is located medially to the cardiac crescent and extend posteriorly (Cai et al. 2003). Formation of SHF is marked by the expression of LIM-homeodomain transcription factor islet 1 (ISL1) (Cai et al. 2003). Sometimes progenitors of FHF and SHF are called cardiogenic or cardiac mesoderm (Dupays et al. 2015; Liu et al. 2014; Kitajima et al. 2000). These distinct heart fields fuse to form heart tube, which eventually develops into functional heart (Fig. 1) (Moorman et al. 2003; Nemer 2008). During this time, the primitive cardiac conduction system, including sinoatrial node (SAN), ventricular conduction system and other, starts to form (van Weerd and Christoffels 2016). The FHF cells develop into the left ventricle, as well as into the atrioventricular canal and part of the atria, whereas SHF cells develop into the right ventricle and outflow tract, with contribution to the formation of atria and inflow vessels (Buckingham et al. 2005). Once the heart fields are formed, they fuse into heart tube and undergo process called heart looping. During this phase the whole heart tube twists in the rightward direction eventually forming clearly visible, but still primitive, heart chambers (Santini et al. 2016). Later on, the heart undergoes septation to fully separated left and right sides of the heart.

There are many factors regulating human and mouse heart development, however only some of them may be considered to be core regulators of cardiogenesis. One of the most important TF is GATA4 which orchestrates expression of multiple transcriptions including other major determinants of cardiomyogenesis like Nkx2-5, T-box transcription factor 5 (Tbx5), heart- and

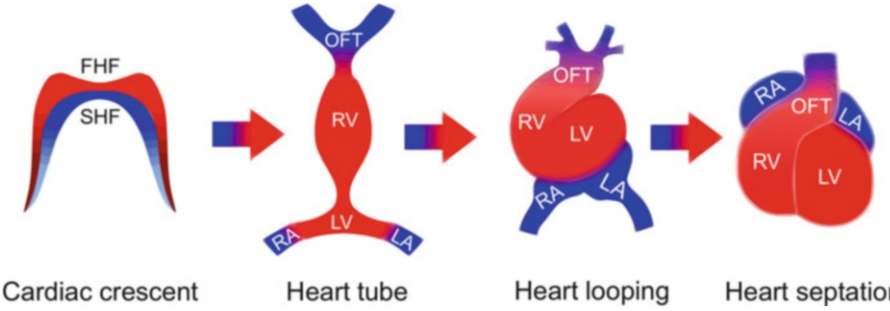

Fig. 1 Schematic representation of heart development in human and mouse. *FGF* first heart field, *SHF* second heart field, *RV* right ventricle, *LV* left ventricle, *RA* right atrium, *LA* left atrium. (Scheme adapted from (Nemer, 2008))

neural crest derivatives-expressed protein 1 (HAND1/2) and others (Bruneau et al. 2001a; Belaguli et al. 2000; Sepulveda et al. 1998). GATA4 integrates bone morphogenetic proteins (BMP) and SMAD, the main signal transducers for receptors of the transforming growth factor beta (TGF-β) superfamily, to ensure cardiac cell survival and stable lineage during cardiac development (Benchabane and Wrana 2003). Of course, there are other factors that promote development of various structures within the heart. For example, it is know that Tbx5 controls atrial gene expression, whereas myocyte-specific enhancer factor 2C (MEF2C) promotes development of ventricle and vasculogenesis (Bruneau et al. 2001a; Lin et al. 1997). Altogether, the development of human heart is a carefully controlled multistep process involving many genes, intracellular and extracellular signalling factors leading to proper cardiac function. The miss-controlled heart development process leads to various inherited or acquired cardiac disorders. This review focuses mostly on the homeodomain proteins, as one of the most important group of transcription factors regulating heart development, function and impairment.

3 Homeobox Genes

Homeodomain proteins are one of the most important group of proteins/transcription factors regulating plan of body structure and organogenesis in eukaryotes including heart development and disorders. DNA binding proteins have been extensively studied, but even today there are no established rules for predicting the specificity of DNA sequence based upon the amino acid sequence of the proteins. Homeodomain proteins are characterized by specific 60 amino acid long helix-turn-helix DNA binding homeodomain motif (Seifert et al. 2015). The homeodomain is a very highly conserved structure and consists of three helical regions folded into a tight globular structure that binds a 5′-TAAT-3′ core motif. The high degree of conservation of homeodomain proteins is an ideal model to study specific protein-DNA interactions. The DNA sequence that encodes the homeodomain is called the “homeobox” and homeobox-containing genes are known as “hox” genes.

Most of the transcription factors belonging to this group are not only structurally but also evolutionary conserved and play crucial roles in embryonic patterning and differentiation (Pearson et al. 2005). The main role of homeodomain proteins *in vivo* is to control the genetic determination of development and implementation of the genetic body plan. There are 102 homeobox gene families that represent 235 active human homeodomain proteins, but only some homeodomain classes have close association with cardiac development and/or diseases (Bürglin and Affolter 2016). This review covers description of around 20 homeobox genes that up today are known to have a major

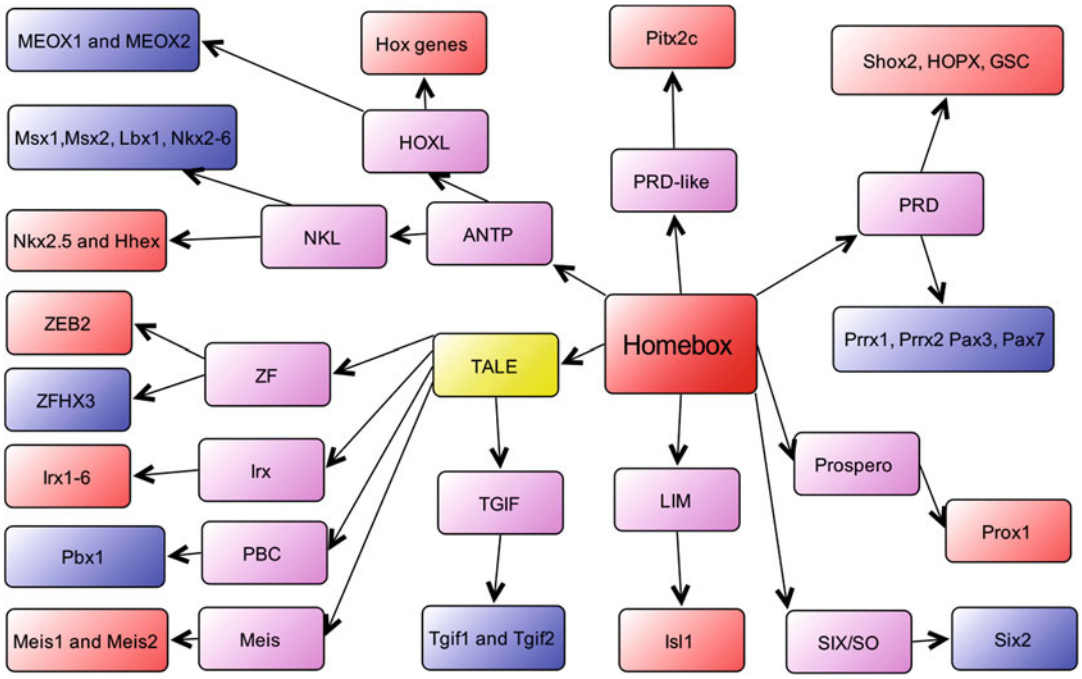

Fig. 2 Hierarchy of homeobox genes involved in heart development. Pink boxes indicate homeobox gene classes, yellow box indicates TALE gene superclass. Red boxes indicate genes with major involvement in heart development and diseases. Blue boxes indicate genes having less important role in heart development and diseases

impact in the regulation of heart development and functioning (Fig. 2).

4 Homeobox Genes in Mouse Heart Development and Human Disease

Humans have more than 67 genes that are important for cardiac hypertrophy and over 92 genes that control cardiovascular system, therefore it is reasonable to assume that some of the genes might be responsible for cardiac development and disease (van der Harst et al. 2016; Smith and Newton-Cheh 2015). Congenital heart disease (CHD) have structural heart anomaly, including atrial or ventricle septal defects, overriding aorta, right atrium isomerism and other structural changes in new born heart. Patients do usually display multiple symptoms, like, rapid breathing, bluish skin, poor weight gain and feeling tired in general (Sun et al. 2015). The main cause of these abnormalities is reduced blood oxygen levels in whole organism, which is a result of improper heart septation leading to the mixture of oxygenated and deoxygenated blood in systemic blood circulation. Additionally, CHD could be caused by genetical or environmental factors like infections during pregnancy such as Rubella, drugs and maternal illness (Sun et al. 2015). Since homeobox genes are important for the heart development, some mutation of the homeobox genes including *MEIS2*, *Nkx2-5* and others can potentially cause CHD (Zakariyah et al. 2017; Johansson et al. 2014). Of course, homeobox gene mutations is not a sole cause of heart defect present at human birth. For example, mutations in *GATA4* and *Tbx5* can also affect integrity of heart tissue (McCulley and Black 2012). In addition, impaired gene functioning might be also related to the other heart diseases like cardiomyopathy, hypertrophy,

defects of the heart rhythm and other abnormalities (Kathiresan and Srivastava 2012). However, the impact of homeobox genes in heart disorders is still not clear and needs further investigation to clarify their role not only in the cardiac development but also in the physiological and pathophysiological conditions.

5 ANTP Class of Homeobox Genes

The Antennapedia (ANTP)-class of homeobox genes are involved in the determination of pattern formation along the anterior-posterior axis of the animal embryo. NK-like (NKL) and homeobox transcription factor Hox-like (HOXL) are an ancient subclasses of homeobox genes that belong to an ANTP homeobox gene class (Holland et al. 2007). It is likely that HOXL gene clusters originate from NKL, since NKL genes are widespread throughout the genome as tight clustered Hox genes (Bürglin and Affolter 2016). Both subclasses of the genes (NKL and HOXL) are evolutionarily conserved and play predetermined roles in heart patterning and disease.

6 Hox Gene Families of HOXL Subclass

Hox gene families belong to the HOXL subclass of ANTP class of homeobox. Hox genes code transcription factors which are important for whole body patterning and development (Pearson et al. 2005). It total, there are 39 human Hox genes grouped in HOXA, HOXB, HOXC and HOXD gene clusters. Hox genes are highly conserved, because they play a vital role in anterior-posterior formation of body axis (Pearson et al. 2005). The precise function of these genes is achieved by their specific temporal and spatial expression over the life course. During early mouse cardiac development, the retinoic acid (RA) might be responsible for the anterior-posterior patterning in SHF (Bertrand et al. 2011). Hoxb1, Hoxa1, and Hoxa3 act as downstream targets of RA and participate in forming outflow tract (OFT) and normal SHF development (Bertrand et al. 2011). *Hoxb1−/−* or *Hoxa1−/−*, *Hoxb1+/−* mouse embryos develop shortened OFT and display abnormal proliferation and premature differentiation of cardiac progenitors (Bertrand et al. 2011). This is probably related to the altered fibroblast growth factor (FGF) and BMP signaling pathways in developing mouse embryo (Roux et al. 2015). Clinical studies have revealed that Hoxa1 mutations might cause congenital human heart defects and other abnormalities like, mental retardation, deafness, horizontal gaze restriction and etc. (Bosley et al. 2008). Several other studies have shown that Hox genes might be also related to the human heart diseases, however more research is needed to unveil exact functions of these genes in human heart development (Gong et al. 2005; Haas et al. 2013).

7 Nk4 Gene Family of NKL Subclass

There are multiple NKL genes in mouse and humans regulating various developmental processes, however only some of them contribute to the development of the heart (Larroux et al. 2007). The *Nkx2-5* and *Nkx2-6* genes are the members of the NK4 homeobox gene family of NKL subclass and are closely related to the Drosophila *tinman* gene (Bürglin and Affolter 2016; Harvey 1996). To our knowledge, only *Nkx2-6* and *Nkx2-5* relate to the mouse and human heart development and disease, whereas *Nkx2-3*, *Nkx2-7*, *Nkx2-8* and *Nkx2-10* might be important for the heart development of zebrafish, frog or chicken (Newman and Krieg 1998; Wang et al. 2014; Tu et al. 2009; Allen et al. 2006; Brand et al. 1997).

During the early stages of embryogenesis, Nkx2-5 is expressed in myocardium and pharyngeal endoderm, whereas Nkx2-6 can be found in sinus venosus, pharyngeal endoderm and myocardium of the outflow tract (Lints et al. 1993).

Moreover, during normal heart development, Nkx2-5 expression is essential for the looping of vertebrate embryonic heart, heart septation and formation of cardiac conduction system, whereas most of the *Nkx2-5* mutations are related to human congenital heart disease and conduction defects (Tanaka et al. 1999). Inactivation of *Nkx2-5* arrested heart formation at the looping stage revealing its critical role in cardiac development (Lyons et al. 1995). However, targeted disruption of *Nkx-2.6* did not cause any abnormalities in the heart suggesting a possible compensatory function of Nkx-2.5 (Tanaka et al. 2000).

It is important to note that *Nkx2-5* mutations lead to an altered spatiotemporal development of human heart, improper heart septation and formation of cardiac conduction system (Dupays et al. 2015; McCulley and Black 2012; McElhinney et al. 2003). Analysis of human *Nkx2-5* mutants and gene truncations showed that most of the mutations affected *Nkx2-5* binding to DNA or its localization but not protein-protein interactions (McCulley and Black 2012; Reamon-Buettner et al. 2004). Several different studies of mice *Nkx2-5* knockout and human embryonic stem cells (ESC) revealed that *Nkx2-5* mutations might alter gene expression of specific transcription factors like SP1, SRY, JUND, STAT6, *MYCN*, *PRDM16*, *HEY2* and others (Anderson et al. 2018; Li et al. 2015). Also some studies support an idea that, *Nkx2-5* mutant proteins might alter space and time specific human cardiac development by dysregulating BMP, Notch and Wnt signalling pathways (Anderson et al. 2018; Wang et al. 2011; Luxán et al. 2016; Cambier et al. 2014). There is a possibility that Nkx2-5 modulates these pathways by interacting with multiple transcription factors in time-dependent mode. For example, in mouse heart Nkx2-5 interacts with Hand2 transcriptions factor to activate Irx4, which is necessary for the ventricular identity (Yamagishi et al. 2001). Conversely, Nkx2-5 expression is also timely regulated since Nkx2-5 overexpression leads to an improper SAN formation in early mouse development (Roux et al. 2015). Mammalian heart development is also regulated by the combination of cardiac transcription factors having specific DNA motifs in their centrally located DNA binding domains. It was also shown that Nkx2-5, GATA4 and Tbx5 can physically interact and synergistically regulate targeted genes (Hiroi et al. 2001; Pradhan et al. 2016). Since these genes are the master regulators of heart development, functional mutations in these genes are linked to various types of congenital heart diseases (Benson 2002; Hatcher et al. 2003). Taken together, Nkx2-5 and other transcription factors like Isl1, GATA4, Tbx5, Hand2, MEF2C, Irx4 form a core of transcription factors essential for the heart development and congenital heart disease (Fig. 3) (McCulley and Black 2012).

8 HHEX Gene Family of NKL Subclass

Proline rich homeodomain protein or homeobox protein (PRH/HHEX) expressed by hematopoietic system is a transcription factor belonging to the family of NKL subclass gene (Bedford et al. 1993). As the name implies, it is important for the development of hematopoietic cell, but not less is essential for the development of other systems, including heart (Bedford et al. 1993). Mouse double *HHex* mutants have multiple developmental issues, including defective vasculogenesis, hypoplasia of the right ventricle, aberrant development of the compact myocardium and other complications related to forebrain, thyroid and liver developmental disorders (Hallaq et al. 1998). Additional studies have revealed that HHex plays distinct role in mouse cardiac mesoderm specification and development. HHex expression is controlled by Sox17 transcription factor, which is known to be essential for the formation of mouse cardiac mesoderm (Liu et al. 2014). Several studies of human population have shown that common variants of *HHex* gene (rs7923837 and rs1111875) may also be associated with diabetes (Karns et al. 2013; Kelliny et al. 2009; Pechlivanis et al. 2010).

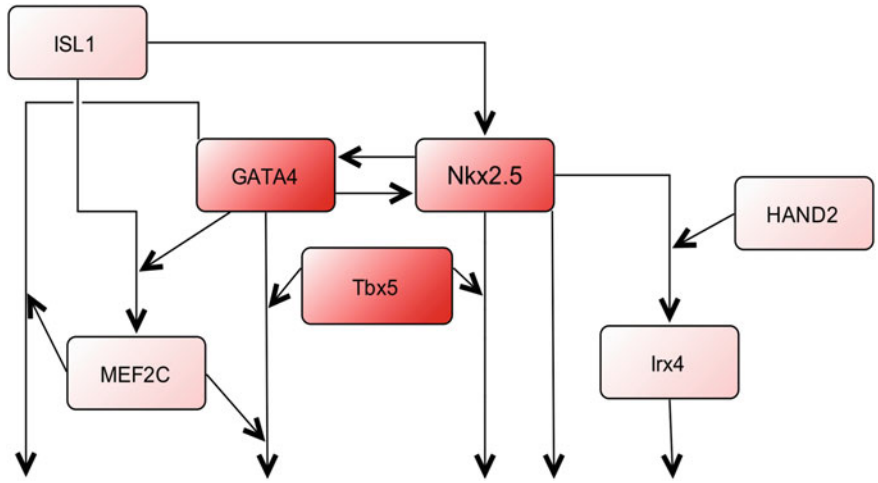

Fig. 3 Core transcription factors important for the heart development and congenital heart disease. (Scheme adapted from (McCulley and Black, 2012))

9 PRD and PRD-Like Homeobox Class

The PRD class is the second largest of the homeobox gene classes in animal genomes and, like the ANTP class, these genes have been found only in animals. The PRD class derives its name from the *Paired* (*Prd*) gene of *Drosophila*. Multiple gene families belong to the PRD homeobox class, including *Shox2*, *Hopx*, *GSC*, *Pitx2* and others, which are important for the heart development and disease.

10 Shox Gene Family of PRD Class

Short stature homeobox 2 (Shox2) is a homeobox gene belonging to the PRD class of homeobox. Shox2 is an essential for the development of limb and cardiac conduction systems, including formation of sinoatrial node (SAN) in mice and humans (Gu et al. 2008; Blaschke et al. 2007; Liu et al. 2011). Studies of Shox2 function during the mouse development revealed several cues how this homeodomain transcription factor in particular controls formation of SAN (Blaschke et al. 2007). Shox2 mice null mutants displayed severe cardiac conduction defects, such as low heart rhythm rate and drastically reduced cell proliferation (Espinoza-Lewis et al. 2009). This phenotype is probably related to the downregulation of *HCN4*, *Tbx3* and the upregulation of natriuretic peptide A (*Nppa)*, gap junction protein alpha 5 (*GJA5)* and *Nkx2-5* gene expressions (Espinoza-Lewis et al. 2009). It is also known that HCN channels play a vital role in autonomic control of heart rate, so it is no surprise why Shox2 null mutants do not develop SAN (Alig et al. 2009). During the normal development of mouse cardiac expression of Shox2 is also tightly controlled by several transcription factors. For example, transcription factor Tbx5 activate Shox2 expression, however transcription factors like Pitx2c and NKX2-5 potentially silence Shox2 expression (Espinoza-Lewis et al. 2011; Puskaric et al. 2010). Paired-like homeodomain transcription factor 2 (Pitx2c) also can potentially inhibit left-sided pacemaker specification by suppressing Shox2 expression in left atrium, therefore SAN develops only in the region of right atrium (Wang et al. 2010). All these results indicate that Shox2 is essential for the maintaining pacemaker cell program during the heart development. On the other hand, in adulthood Nkx2-5

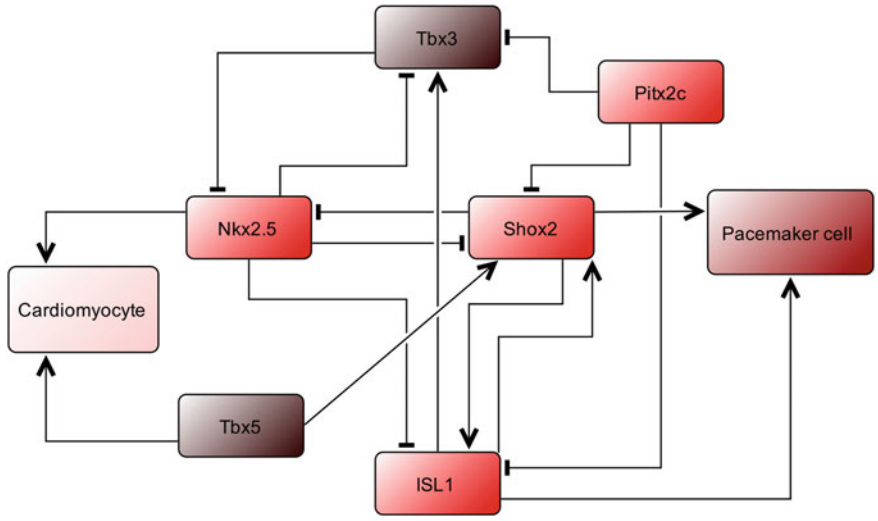

Fig. 4 Signaling networks governing pacemaker and atrial differentiation of cardiomyocyte in developing mouse heart. (Scheme adapted from (Liang et al. 2017))

antagonizes Shox2 and promotes cardiomyocyte formation (Fig. 4) (Liang et al. 2017).

Taken together, these studies indicate that Shox2 might be a good candidate to develop biological pacemaker. Results from mouse ESCs and canine mesenchymal stem cells (MSCs) have shown that cells overexpressing Shox2 induce expression of SAN markers such as HCN4, Cx45 and Tbx3 (Ionta et al. 2015; Feng et al. 2016). In addition, mouse embryonic bodies overexpressing Shox2 showed better contractile phenotype compared to the control group of embryonic bodies (Ionta et al. 2015). Moreover, human patients with an early-onset atrial fibrillation had significantly downregulated expression of *Shox2* gene (Hoffmann et al. 2016). These results are promising for patients suffering from heart rhythm defects, however, more studies are needed to test functions of biological pacemaker in order to treat human arrhythmias.

11 HOPX Gene Family of PRD Class

HOPX is another PRD-class homeobox gene family important for the multiple organ development and tissue homeostasis in adults (Chen et al. 2015; Schneider et al. 2015; Mariotto et al. 2016). Since it lacks a DNA binding domain, HOPX can only modulate gene expression by forming complexes with other regulatory proteins (Kook et al. 2006). In general HOPX acts as a cell proliferation inhibitor in humans cancer cells, however its function in mouse cardiac cell differentiation is not entirely clear (Chen et al. 2015; Waraya et al. 2012; Yap et al. 2016). Studies of mouse development have shown that HOPX plays a critical function in early formation of cardiomyocyte progenitors. HOPX integrates BMP and WNT signalling in developing mouse heart by interacting with SMAD proteins and inhibiting WNT signalling pathway leading to the formation and differentiation of cardiomyocyte progenitors (Jain et al. 2015).

On the other hand, there are some cues that HOPX might act as a negative regulator of cardiac differentiation in mice. HOPX interacts with HDAC2, thus reducing GATA4 transcriptional activity by deacetylation (Trivedi et al. 2010). These findings are consistent with previous reports that overexpression of HDAC2 inhibits the development of cardiomyocytes by downregulating the expression of *GATA4* and *Nkx2-5*

genes (Kawamura et al. 2005; Karamboulas et al. 2006). All these results highlight the complex nature of HOPX and its partners in heart development. HOPX undeniably plays a critical role in early heart development, because some mouse mutants cannot develop a functional myocardium and display cardiac conduction defects (Chen et al. 2002; Ismat et al. 2005). HOPX also might be related to the human heart failure, since HOPX is downregulated in patients having cardiac hypertrophy (Güleç et al. 2014; Trivedi et al. 2011).

12 Goosecoid Gene Family of PRD Class

Goosecoid (GSC) is another protein that belongs to the bicoid related paired (PRD) homeobox class of genes. Goosecoid is often associated with limb, skeletal and craniofacial development, although, it might be important for cardiac mesoderm formation, since its expression is controlled by Mesp1 (Zhu et al. 1998). Mesoderm posterior BHLH transcription factor 1 (MESP1) preferentially binds to two variations of E-box sequences and activates critical mesoderm modulators, including Gata4, mix paired-like homeobox (Mixl1) and GSC homeobox (Soibam et al. 2015). In addition, mesoderm formation can be induced with l-proline and trans-4-hydroxy-l-proline resulting in increased expression of Mixl1 and GSC (Date et al. 2013). GSC also is important for the cell migration in early embryonic development, therefore the overexpression of goosecoid enhances oncogenic cell growth and metastasis (Kang et al. 2014).

13 Pitx Gene Family of PRD-Like Class

Paired like homeodomain 2 *(Pitx2)* is a PRD-like homeobox class gene which is important for the establishment of the left-right axis and for the asymmetrical development of the mouse and probably human heart, lung, and spleen, twisting of the gut and stomach, as well as the development of the eyes (Campione et al. 1999; Shiratori et al. 2006; Evans and Gage, 2005). There are several alternative *Pitx2* transcripts, however only Pitx2c isoform plays determined role in the asymmetric development of mouse heart (Liu et al. 2002). Higher vertebrates, at an early heart development stage and after the heart tube formation, undergo embryonic heart looping, which is the first visual evidence of embryo asymmetry (Harvey 2002). Transcription factors like nodal growth differentiation factor (Nodal) and Cbp/P300 interacting transactivator with Glu/Asp Rich Carboxy-Terminal Domain 2 (Cited2) activate Pitx2 transcription leading to the rightward twist of the heart tube and forming prospective embryonic atrial and ventricular chambers. Deletion of *Pitx2c* in mouse caused drastic alteration of looping process leading to various heart defects including the isomerism of right atrium and ventricle (Lin et al. 1999; Yu et al. 2001).

Humans with Pitx2c mutations develop various heart abnormalities, including an improper formation of ventricle and atrial chambers septa, atrial fibrillation and others. It is quite likely that septation defects are caused by the downregulation of transcription factors downstream of Pitx2, since certain *Pitx2* mutants displayed reduced cardiac transcriptional activity in human patients (Wang et al. 2013; Wei et al. 2014). Surprisingly, the overexpression of Pitx2c in mouse R1-embryonic stem cells results in elevated gene expression of essential cardiac transcription factors like GATA4, MEF2C, Nkx2-5 and others (Lozano-Velasco et al. 2011). Consequently, *Pitx2c* might be a good candidate for heart regeneration, since it positively regulates multiple transcription factors important for cardiac development. Recently it was shown that mouse embryonic stem cells overexpressing Pitx2c could restore mouse heart function after a myocardium infarct through the multiple mechanisms including efficient terminal

differentiation, regulation of action potentials of cardiomyocytes and positive paracrine effects (Guddati et al. 2009). However, more studies need to be done to determine the utility of Pitx2c in human heart regeneration strategies.

14 TALE Homeobox Superclass

Three-amino-acid loop extension (TALE) is another superclass of homeobox genes, which codes for highly conserved transcription regulators essential for various developmental programs. These genes encode proteins with atypical homeodomain structure, defined by having three additional amino acids in homeodomain. TALE homeobox gene superclass includes the main zinc finger (ZF), PBC and Meis homeobox 1 (Meis) classes. Out of 20 human homeodomains only Meis1, Meis2 and Iroquois homeobox proteins 1-6 (Irx1-6) have their clearly defined function in heart development and disease.

15 Meis Genes Family of Meis Class

Meis1 encodes the TALE superclass homeobox transcription factor implicated in cardiac, hematopoietic and neural development (Mariotto et al. 2013; Azcoitia et al. 2005; Hisa et al. 2004). *Meis1* deficient mice have malformed cardiac outflow tracts with overriding aorta and ventricular septal defect (Stankunas et al. 2008). Downregulation of Meis1 leads to cardiac hypertrophy in humans and mice. Meis1 binds poly (rC)-binding protein 2 (*PCBP2*) gene promoter and activates its expression in order to suppress human or mouse heart hypertrophy (Zhang et al. 2016). In turn, PCBP2 represses angiotensin II, which enhances hypertrophic human or mouse cardiac growth (Zhang et al. 2015). There are around 79 cardiac specific genes that have Meis1 and NKX2-5 binding sites in developing mouse heart, some of them are associated with cell signaling and cardiac progenitor differentiation, like Tbx20, myocardin, cadherin 2 (Cdh2), Wnt11, and Wnt2 (Dupays et al. 2015). Adult mouse cardiomyocytes with mutant *MEIS1* exhibit increased proliferation and progression of the cell cycle (Mariotto et al. 2013). This function is emphasized in adult mouse hearts since Meis1 activates inhibitors of cyclin-dependent kinases (CDK) like p15, p16 and p21 (Mariotto et al. 2013). In humans non-synonymous *Meis1* gene variants might be associated with congenital heart defects, whereas patients carrying 2p14 microdeletions show symptoms of deafness and cardiomyopathy (Mathieu et al. 2017; Arrington et al. 2012). It is likely that Meis1 is required for the control of spatiotemporal cell proliferation in early developing heart to prevent hypertrophy, however more studies need to be done to fully understand the role of Meis1 in cardiac development and disease.

Meis2 encodes TALE homeobox superclass transcription factor essential for the development of mouse cranial and cardiac neural crest (Machon et al. 2015). Recent findings indicate that Meis2 might be an important factor for the proliferation of fetal human cardiomyocyte cells (Wu et al. 2015). Reduction of *Meis2* gene expression by miR-134 results in slowed progression of human cardiomyocyte progenitor cell cycle (Wu et al. 2015). A clinical and genetic study also revealed that small *Meis2* deletion can negatively affect several developmental processes: human patients with small *Meis2* non-frame shift deletion (c.998_1000del:p.Arg333del) had serious cleft palate and cardiac septal defects (Louw et al. 2015). It is known that Meis2 interacts with DNA and forms multimeric complexes with Hox and Pbx proteins (Louw et al. 2015). Single deletion of arginine residue affects the ability of Meis2 to bind DNA leading to serious developmental problems of human heart (Louw et al. 2015). Clinical studies have shown that patients having only one functioning Meis2 gene copy survive, however they have similar phenotype such as clefting and ventricular septal defects leading to delayed

motor development and learning disability (Johansson et al. 2014).

16 Irx Gene Family of IRX Class

Iroquois homeobox genes and their coded homeodomain proteins are another class of transcription factors belonging to TALE superclass of homeobox genes. Iroquois-class homeodomain TF (Irx) defining feature is atypical homeodomain structure and specific Iroquois (IRO) homeodomain family sequence motif, which is important for the recognition of DNA sequence (Gómez-Skarmeta and Modolell 2002; Cavodeassi et al. 2001). Humans and mice have six Irx proteins, which are important for the development of lung, nervous system, eye, pancreas, female gonad, early limb and, of course, heart patterning (Cavodeassi et al. 2001; Cheng et al. 2005; Schwab et al. 2006; van Tuyl et al. 2006; Ragvin et al. 2010; Jorgensen and Gao 2005; McDonald et al. 2010). Irx1 and Irx2 are expressed in interventricular septum from E14.5 onward, however, mouse *Irx2* mutants are viable and display no notable phenotype defects in the developing heart (Christoffels et al. 2000; Lebel et al. 2003). *Irx1* gene variants might be related to the congenital heart disease in humans (Guo et al. 2017).

Irx3 gene in mice seems to be very important for the ventricular conduction system (VCS) (Christoffels et al. 2000). Various studies suggest that Irx3 is required to maintain rapid electric conduction through the VCS for proper ventricular activation, via antithetical regulation of Cx40 and Cx43 expression (Zhang et al. 2011; Kasahara et al. 2003). Clinical studies have revealed that defects of *Irx3* gene can cause lethal cardiac arrhythmias in human patients (Koizumi et al. 2016). Irx3 function appears to be evolutionary conserved, since expression of Ziro3a, a Irx3 homologue in zebrafish, is detected in developing fish heart (Zhang et al. 2011).

Irx4 is associated with the formation of ventricular myocardium in mouse and humans (Christoffels et al. 2000; Cheng et al. 2011). Data from mouse and chicken indicate that Irx4 suppresses atrial gene expression by down regulating atrial myosin heavy chain-1 (AMHC1) (Bao et al. 1999; Bruneau et al. 2001b). Several *Irx4* mutations have been identified that might be associated with human congenital heart disease, particularly ventricular septal defect (Cheng et al. 2011).

Irx5 is expressed in adult mouse heart and maintains proper action potentials, particularly regulates T-wave seen in ECG (Costantini et al. 2005). Mice lacking Irx5 develop properly without any structural abnormalities in the heart (Costantini et al. 2005). This indicates that *Irx5* is not required for cardiac development or that other *Irx* genes can compensate for the loss of Irx5.

Irx6 is detectable in mouse developing heart, however its expression is relatively weak compared to other *Irx* genes (Christoffels et al. 2000).

17 Zeb Gene Family of ZF Class

ZEB2 or zinc finger E-box binding homeobox 2 is a gene coding transcription factor belonging to class of ZF homeobox gene and homeodomain class of ZN proteins (Bürglin and Affolter, 2016). It has multiple functional domains (E-box, Zinc finger, homeobox), so naturally it can control gene expression with a variety of transcription factors (Gheldof et al. 2012). The complex nature of Zeb2 shows that it drives multiple processes including the development of heart and neural systems, however, it usually acts as a transcription repressor rather than activator (Hegarty et al. 2015). Systematic study of mouse and human ESC transcriptome differentiation profiles revealed that Zeb2 might play important role in cardiac specialization. Human ESC with silenced *Zeb2* gene proliferate more slowly and fail to differentiate into mature cardiomyocytes compared to the wild cells (Busser et al. 2015). In addition, cardiomyocytes with silenced *Zeb2* do not show any contractile properties, although cardiac differentiation program is activated. More

detailed analysis has revealed that silencing of *Zeb2* gene negatively affects human striated muscle contraction program, including genes related to calmodulin pathway, HCN and potassium channels (Busser et al. 2015). Targeted regulation of Zeb2 gene expression improves cardiomyogenic processes and heart regeneration.

Zeb2 mutation is also often associated with Mowat-Wilson syndrome (Garavelli and Mainardi 2007). Major signs of this disorder frequently include distinctive facial features, intellectual disability, delayed development, an intestinal disorder called Hirschsprung disease, Congenital Heart Disease and other types of birth defects (Garavelli and Mainardi 2007). All mentioned disorders are related to the improper heart development caused by the Zeb2 defective heart cells. In addition, Zeb2 repress epithelial genes (claudins, tight junction protein 3 (ZO-3), connexins, E-cadherin, plakophilin 2, desmoplakin, and crumbs3) in order to induce epithelial to mesenchymal transition (EMT), which is crucial for the developmental processes such as gastrulation, neural crest formation, heart morphogenesis, formation of the musculoskeletal system, and craniofacial structures (Vandewalle et al. 2009; Garavelli et al. 2017).

18 LIM Homeobox Class

LIM homeobox class genes encode two Lim domains and one homeodomain. Lim domain is a 50–60 amino acid length zinc finger motif, which is primarily involved in protein-protein interactions, so naturally LIM transcription factors can interact with multiple proteins in cell, thus regulating its phenotype.

19 Isl Gene Family of LIM Homeobox Class

Isl1 is a LIM homeobox class member that encodes a homeodomain transcription factor important for cell differentiation, fate determination and generation of cell diversity in multiple mouse and human tissues including central nerve system (CNS), pancreas and heart (Zhuang et al. 2013). During early cardiac development, Isl1, Nkx2-5 and fetal liver kinase 1 (Flk1) support the formation of SHF, which gives rise to the right ventricle, outflow tract and part of the atria (Dyer and Kirby, 2009). Isl1 promotes expansion, migration and proliferation of SHF progenitor cells during the development of the mouse heart (Witzel et al. 2012). Additionally, Isl1+ mouse heart cells have potential to differentiate into multiple cell types within the heart, including cardiomyocytes, smooth muscle, pacemaker and endothelial cells (Laugwitz et al. 2007).

There are multiple mechanisms explaining how Isl1 can promote expression of target genes, which suggests the expression of Isl1 is tightly controlled during the mouse heart development. For example, Nkx2-5 homeodomain transcription factor downregulates Isl1 expression in order to promote ventricular development in mouse heart (Witzel et al. 2012; Prall et al. 2007). The newest studies indicate, that Isl1 may repress development of mouse heart ventricle in order to promote the development of SAN (Dorn et al. 2015). Mouse embryos overexpressing Isl1 develop SAN-like cells instead of ventricle myocardium (Dorn et al. 2015). It is likely that the expression of Isl1 activates Nkx2-5 expression in SHF progenitor cells, however, in later staged of heart development Nkx2-5 shuts down ISL1 expression to promote ventricular development (Dorn et al. 2015). Isl1 orchestrates the expression of hundreds of potential genes implicated in cardiac differentiation, mainly through epigenetic mechanisms (Wang et al. 2016). Isl1 in mouse ESCs acts together with JmjC domain-containing protein 3 (JMJD3) histone demethylase to promote the demethylation or tri-methylation of core histone H3 on the amino (N) terminal tail (H3K27me3) at the enhancer's place of key downstream target genes, such as myocardin (*Myocd*), *MEF2C* and others (Wang et al. 2016). In addition, Isl1 may reduce histone methylation near *GATA4* and *Nkx2-5* genes after the expression of Isl1 lentiviral gene, and can also recruit p300 histone acetyltransferase to the promoter of

MEF2C gene in order to promote Mef2c expression in developing mouse embryo (Yu et al. 2013). Other data suggest that lentiviral-induced overexpression of *Isl1* gene promotes not only *MEF2C* gene acetylation, but also *GATA4* and *Nkx2-5* in C3H10T1/2 mouse cell line (Xu et al. 2016). The tight control of *Ils1* gene expression is required, since it acts as a positive cardiomyogenic gene regulator reducing methylation and increasing acetylation levels of genes and histones by direct and indirect methods. Most of the published data concerning Isl1 function have come from the studies of mouse development, however there are some studies that link *Isl1* gene expression with the susceptibility to human congenital heart disease (Luo et al. 2014; Stevens et al. 2010). Development of mechanisms that could control expression of Isl1 might be important target in further regulation of heart regeneration.

20 PROS Homeobox Class

Homeobox prospero (PROS) genes code atypical C terminal prospero domain and belongs to a distinctive class of Prospero homeodomain proteins (Yousef and Matthews, 2005). The PROS domain is a DNA binding domain of approximately 100 amino acids. In addition, PROS homeobox genes code additional three amino acids in their HD domain (Yousef and Matthews 2005).

21 PROX Gene Family of PROS Homeobox Class

Prospero homeobox 1 or Prox1 is a gene coding a transcription factor that plays important role in the development of mouse heart, CNS, eye, liver and lymphatic system (Elsir et al. 2012). Firstly, it was discovered in Drosophila as an important player in the development of central nervous system in insects. However, later Prox1 homologues were found in vertebrates and mammals (Elsir et al. 2012). In mouse heart development of Prox1 is important for the sarcomere formation and muscle contraction (Risebro et al. 2009). Mouse Prox1 conditional mutants show increased number of fast twitch fibers compared to slow twitch fibers. *Prox1* mutant mice develop fatal dilated cardiomyopathy and die around 7–14th week (Petchey et al. 2014). It was shown that in mice Prox1 acts as a transcriptional repressor of genes like *Tnnt3*, *Tnni2* and *Myl1* that are essential for the formation of fast twitch fibbers (Petchey et al. 2014). Prox1 might also be important for the maintenance of cardiac conduction system in adult mice. It was also shown that uncontrolled Nkx2-5 expression led to cardiac conduction defects, surprisingly suggesting that Prox1 might act as a direct upstream modifier of *Nkx2-5* gene expression (Risebro et al. 2012). In humans dysregulation of *Prox1* gene expression might also lead to congenital heart disease, like, hypoplastic left heart (Gill et al. 2009). Thus, the close connection of Prox1 with Nkx2-5 and other heart development and diseases regulating genes makes it an attractive target in cardiac regeneration field.

22 Role of Homeobox Genes in Cardiomyogenesis

The summarized and reviewed data of estimated involvement of homeobox genes in the heart development, diseases and/or regeneration processes suggest that some homeobox genes play more important role than the other. Data summarized in Table 1 show the homeobox genes that have been most commonly investigated with important roles in cardiomyogenesis.

It is quite evident that dozens of homeobox genes are required for early cardiomyogenesis, heart septation, formation of pacemaker cell, cardiomyocyte and etc. Some of the homeobox genes are directly related to the development of CHD, atrial fibrillations and other cardiac pathologies. However, there are much more

Table 1 Homeobox genes with major involvement in heart development and diseases

Gene	Development	Disease	Reference
Hox	Hoxa1, Hoxb2 and Hoxb2 is important for anterior-posterior patterning in SHF. Integrating FGF and BMP signalling.	Hoxa1 mutations might cause CHD.	Pearson et al. (2005), Bertrand et al. (2011), Bosley et al. (2008), Gong et al. (2005) and Haas et al. (2013)
		HOXB13, and HOXC5 mutations might be related to heart disease.	
Nkx2–5	Heart looping, heart septation and cardiac conduction system formation. Integrates BMP, notch and WNT signaling during development.	Multiple gene variants and truncations are related to CHD.	McCulley and Black (2012), Tanaka et al. (1999), McElhinney et al. (2003), Anderson et al. (2018), Wang et al. (2011), Luxán et al. (2016) and Cambier et al. (2014)
Hhex	Cardiac mesoderm specification.	HHex gene variants might be associated with diabetes.	Liu et al. (2014), Karns et al. (2013), Kelliny et al. (2009) and Pechlivanis et al. (2010)
Shox2	Cardiac conduction system development.	Downregulation during early-onset atrial fibrillation.	Blaschke et al. (2007) and Hoffmann et al. (2016)
Hopx	Cardiomyocyte progenitor formation in mouse early heart. Negative regulator of GATA4 expression.	Downregulated in patients having cardiac hypertrophy.	Jain et al. (2015), Trivedi et al. (2010) and Trivedi et al. (2011)
GSC	Cardiac mesoderm specification.		Zhu et al. (1998)
Pitx2c	Establishment of the left-right axis in heart development. Heart looping and chamber septation.	Mutations cause improper ventricle and atrial chambers septa formation, atrial fibrillation.	Liu et al. (2002), Wang et al. (2013) and Wei et al. (2014)
Meis1 and *Meis2*	Meis1 and Meis2 control of cell cycle progression during heart development.	Meis1 and Meis2 gene variants might be associated with CHD.	Mariotto et al. (2013), Arrington et al. (2012), Wu et al. (2015) and Louw et al. (2015)
Irx1-6	Irx3 very important for ventricular conduction system.	Irx1 gene variants might be associated with CHD.	Christoffels et al. (2000), Guo et al. (2017), Koizumi et al. (2016) and Cheng et al. (2011)
	Irx4 is associated with the formation of ventricular myocardium in mouse and humans.	Irx3 gene defects can cause lethal cardiac arrhythmias in human patients.	
ZEB2	Controls striated muscle development and contraction.	Gene variants cause Mowat-Wilson syndrome. Patients display CHD and other defects.	Busser et al. (2015) and Garavelli and Mainardi (2007)
Islet1	Cell expansion, migration and proliferation. Marks formation of SHF. Repress ventricular fate in order to promote sinoatrial node development. Positive gene regulator, which reduces gene and histone methylation levels and increase acetylation.	Gene variants might be related to CHD.	Witzel et al. (2012), Dorn et al. (2015), Wang et al. (2016) and Stevens et al. (2010)
Prox1	Important for sarcomere formation and muscle contraction.	Dysregulation of Prox1 might lead to CHD.	Elsir et al. (2012) and Petchey et al. (2014)

homeobox genes related to the heart development, that so far have been less investigated or in one or another model system showed less direct involvement in cardiomyogenic processes (Table 2). Data summarized in Table 2 also highlight the fact that many more studies are needed to understand regulation of homeobox genes and their role in cardiomyogenic processes.

Table 2 Homeobox genes having less important role in heart development and diseases

Class	Subclass	Gene	Development and disease	Reference
ANTP	HOXL	*MEOX1* and *MEOX2*	Control of vascular endothelial cells proliferation in mice. Dysregulation might be associated with heart disease in mouse.	Lu et al. (2018), Douville et al. (2011)
ANTP	NKL	*Msx1* and *Msx2*	Regulate survival of secondary heart field precursors and post-migratory proliferation of cardiac neural crest in the outflow tract	Chen et al. (2007)
ANTP	NKL	*Lbx1*	Specification of a subpopulation of cardiac neural crest necessary for normal heart development.	Schäfer et al. (2003)
ANTP	NKL	*Nkx2-6*	NKX2-6 mutation predisposes to familial atrial fibrillation.	Wang et al. (2014)
PRD		*Prrx1* and *Prrx2*	Formation of cardiovascular system and connective tissues of the heart and in the great arteries and veins.	Bergwerff et al. (2000)
PRD		*Pax3, Pax7*	Involved in neural crest and cardiac development	JA (1996)
ZF (TALE)		*ZFHX3*	Genetic polymorphisms in are associated with atrial fibrillation in a Chinese Han population.	Liu et al. (2014)
PBC (TALE)		*Pbx1*	Patterning of the great arteries and cardiac outflow tract.	Stankunas et al. (2008) and Arrington et al. (2012), Chang et al. (2008)
			Pbx acts with Hand2 in early myocardial differentiation in zebrafish. Non-synonymous variants in PBX genes are associated with congenital heart defects.	
TGIF (TALE)		*Tgif1* and *Tgif2*	Left-right asymmetry formation and embryonic heart looping.	Powers et al. (2010)
SIX/SO		*Six2*	Six2 marks a dynamic subset of second heart field progenitors.	Zhou et al. (2017)

23 Concluding Remarks

Heart development is a complex process requiring strict spatiotemporal development to form a healthy organ providing properly functioning organism. Multiple transcription factors, signalling pathways, morphogens and other stimuli govern the heart development process. However, it is possible to state that multiple homeobox genes and their coded transcription factors come into the heart developmental stages when their function is needed (Tables 1 and 2). None of these homeodomain transcription factors can be separated from each other, since their ability to bind DNA affects patterns of multiple gene thus resulting in changed transcriptome level of multiple cells.

Homeodomain proteins are not the only transcription factors important for cardiac development. The transcription factors of other gene families also significantly contribute heart development. For example, GATA, Tbx, HAND, Mef2c and other accompany homeodomain factors like Nkx2-5, Isl1, etc. (Hiroi et al. 2001; Gao et al. 2011; Maves et al. 2009; Skerjanc et al. 1998). Most of these homeodomain transcription factors are conserved and display coexistence and codependence in heart development of human as well as simple invertebrates like fruit fly or ascidians (Jensen et al. 2013a; Olson 2006). Only birds and mammals display fully separated heart, however reptilians still have no septum between right and left ventricles (Jensen et al. 2013b). Deeper further insights into septum formation of lower vertebrates like snakes, lizards and turtles could also help to understand signalling networks of human congenital heart diseases. Maybe in the future will be possible to engineer a reptile with four chambered heart, thus leading to better understanding of cardiac regeneration process and allowing to develop new therapeutic

strategies for human cardiac congenital and other types of diseases.

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