Part V

The Second Heart Field and Outflow Tract

Perspective

Seema Mital

Outflow tract defects account for around 30 % of all congenital heart disease and are associated with a significant health burden and ongoing late mortality despite complete repair. Although outflow tract defects manifest as a range of anatomically distinct and apparently heterogeneous lesions – tetralogy of Fallot, double-outlet right ventricle, interrupted aortic arch, transposition of great arteries, truncus arteriosus, and other related defects – embryologically they appear to have a common origin related to abnormal development of the embryonic conotruncus during the septation of the arterial outflow tract. In the vast majority of cases (80 %), the genetic etiology is not known. The most common defect causing outflow tract defects is the 22q11del that only accounts for 10–15 % of cases overall.

In the past decade, major advances using mouse models have created a paradigm shift in our understanding of cardiac development through the discovery of the second heart field. While the primary heart field gives rise to the left ventricle and most other cardiac structures, the right ventricle and the outflow tract arise from the embryologically distinct second heart field. Proliferation and differentiation of the second heart field are controlled by a complex transcriptional network. Pioneering work in the understanding of the second heart field including lineage-tracing studies has shown that perturbations in genes of the second heart field result in abnormal formation and septation of the arterial outflow tract resulting in outflow tract defects. Computer-assisted 3D reconstruction analysis to assess spatial and

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developmental gene expression patterns, morphogenesis, and proliferation in situ has further helped understand outflow tract morphogenesis and effect of dysregulation of genes regulating its development.

The foregoing chapters discuss the history of the second heart field, the central role of multipotent second heart field progenitors in cardiac development and their contribution to the definitive arterial pole, the major signaling pathways that control second heart field progenitors including the role of genes like *Mef2c*, *Cadm4*, and *Foxc2*, as well as the environmental factors in outflow tract defect development. Eventually, knowledge gained from these model organisms can be used to guide genetic screening of human subjects to identify causal genes associated with outflow tract defects in humans.

Properties of Cardiac Progenitor Cells in the Second Heart Field

23

Alexandre Francou and Robert G. Kelly

Abstract

Heart tube elongation occurs by progressive addition of cells from pharyngeal mesoderm to the poles of the heart. These progenitor cells, termed the second heart field, contribute to right ventricular and outflow tract myocardium at the arterial pole of the heart and to atrial myocardium at the venous pole. Perturbation of this process results in congenital heart defects. Since the discovery of this progenitor cell population, much has been learned about the signaling pathways and transcription factors regulating second heart field deployment. However, fundamental questions about the molecular and cellular mechanisms underlying heart tube elongation remain. Here we briefly review a selection of recent findings in the area of second heart field biology and discuss the clinical implications of these new studies for our understanding of the etiology of congenital heart defects.

Keywords

Cardiac progenitor cells • Congenital heart defect • Second heart field

23.1 Introduction

In 2001 a cardiac progenitor cell population situated in pharyngeal mesoderm was found to give rise to myocardium of the right ventricle and outflow tract. In the intervening period, it has become apparent that this progenitor cell population (1) is part of a larger population of cardiac progenitor cells termed the second heart field (SHF) that also contributes to atrial myocardium, (2) corresponds to a genetic

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lineage distinct from the first heart field (FHF) that gives rise to the cardiac crescent and left ventricle, and (3) is progressively deployed in the pharyngeal region during embryonic heart development through the activity of multiple signaling pathways and transcription factors. Of particular relevance is the discovery that perturbation of SHF development in animal models and human patients results in a spectrum of congenital heart defects (CHD) affecting the poles of the heart, including conotruncal and atrial septal defects. Details of these features of SHF development have been documented in a series of recent reviews [1, 2]. Here we will discuss a selection of recently published studies that impact on our understanding of second heart field biology, with focus on mechanistic insights into the etiology of CHD.

23.2 Demarcating the First and Second Heart Fields and Their Contributions to the Heart

The distinction between the FHF and SHF has been controversial, despite evidence from clonal analysis and genetic lineage studies that these progenitor populations correspond to separate lineages [1, 2]. Further support for a two-lineage model of heart development has been provided by a study involving Boolean modeling of gene regulatory networks in heart development [3]. This work identified two stable states corresponding to the FHF and SHF and suggests that the differences between these states are hardwired into the signaling and transcription factor interactions operative in the early embryo. This modeling approach highlights the temporal distinction between the FHF and SHF and can be used to predict gene function, providing an important step toward integrated understanding of regulatory networks during early heart development.

The discovery that *Hcn4*, encoding a nucleotide-gated channel protein, is expressed in the FHF, in a complementary pattern to the SHF gene *Isl1*, has reinforced the concept that the vertebrate heart is built from distinct progenitor cell populations [4, 5]. An inducible Cre allele of *Hcn4* has allowed evaluation of the contribution of the FHF to the definitive heart. Unlike the SHF, which contains multipotent cardiovascular progenitor cells, FHF derivatives appear to be restricted to myocardium [4]. Interestingly, the two lineages contribute differently and in a complementary manner to different components of the cardiac conduction system [5]. In particular, the right bundle branch and majority of the right Purkinje fiber system have a SHF origin. Similar observations have recently been made based on retrospective clonal analysis and regionalized transgene expression data, supporting dual contributions of the FHF and SHF to the conduction system [6]. These findings are of relevance in understanding the origins of arrhythmias resulting from perturbed development of particular segments of the conduction axis.

23.3 New Insights into the Role and Regulation of Noncanonical Wnt Signaling in the Second Heart Field and the Origins of Conotruncal CHD

Continued proliferation and delayed differentiation are defining properties of the SHF; indeed, separation of the sites of proliferation and differentiation provides a mechanism allowing rapid growth of the embryonic heart. The role and regulation of the noncanonical Wnt ligands Wnt5 and Wnt11 in these processes have been the focus of a number of recent studies. While both ligands were known to be individually required for outflow tract morphogenesis, they have now been shown to be co-required for SHF development [7], by downregulating the canonical Wnt pathway during myocardial differentiation and activating noncanonical Wnt planar cell polarity (PCP) signaling. *Wnt5* is expressed before *Wnt11* in SHF cells in the posterior dorsal pericardial wall and has been shown to be directly activated by the DiGeorge syndrome candidate gene *Tbx1*, a key regulator of proliferation and differentiation in the SHF [8]. The severity of conotruncal defects in *Wnt5a* null mice is increased in the presence of a *Tbx1* null allele, while double mutant embryos lack the right ventricle and outflow tract and die at midgestation. TBX1 directly regulates *Wnt5a* through interaction with BAF60a, a progenitor cell-specific component of the BAF chromatin remodeling complex, as well as the histone methyltransferase SETD7, interactions shown to be necessary for activation of a number of TBX1 transcriptional targets in the SHF [8]. *Tbx1* itself is regulated by the activity of a histone acetyltransferase, MOZ, loss of function of which partially phenocopies DiGeorge syndrome [9]. The intersection of *Tbx1* with chromatin regulators is highly significant given the recent finding that de novo mutations in genes encoding such molecules are overrepresented in human CHD patients [10].

In support of a role for Tbx1 upstream of noncanonical Wnt signaling, *wnt11r* is downregulated in *tbx1* mutant zebrafish; heart looping and differentiation defects in the absence of *tbx1* can be partially rescued by ectopic wnt11r or a wnt11r target gene encoding a cell adhesion molecule, alcama [11]. Altered cell shape in *tbx1* mutant fish hearts suggests that noncanonical Wnt signaling regulates cardiomyocyte cell polarity downstream of tbx1 [11]. Whether Tbx1 and noncanonical Wnt signaling also regulate cell polarity in the SHF remains to be seen. SHF and conotruncal development is impaired in mice lacking Dvl1 and Dvl2, regulators of both canonical Wnt signaling and the noncanonical Wnt PCP pathway [12]. In this study, the PCP signaling function of *Dvl* genes was shown to be specifically required in the SHF lineage and the cardiac phenotype to resemble that of embryos lacking the core PCP gene *Vangl2*; furthermore, *Wnt5a* and *Vangl2* interact to increase the severity of outflow tract defects [12]. Loss of PCP gene function results in disorganization of progenitor cells in the posterior dorsal pericardial wall, potentially resulting in impaired SHF deployment toward the outflow tract and conotruncal anomalies.

Among the last myocardial derivatives of the SHF to be added to the elongating heart tube is future subpulmonary myocardium, a cell population specifically affected in *Tbx1* mutant embryos [13]. Asymmetric addition of SHF progenitor

cells giving rise to subpulmonary myocardium continues on the left side of the outflow tract up until embryonic day 12.5 in the mouse; furthermore, this late contribution drives rotation of the outflow tract, positioning subpulmonary myocardium on the ventral side of the heart and aligning the ascending aorta with the left ventricle [14]. Failure of this process, termed the "pulmonary push," results in outflow tract alignment defects such as double outlet right ventricle. Underdevelopment of subpulmonary myocardium has been implicated in the etiology of tetralogy of Fallot [15], and further analysis of the regulation of future subpulmonary myocardium is an important step toward deciphering the etiology of CHD.

23.4 Involvement of the Second Heart Field in Atrial and Atrioventricular Septal Defects

At the venous pole of the heart, SHF cells contribute to the dorsal mesenchymal protrusion that bridges the atrioventricular cushions and primary atrial septum. Failure of proliferation or precocious differentiation of these cells results in primum atrial septal defects when canonical Wnt or hedgehog signaling, respectively, is compromised [1, 2]. BMP signaling has now been shown to promote the proliferation of DMP progenitor cells, in contrast to the pro-differentiation role of this signaling pathway during SHF addition at the arterial pole [16]. Isl1, Wnt2, and the transcription factor Gli1 are expressed in this region of the SHF and have been shown to identify a multipotent cardiopulmonary progenitor cell population that contributes not only to the venous pole of the heart but also to diverse pulmonary lineages, including smooth muscle and endothelium [17]. The development of these cells that ensure the vascular connection between the heart and lung is coordinated by hedgehog signaling from future pulmonary endoderm. The Holt-Oram syndrome gene *Tbx5* also operates in this posterior component of the SHF and is required for normal atrial septation through regulating proliferation and hedgehog signal reception by direct activation of cell cycle progression genes such as *Cdk6* and the hedgehog signaling component Gas1 [18]. Genetic and retrospective lineage studies have revealed that venous pole and future subpulmonary myocardium are clonally related, suggesting that a population of common SHF progenitor cells segregates to the arterial and venous pole of the heart [19]. Where such common progenitor cells are located and how their segregation to the poles is regulated remains unknown.

23.5 Future Directions and Clinical Implications

The importance of perturbed SHF development in the etiology of common forms of CHD affecting both poles of the heart is now clear. The studies discussed here provide new insights into the underlying mechanisms, although much remains to be learnt about the regulatory interactions between signaling pathways and

transcription factors controlling cellular properties of the SHF and how different regions of the heart are prepatterned and segregate within the progenitor cell population. Future research will address these questions in the context of dynamic heart tube elongation. The human SHF, as defined by ISl1 expression, coincides with that observed in avian and mouse embryos [20], and thus findings from these models are directly relevant to a better understanding of the origins of CHD in man. Furthermore, insights into how the differentiation of cardiac progenitor cells is controlled are essential for future regenerative therapies.

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Nodal Signaling and Congenital Heart Defects

24

Ralston M. Barnes and Brian L. Black

Abstract

Nodal is a TGF-β family member ligand that is critical for early embryonic patterning in vertebrates. Nodal signaling functions through core TGF-β receptors to activate a Smad transcription factor signaling cascade. However, unlike other TGF-β ligands, Nodal signaling requires an additional co-receptor of the EGF-CFC family to activate intracellular signaling. Nodal signaling is also subject to extensive negative regulation by Lefty and other factors. Work in numerous model organisms, including mouse, chicken, and zebrafish, established that Nodal signaling plays an essential role during germ layer formation, anterior-posterior axis patterning, and left-right axis determination. Incomplete or delayed loss of Nodal signaling results in defective organogenesis and birth defects, including congenital heart defects, and clinical studies have linked aberrant Nodal signaling in humans with many common congenital malformations, including congenital heart defects. Congenital heart defects associated with disrupted Nodal signaling in mammals include those that arise due to global defects in left-right patterning of the embryo, such as heterotaxy. Other Nodal-associated heart defects appear to occur as more subtle isolated malformations of the great arteries and atrioventricular septum, which may not be related to overall perturbations in laterality. A more detailed understanding of the Nodal signaling pathway and its targets in the heart is required to more fully understand the etiology of Nodal signaling pathway-associated congenital heart defects.

Keywords

Nodal signaling • *Tdgf1* • Cripto • Congenital heart defects • TGF-β • Laterality defects

Note: This chapter is also related to Part II.

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24.1 Introduction

The Nodal signaling pathway is critical for early embryonic patterning in all vertebrates. Nodal is a secreted signaling molecule that belongs to the transforming growth factor-β (TGF-β) family and functions primarily to activate downstream signaling through a receptor-mediated response [1–3]. The Nodal pathway plays a critical role in mesoderm specification and anterior-posterior patterning during gastrulation and is also essential for establishing the left-right axis in the developing embryo [4–7]. Changes in Nodal expression or dosage can disrupt left-right patterning and result in a range of congenital defects that affect development of the forebrain, the craniofacial skeleton, and several other organs, including the heart.

24.2 The Nodal Signaling Pathway

The Nodal signaling pathway shares many similarities with other TGF-β signaling pathways in that it utilizes core Smad-dependent signaling components (Fig. 24.1). Nodal is secreted extracellularly as a proprotein homodimer like other TGF-β ligands [8]. Once cleaved into the mature ligand, the Nodal homodimer binds tightly to a TGF-β receptor heterodimer consisting of both Type I and Type II receptors [9]. Nodal ligand binding causes the constitutively active Type II receptor serine/threonine kinases to associate with the inactive Type I receptor kinases and leads to phosphorylation and the subsequent dissociation of R-Smad from the TGF-β receptor [10]. Following formation of a trimeric complex composed of two R-Smads and the common partner Smad, Smad4, the Smad oligomer translocates to the nucleus where it regulates gene expression through direct and indirect DNA binding (Fig. 24.1) [1, 11, 12].

The Nodal signaling pathway has important distinctions from other TGF-β-mediated signaling pathways. Nodal can only activate TGF-β receptor signaling in the presence of an EGF-CFC protein (Fig. 24.1). There are two EGF-CFC proteins, Cripto and Cryptic, which function as co-receptors for Nodal [13]. Cripto and Cryptic are extracellular proteins that contain an epidermal growth factor-like motif and a novel cystine-rich domain named the CFC [13]. EGF-CFC proteins function primarily by binding to the Type II TGF-β receptor through the CFC domain and by binding to Nodal through the EGF domain [14].

In response to Nodal signaling, the Smad complex cooperates specifically with FoxH1, a winged helix transcription factor, or Mixer, a member of the Mix subclass of homeodomain proteins [15, 16]. These cofactors are critical for Nodal-dependent downstream gene activation and act to stabilize Smad-DNA interactions, since Smads have relatively weak DNA-binding affinity [17, 18]. FoxH1 and Mixer recruit the Smad complex to promoter and enhancer elements and help to establish temporal and spatial regulation of Nodal-dependent target genes.

In addition, the Nodal signaling pathway is subject to specific negative regulation not found for other TGF-β family members. Proteins of the Lefty family, specifically Lefty1 and Lefty2, inhibit Nodal-dependent activation of TGF-β

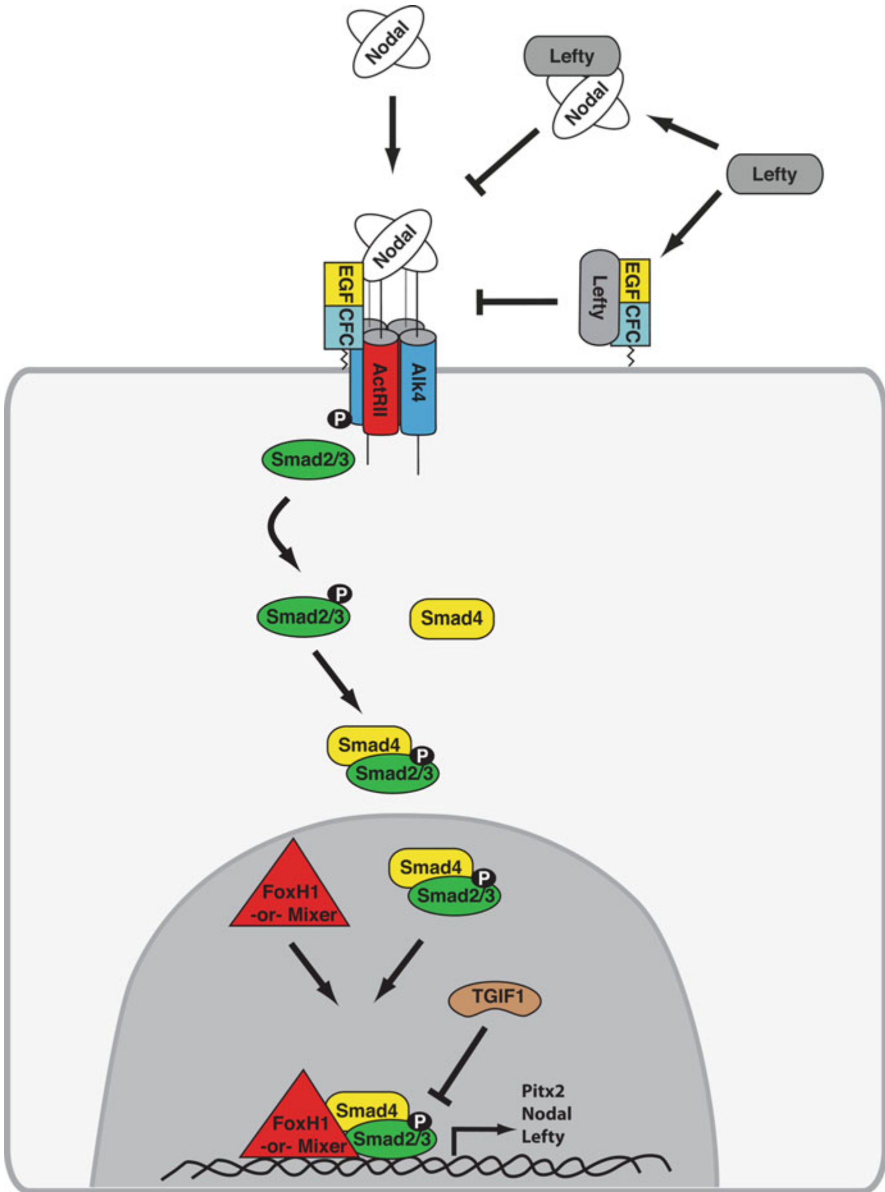

Fig. 24.1 The Nodal signaling pathway. The Nodal ligand binds to a dimer of the TGF-β Type I and Type II receptors. In association with an EGF-CFC co-receptor (Cripto or Cryptic), Nodal activates the receptor complex causing the phosphorylation of either Smad2 or Smad3 followed by oligomerization with the common partner Smad4. The Smad2/Smad3-Smad4 complex then translocates to the nucleus where it binds to DNA with the transcriptional cofactor FoxH1 or Mixer, leading to transcription of downstream target genes. The Nodal signaling pathway is negatively regulated by proteins such as Lefty, which can bind to either EGF-CFC or the Nodal dimer to prevent activation of the receptor complex, or TGIF1, which recruits histone deacetylases to Smad2/Smad3 and represses transcriptional activation

receptors by interacting directly with the Nodal ligand and preventing binding to the TGF-β receptor heterodimer and by binding to the EGF-CFC Nodal co-receptors Cripto and Cryptic and preventing their association with the TGF-β receptor complex (Fig. 24.1) [19–21].

24.3 Requirement for Nodal in Development

Nodal signaling is critical for the patterning of the developing embryo [4, 22]. Loss-of-function studies in vertebrate model organisms indicate that Nodal signaling is first required during gastrulation. Germline loss of *Nodal* in mice results in severe patterning and differentiation defects and embryonic lethality due to a failure to induce the primitive streak from the ectoderm and to disrupted specification of mesoderm and endoderm from the epiblast [5]. Additionally, loss of *Nodal* in mice results in impaired anterior-posterior axis formation due to the lack of anterior visceral endoderm formation [22]. The requirement of Nodal signaling for early embryonic pattern formation was further highlighted by loss-of-function studies of the EGF-CFC Nodal co-receptor Cripto. Inactivation of *Tdgf1*, the gene encoding Cripto, in mice results in a phenocopy of early Nodal defects, including lethality shortly after gastrulation. *Tdgf1* mutants lack a primitive streak, fail to form embryonic mesoderm, and exhibit anterior-posterior axis defects [6]. The *one-eyed pinhead* (*oep*) mutation in zebrafish results in a complete loss of function of the fish ortholog of the Nodal co-receptor Cripto [23]. These mutants exhibit a phenocopy of the early Nodal defects seen in mice, including an absence of mesoderm and anterior-posterior axis abnormalities [23]. Together, both animal models establish that EGF-CFC proteins are required for Nodal signaling and support an early requirement for the Nodal pathway in embryo morphogenesis.

Following gastrulation, Nodal signaling is indispensable for the establishment of left-right asymmetry. Conditional deletion of *Nodal* in the lateral plate mesoderm in mice circumvents the early requirement for Nodal signaling during gastrulation and results in heterotaxy, a condition characterized by left-right ambiguity of thoracic and abdominal visceral organs [24]. These mice exhibit transposition of the great arteries of the heart, right-sided isomerism of the lungs, and right-sided stomach [25]. Similarly, germline deletion of *Cfc1*, the gene encoding Cryptic, results in left-right laterality defects with mutants exhibiting heterotaxy [26].

In addition to playing a role in the establishment of laterality following gastrulation, Nodal signaling is also required for midline patterning of the ventral forebrain [27]. Zebrafish mutants for the Nodal ligands and for the ortholog of Cripto result in holoprosencephaly, a condition where bifurcation of the ventral forebrain fails to occur and results in fusion of the two brain hemispheres [28]. Similarly, mice heterozygous for germline knockout alleles of both *Nodal* and *Smad2* have cyclopia, a rare and severe form of holoprosencephaly [29], further supporting the relationship between Nodal signaling and forebrain development. Mechanistically, this phenotype is thought to occur due to the patterning of *Sonic Hedgehog (Shh*) expression in the forebrain by Nodal [30, 31].

24.4 Congenital Heart Defects Associated with Perturbations in Nodal Signaling

It is perhaps not surprising to find that the Nodal signaling pathway is associated with pathogenesis in humans, given its critical role in patterning during embryonic development. Nodal signaling was first linked to human congenital defects through the identification of mutations associated with left-right laterality defects. These include mutations in genes encoding Lefty family members, the EGF-CFC co-receptor Cryptic, and the Type II TGF-β receptors [32–34]. In addition, mutations in genes encoding transcriptional inhibitors of Smad2, such as TGIF1, are associated with holoprosencephaly, a defect strongly associated with disrupted Nodal signaling [30, 35]. Interestingly, these human pathologies are similar to the defects observed in animal models with defective (but not incomplete) Nodal signaling.

Congenital heart defects have also been linked to aberrant Nodal signaling (Table 24.1) [36]. Loss-of-function mutations in genes encoding numerous Nodal signaling components, including Nodal, Cripto, Cryptic, and FoxH1, have been identified in patients with heart defects [37, 38]. The spectrum of heart defects in these patients can be roughly grouped into two broadly defined classes: (1) those that occur as a result of overall isomerism or heterotaxy and (2) those that occur as isolated congenital heart defects. The isomerisms of the heart can be classified as *situs inversus totalis*, a complete mirror image of the visceral organs of the body including the heart, or *situs inversus ambiguous*, where the abdominal and visceral organs are distributed abnormally and randomly in a condition more commonly called heterotaxy [39]. Both of these conditions can be linked to aberrant Nodal signaling [40]. Heterotaxy often results in complex congenital heart defects [41, 42]. These defects include levo-transposition of the great arteries (l-TGA) and atrial isomerism [36, 39]. The feature characteristics of l-TGA are improper positioning of the aorta and the pulmonary artery such that the arteries are switched in conjunction with the ventricles such that they still have the normal relationship between the ventricles and the arteries [43].

Isolated congenital heart defects associated with Nodal mutations can result in structural and functional abnormalities that appear to be independent of overall left-right ambiguity. These isolated defects include dextro-transposition of the great arteries (d-TGA), double outlet right ventricle (DORV), tetralogy of Fallot, and isolated ventricular septal defects [37, 38]. Unlike l-TGA, which results in a proper alignment of the arteries with respect to the ventricles, d-TGA results in a switching of aorta and the pulmonary artery such that the pulmonary artery connects to the left ventricle and the aorta emanates from the right ventricle [43]. DORV, as the name implies, occurs when the aorta and the pulmonary artery connect to the right ventricle. Tetralogy of Fallot is essentially a milder form of DORV in which the aorta overrides the ventricular septum and empties blood from both ventricles [44]. There is also pulmonary artery stenosis and a hypertrophic right ventricle secondary to pulmonary artery blockage associated with tetralogy of Fallot [44]. These defects appear to be independent of overall laterality, although the

Table 24.1 Some of the congenital defects associated with altered Nodal signaling in mammals

Component	Species	CV defects	Other defects	References
Nodal	Human	Dextrocardia, d-TGA, DORV, VSD, ASD, l-TGA, DILV, PA, TOF	Asplenia, bilateral trilobed lungs, hydronephrosis, HPE, intestinal malrotation	[36, 38]
	Mouse	l-TGA, VSD	Heterotaxy, asplenia, isomerisms, HPE, cyclopia, disrupted endoderm and mesoderm specification, defective A-P axis	[5, 22, 25, 27]
Cryptic	Human	Dextrocardia, TGA, d-TGA, PA, VSD, ASD, TOF, DORV	Heterotaxy, isomerisms, polysplenia, asplenia	[32, 37, 49]
	Mouse	l-TGA, ASD	Heterotaxy, r-isomerism of the lung, hyposplenia	[26]
Cripto	Human	TOF, VSD, ASD	HPE	[37, 50, 51]
	Mouse		HPE, disrupted mesoderm specification, defective A-P axis	[6, 52]
Smad2	Human	Dextrocardia, d-TGA, DORV, ASD	Heterotaxy, asplenia, HPE	[37, 53]
TGF-βR2	Mouse	TGA, DORV, dextrocardia, levocardia, VSD, ASD, arch artery defects	R-isomerism of the lung, axial skeleton abnormalities	[54]
FoxH1	Human	VSD, TGA, TOF	HPE	[37, 55]
	Mouse		Defective elongation of the primitive streak, defective A-P axis	[16]

mechanistic basis for why these defects occur when Nodal signaling is disrupted is unclear. Interestingly, *Nodal* expression occurs well prior to the patterning and final alignment of the aorta and pulmonary artery [40]. Furthermore, the temporal expression pattern of *Nodal* observed during development is tightly regulated within a narrow developmental window due, at least in part, to the extensive negative feedback by Lefty proteins and other factors. The diverse nature of heterotaxy in humans suggests that some isolated congenital heart defects associated with perturbed Nodal signaling may still be secondary to overall laterality defects.

A role for Nodal signaling in isolated congenital heart defects is also supported by studies in mice in which subtle perturbations of Nodal signaling result in less severe defects (Table 24.1), suggesting that the Nodal ligand functions in a dosage-dependent manner. For example, deletion of an intronic enhancer of *Nodal* resulted in decreased expression of *Nodal* in the lateral plate mesoderm. These mouse

embryos developed laterality defects that were less severe than the heterotaxy observed in *Nodal* conditional knockouts where *Nodal* expression in lateral plate mesoderm was completely abolished [25, 45].

Consistent with Nodal function in left-right patterning, Nodal-dependent target genes are also critical for left-right patterning and heart development [21]. *Pitx2* is perhaps the best-described target gene of Nodal signaling and is expressed asymmetrically on the left side after gastrulation [46]. Misexpression of Pitx2 on the embryonic right side in mice results in heterotaxia with conditions such as aberrant heart looping, cardiac isomerism, and visceral organ laterality defects [46]. In the mouse, germline loss of function of *Pitx2* results in left-right asymmetry defects in specific organs, such as the lung [47]. Interestingly, *Pitx2*-null and isoform-specific *Pitx2c*-null embryos undergo normal heart looping but have a subset of congenital cardiovascular anomalies such as DORV and ventricular and atrial septal defects [47, 48]. These observations suggest that Pitx2 functions in heart development after left-right determination and that other Nodal-dependent target genes may be required for cardiac laterality. Together, these observations suggest that other unappreciated Nodal-dependent target genes are involved in the establishment of left-right identity and cardiac development. A more detailed elucidation of this fundamental pathway, including target genes in the cardiac mesoderm, is required to more fully understand the role of Nodal signaling in heart development and in congenital heart defects.

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Utilizing Zebrafish to Understand Second Heart Field Development

25

H.G. Knight and Deborah Yelon

Abstract

Heart formation relies on two sources of cardiomyocytes: the first heart field (FHF), which gives rise to the linear heart tube, and the second heart field (SHF), which gives rise to the right ventricle, the outflow tract, parts of the atria, and the inflow tract. The development of the SHF is of particular importance due to its relevance to common congenital heart defects. However, it remains unclear how the SHF is maintained in a progenitor state while the FHF differentiates. Likewise, the factors that trigger SHF differentiation into specific cardiac cell types are poorly understood. Investigation of SHF development can benefit from the utilization of multiple model organisms. Here, we review the experiments that have identified the SHF in zebrafish and investigated its contribution to the poles of the zebrafish heart. Already, zebrafish research has illuminated novel positive and negative regulators of SHF development, cementing the utility of zebrafish in this context.

Keywords

Second heart field • Zebrafish • Outflow tract • Inflow tract

25.1 Introduction

The embryonic origins of the heart have been a topic of intense interest due to the prevalence of congenital heart defects [1]. Cardiac progenitors (CPs) from the first heart field (FHF) form the initial heart tube, and CPs from the second heart field (SHF) contribute to most of the structures of the mature heart including the outflow tract, right ventricle, and much of the atria [2]. The SHF is generally defined as a

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population of CPs that originates adjacent to the FHF, differentiates after the initial heart tube has formed, and is responsible for cardiomyocyte accretion at both poles of the heart tube [2]. The SHF is particularly significant to congenital heart disease; many common cardiac abnormalities are caused by defects in SHF-derived tissues, including ventricular and atrial septal defects, transposition of the great arteries, and double outlet right ventricle [3]. Despite the importance of the SHF, the mechanisms that distinguish FHF and SHF development remain unclear. What signals or factors prevent the SHF from differentiating while the FHF is deployed, and what eventual change triggers SHF differentiation? Recent advances in zebrafish research offer new approaches that can complement work in mice to deepen our comprehension of SHF regulation.

Several lines of evidence indicate the presence of a population of late-differentiating CPs in zebrafish that is likely to be analogous to the mammalian SHF. The conservation of the SHF provides exciting opportunities to advance our understanding using the distinct advantages of zebrafish embryos [4]. Zebrafish embryos develop rapidly and have small hearts that are particularly tractable for cellular resolution of cardiogenesis. Furthermore, the transparency of the zebrafish embryo facilitates exceptional opportunities for time-lapse imaging of heart formation and tracking of cardiac cell fates. Finally, zebrafish are particularly well suited for conducting both genetic and chemical screens, which have the potential to identify novel regulators of heart development. Here, we review the studies that support the existence of a zebrafish SHF and demonstrate the utility of the zebrafish for opening new avenues in SHF research.

25.2 Late-Differentiating Cardiomyocytes Originate from the SHF in Zebrafish

Two types of assays have demonstrated that late-differentiating cardiomyocytes are recruited to the poles of the zebrafish heart tube. First, a developmental timing assay that relies on the different kinetics of GFP and DsRed fluorescence was used to visualize the dynamics of cardiomyocyte differentiation. Analysis of *Tg(myl7:GFP); Tg(myl7:DsRed)* embryos showed that newly differentiated cardiomyocytes populate the cardiac poles at 48 h postfertilization (hpf), whereas cardiomyocytes in the middle of the heart differentiate at an earlier stage (Fig. 25.1a; [5]). Second, photoconversion assays have consistently revealed late-differentiating cardiomyocytes in the outflow tract. UV exposure of *Tg(myl7:kaede)* or *Tg(myl7:KikGR)* embryos after the heart tube has formed, followed by imaging at 48 hpf, showed addition of cardiomyocytes to the outflow tract after the time of photoconversion (Fig. 25.1b, [5, 6]). Together, these experiments revealed the existence of late-differentiating cardiomyocytes at the arterial pole of the zebrafish heart that seem to be analogous to SHF-derived cardiomyocytes in mammals.

Fate mapping in zebrafish has shown that early SHF precursors seem to neighbor the FHF. Prior to gastrulation, arterial pole progenitors are found adjacent to ventricular progenitors at the embryonic margin (Fig. 25.1c; [7]). After

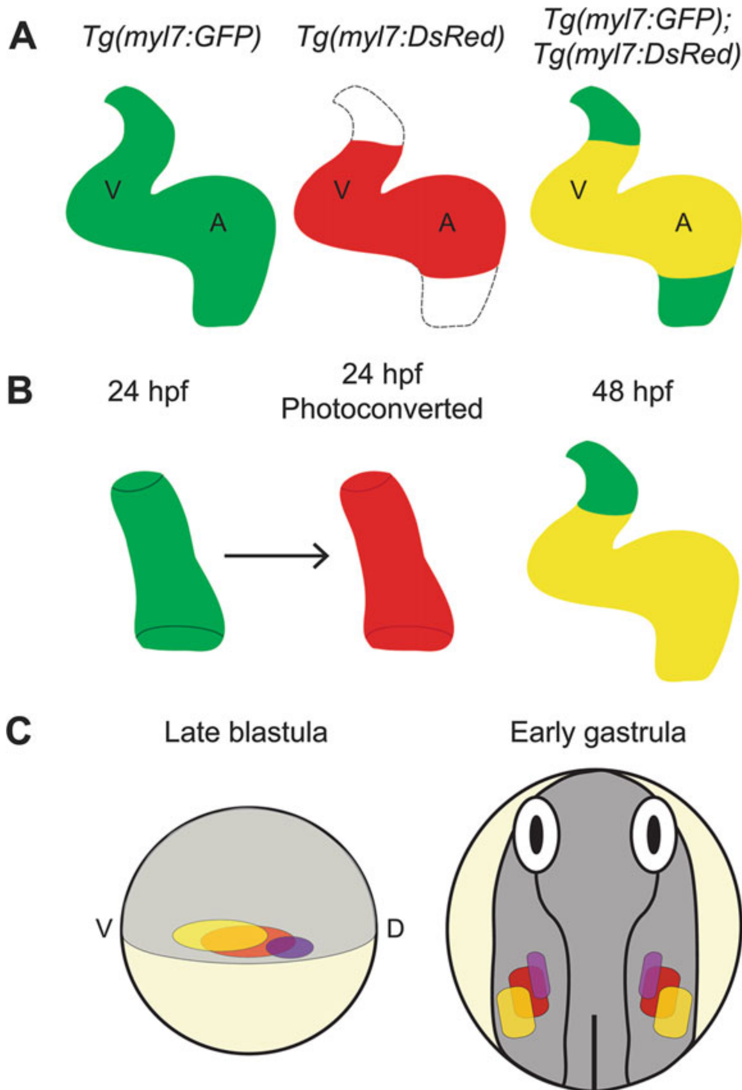

Fig. 25.1 Late-differentiating cardiomyocytes originate from the zebrafish SHF. (**a**) A developmental timing assay reveals late-differentiating cardiomyocytes displaying GFP, but not DsRed [5]. (**b**) Green-to-red conversion of photoconvertible proteins expressed in differentiated cardiomyocytes at 24 hpf, followed by imaging at 48 hpf, reveals newly added green cardiomyocytes in the outflow tract [6]. (**c**) Fate mapping in the late blastula shows that outflow tract progenitors (*purple*) are located close to the margin, adjacent to ventricular progenitors (*red*), and separate from atrial progenitors (*yellow*) [7]. In the early gastrula, outflow tract progenitors are located in a medial cranial portion of the ALPM [7]

gastrulation, arterial pole progenitors map to a medial cranial region next to the FHF in the anterior lateral plate mesoderm (ALPM) (Fig. 25.1c; [7]). Finally, DiI labeling has shown that the SHF resides adjacent to the heart tube in older embryos: pericardial cells just outside the outflow tract at 24 hpf move into the arterial pole at later stages [7]. The SHF has also been identified using Cre-mediated lineage tracing. This technique has shown that arterial pole progenitors express both *gata4* and *nkx2.5* during somitogenesis, confirming that SHF progenitors originate in the ALPM [8]. Furthermore, Cre-mediated lineage tracing has confirmed that cells from the pericardial mesenchyme adjacent to the heart tube migrate into the outflow tract [9]. Taken together, these analyses show that the late-differentiating cardiomyocytes at the zebrafish arterial pole meet the criteria that define the SHF. Outflow tract cells remain undifferentiated until after the linear heart tube has formed, are recruited to the arterial pole from outside the heart, and map to an area adjacent to the FHF. These data, combined with conserved molecular mechanisms regulating mouse and zebrafish arterial pole development, suggest that the SHF is a conserved vertebrate feature.

25.3 Mechanisms Regulating Outflow Tract Development in Zebrafish

Studies of the regulation of outflow tract formation have demonstrated conservation of the transcription factors utilized in zebrafish and mice. Zebrafish embryos deficient in *mef2cb* lack late-differentiating cells that form the outflow tract [6], which is strikingly similar to the phenotype of *Mef2c* mutant mice that lack the SHF-derived outflow tract and right ventricle [10]. Zebrafish *tbx1* mutants have several outflow tract defects, including reduced migration of cells into the heart [7] and reduced proliferation of cells at the arterial pole, resulting in a small outflow tract [11]. This phenotype is reminiscent of mouse *Tbx1* mutants, which also display outflow tract abnormalities due to severely reduced proliferation in the SHF [12].

Signaling pathways also seem to have conserved roles in the mouse and zebrafish SHF. Hedgehog signaling is important for zebrafish SHF development; migration of cells into the heart is impaired in *smoothened* mutants, resulting in a small outflow tract [7]. Similarly, hedgehog signaling is crucial for mammalian SHF survival and outflow tract septation [13]. In zebrafish, reduced FGF signaling eliminates accretion of cardiomyocytes at the arterial pole [5] and blocks *mef2cb* expression in the SHF [6]. This requirement for FGF signaling mimics mouse *Fgf8* mutants, which have a severely hypoplastic outflow tract and right ventricle [13]. These findings underscore the conserved mechanisms regulating outflow tract development and suggest that new discoveries in the zebrafish SHF are likely to be relevant to mammals.

Importantly, novel insights into outflow tract development have emerged through studies in zebrafish. The role of Ltbp3, a secreted protein that regulates TGF-β ligand availability, has been of particular interest. *ltbp3* is expressed in the

zebrafish SHF, and Cre-mediated lineage tracing has shown that *ltbp3*-expressing cells give rise to outflow tract cardiomyocytes [9]. Ltbp3-deficient embryos lack an outflow tract due to reduced SHF proliferation, a consequence of reduced TGF-β signaling [9]. This work not only illuminated Ltbp3 as a new SHF regulator but also uncovered a novel role for TGF-β signaling in SHF development. Additional studies have revealed that Nkx2.5 promotes maintenance of *ltbp3* expression [8]. This is exciting, as it elucidates a new pathway downstream of Nkx2.5: Nkx2.5 facilitates the activation of TGF-β signaling through regulation of *ltbp3* and thereby drives SHF proliferation. Since Nkx2.5 is highly relevant to congenital heart disease, factors downstream of Nkx2.5 are excellent candidates for translational research. Thus, investigations in zebrafish can lead to the discovery of novel regulators of SHF development and provide new insight into connections between important factors.

25.4 Mechanisms Regulating Inflow Tract Development in Zebrafish

In mice, the SHF has been shown to contribute to the venous pole in addition to the arterial pole [2]. The mammalian SHF is thought to be subdivided into the anterior SHF, which gives rise to the right ventricle and outflow tract, and the posterior SHF, which gives rise to the atria and the inflow tract [2]. The zebrafish heart has a distinct population of inflow tract cells that express the canonical SHF marker Isl1 [14]. In addition, developmental timing assays have shown that the zebrafish inflow tract contains a population of late-differentiating cardiomyocytes (Fig. 25.1a; [5]). However, the degree of overlap between these two populations has not been examined, and the precise timing of when inflow tract cells are added to the heart is unclear. Furthermore, it is not known where zebrafish inflow tract cells originate in the early embryo and if inflow and outflow tract progenitors share a common lineage. Future experiments will be valuable to elucidate the zebrafish equivalent of the mammalian posterior SHF.

Studies of inflow tract development in zebrafish have revolved around the role of Isl1. Zebrafish Isl1 mutants lack late-differentiating cardiomyocytes at the venous pole [5]. This phenotype is similar to that of Isl1 null mouse embryos, which lack SHF-derived atrial cardiomyocytes [15]. Interestingly, studies in zebrafish have identified a novel requirement for the LIM domain protein Ajuba, which directly interacts with Isl1 [14]. Ajuba-deficient embryos have large hearts with an excess of Isl1-expressing cells and an expansion of SHF markers in the ALPM. Conversely, Ajuba overexpression eliminates Isl1 in the inflow tract [14]. Ajuba is one of the first factors that has been shown to limit SHF development, and the presence of Ajuba may determine whether Isl1 activity promotes or limits cardiomyocyte formation. The identification of Ajuba as a negative regulator of inflow tract formation further illustrates the utility of zebrafish for the discovery of novel factors involved in SHF development.

25.5 Future Directions and Clinical Implications

Altogether, the studies summarized here support the value of the zebrafish for the investigation of SHF development. It will be particularly exciting for future work in zebrafish to probe important open questions in this area. For example, zebrafish studies may be valuable for elucidating the mechanisms that pattern the SHF into its anterior and posterior subdivisions. In addition, it will be interesting to use zebrafish to examine the factors that control differentiation of multipotent SHF cells into myocardial, endocardial, and smooth muscle lineages [9]. Zebrafish will also be valuable for exploring whether multipotent SHF cells are maintained after embryogenesis, perhaps to be deployed after injury. In the long term, use of the zebrafish for analysis of SHF development is likely to illuminate pathways that facilitate our understanding of the etiology of congenital heart disease.

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A History and Interaction of Outflow Progenitor Cells Implicated in "Takao Syndrome"

26

Hiroyuki Yamagishi, Kazuki Kodo, Jun Maeda, Keiko Uchida, Takatoshi Tsuchihashi, Akimichi Shibata, Reina Ishizaki, Chihiro Yamagishi, and Deepak Srivastava

Abstract

Progenitor cells, derived from the cardiac neural crest (CNC) and the second heart field (SHF), play key roles in development of the cardiac outflow tract (OFT), and their interaction is essential for establishment of the separate pulmonary and systemic circulation in vertebrates. 22q11.2 deletion syndrome (22q11DS) or Takao syndrome is the most common human chromosomal deletion syndrome that is highly associated with OFT defects. Historically, based on the observations in animal models, OFT defects implicated in the 22q11/Takao syndrome are believed to result primarily from abnormal development of CNC that populate into the conotruncal region of the heart. In the twenty-first century, elegant efforts to model 22q11/Takao syndrome in mice succeeded in the identification of T-box-containing transcription factor, Tbx1, as an etiology of OFT defects in this syndrome. Subsequent investigations of the Tbx1 expression pattern revealed that Tbx1 was surprisingly not detectable in CNC but was expressed in the SHF and provided a new concept of molecular and cellular basis for OFT defects associated with 22q11/Takao syndrome. More recently, it was reported that mutations in the gene encoding the transcription factor GATA6 caused CHD characteristic of OFT defects. Genes encoding the neurovascular guiding molecule semaphorin 3C (SEMA3C) and its receptor plexin A2 (PLXNA2) appear to be regulated directly by GATA6. Elucidation

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of molecular mechanism involving GATA6, SEMA3C, PLXNA2, and TBX1 in the interaction between the CNC and the SHF would provide new insights into the OFT development.

Keywords
Congenital heart disease • 22q11.2 deletion syndrome • Neural crest • Second heart field • GATA6

26.1 Introduction

Cardiac outflow tract (OFT) defects account for approximately 30 % of congenital heart disease (CHD) and usually require an intervention during the first year of life [1, 2]. A variety of OFT defects results from disturbance of the morphogenetic process for the establishment of separated systemic and pulmonary circulation. Despite their clinical importance, the etiology of most OFT defects remains unknown because of the multifactorial nature of the diseases.

Progenitor cells derived from the cardiac neural crest (CNC) and the second heart field (SHF) play key roles in development of the OFT. The SHF cells give rise to the OFT myocardium along with subpulmonary conus, and CNC cells give rise to the OFT septum during development. Defects of these progenitor cells may lead to a variety of OFT defects, including tetralogy of Fallot (TOF), characterized by malalignment of the major vessels with the ventricular chambers; interrupted aortic arch type B (IAA-B), resulting from maldevelopment of the left fourth pharyngeal arch artery; and persistent truncus arteriosus (PTA), resulting from failure of septation of the OFT into the aorta and pulmonary artery [3, 4].

26.2 The 22q11.2 Deletion Syndrome (Takao Syndrome)

The 22q11.2 deletion syndrome (22q11DS) is the most common genetic cause of a spectrum of OFT defects with an incidence of 1 in 4000–5000 births [5, 6]. Most are sporadic in origin, while 10–20 % of deletions are inherited as an autosomal dominant trait. 22q11DS involves three distinct syndromes, namely, DiGeorge syndrome (DGS; OMIM#188400), velocardiofacial syndrome (VCFS; OMIM#192430), and conotruncal anomaly face syndrome (CAFS; OMIM#217095) which is so-called Takao syndrome. Historically, DGS was originally characterized by CHD, hypoparathyroidism, and immune deficiency reported in 1965 from the field of immunology [7]; VCFS was associated with cleft palate, CHD, a distinct facial appearance, and learning difficulties reported in 1978 from the field of plastic surgery [8]; and CAFS or Takao syndrome was characterized by conotruncal CHD (OFT defects), a distinct facial appearance and hyper-nasal voice reported in 1976 (in Japanese) from the field of pediatric cardiology [9]. In 1993, clinical genetics revealed that these syndromes shared a common heterozygous deletion of 22q11.2 region and thus had overlapping phenotype [10–12].

Although the acronym “CATCH22 (cardiac defects, abnormal facies, thymic hypoplasia, cleft palate, hypocalcemia, and 22q11 deletions)” was proposed to encompass these syndromes in 1993 [13], clinical use of this term is restricted today, because (1) the term “CATCH22” has a negative meaning which represents a situation where it is impossible for you to do anything, originally from a novel entitled “Catch-22” by Heller [14]; (2) the term “abnormal facies” represented by “A” is difficult to be accepted by patients and their family; and (3) the clinical spectrum associated with 22q11DS is much wider than was previously recognized as “CATCH” [15].

Approximately 75 % of patients with 22q11 DS have CHD. The type of CHD are characterized as OFT defects including TOF, estimated about 30 %; IAA-B, estimated about 15 %; ventricular septal defect (VSD), estimated about 15 %; PTA, estimated about 10 %; and others, estimated about 5 %. Alternatively, 22q11.2 deletion is present in approximately 60 % of patients with IAA-B, 35 % of patients with PTA, and 15 % of patients with TOF. Specifically, it is detected in 55 % of patients with TOF plus pulmonary atresia and major aortopulmonary collateral arteries (MAPCA) [16–18].

Although CHD are the major cause of mortality in 22q11DS, survivors have an exceptionally high incidence of psychiatric illness, including schizophrenia and bipolar disorder, in adolescents and adults, making del22q11 the most frequent genetic cause of such psychiatric disorders [16, 19, 20]. In our experience of 18 adults with 22q11DS, common school and employment were observed in 11 of 18 cases, and 2 females got married; however, difficulties with social interaction and employment were observed in 7 cases. The main reason of difficulties for social interaction and employment was incomplete repair of CHD in four cases, and all of them had TOF with pulmonary atresia and MAPCA. One case was also diagnosed as schizophrenia. Other three cases had repaired VSD and are away from hospital care. Taken together, lifelong comprehensive evaluation and management of patients with 22q11DS, like as shown in Table 26.1, are required for multisystem disorders [6]. The primary care physician, a pediatric cardiologist in most cases, has an important role in the follow-up for the patients and their families and needs to collaborate with many specialists for the associated abnormalities.

26.3 Identification of TBX1

Because of the high incidence and association with OFT defects, 22q11DS has attracted attention as a model for investigating the genetic basis for OFT defects [21, 22]. The structures primarily affected in patients with 22q11DS are derivatives of the embryonic pharyngeal arches, or neural crest cells, suggesting that haploinsufficiency of the gene(s) on the 22q11.2 deleted region is essential for pharyngeal arch and/or CNC development [1, 2, 21, 22]. Extensive gene searches have been successful in identifying more than 30 genes in the deleted segment.

Table 26.1 Management program for patients with 22q11.2 deletion syndrome at Keio University Hospital

	Newborn and infant		Toddler		School age	Puberty
Congenital heart diseases	Palliative operation	Regular follow-up	Corrective operation	Regular follow-up	Exercise guidance (if necessary)	
Immunodeficiency	Blood exam (immunodeficiency screening)		Vaccination		Vaccination	
	Immune reconstruction (severe cases)					
Velopharyngeal dysfunction	Otolaryngeal exam		Assessment of velopharyngeal function and ear problem		Continuous speech therapy	
	Plastic surgery (if necessary)			Pharyngoplasty (if necessary) and speech therapy		
				Assessment of speech (preschool)		
Hypocalcemia	Periodic serum calcium exam				Evaluation of latent hypocalcemia	
	Oral administration of active vitamin D (if necessary) → urinary calcium (and renal ultrasound) check					
		Calcium supplement at perioperative periods				
Developmental delay		Assessment of developmental quality		Assessment of intelligence quality (preschool)		
		Intervention for developmental delay			Intervention for learning disabilities	
Psychiatric disorder						Psychiatrist consultation
Short stature	Regular measurements of body size			Hormonal evaluation		
Others			Screening, renal ultrasound			
			Teeth care (dentist consultation)			
		Pediatric surgery (anal atresia, inguinal hernia, etc.)				
			Ophthalmology (squint, etc.)			
			Orthopedics (scoliosis, talipes equinovarus, etc.)			

Although standard positional cloning has failed to demonstrate a role for any of these genes in the syndrome, elegant efforts by several groups to model 22q11DS in mice by creating orthologous chromosomal deletions were successful in revealing the T-box-containing transcription factor, Tbx1, as the etiology of OFT defects associated with 22q11DS [23–26]. Heterozygosity of Tbx1 in mice alone also caused aortic arch defects, while homozygous mutation of Tbx1 in mice resulted in most main clinical presentations of 22q11DS, including OFT defects, abnormal facial features, cleft palate, and hypoplasia of the thymus and parathyroid glands.

26.4 Expression of TBX1

The delineation of the expression pattern of Tbx1 provided a new concept on the molecular and cellular basis of normal and abnormal development of the OFT. We and other group found that Tbx1 was expressed in the SHF but not in the CNC [27–29]. This finding was surprising because CHD associated with 22q11DS had been believed to result primarily from abnormal development of CNC as mentioned above. Interestingly, in mouse and chick embryos, Tbx1 is preferentially expressed in the pharyngeal arches, in the ventral half of the otic vesicle, and in the head (Fig. 26.1) [27, 28]. Within the pharyngeal arch region, Tbx1 is expressed in the pharyngeal mesoderm, including the SHF, the pharyngeal endoderm, and the head mesenchyme. These results suggest that defects of neural crest-derived tissues in 22q11DS may occur in a non-cell autonomous fashion. Our cre-mediated murine

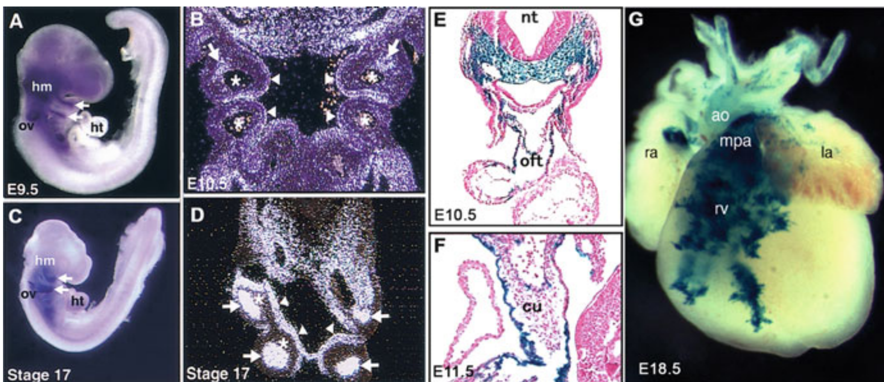

Fig. 26.1 Expression patterns of Tbx1. (**a–d**) RNA in situ hybridizations for whole mount (**a**–**c**) and section (**b**, **d**) in mouse (**a**, **b**) and chick (**c, d**) embryos demonstrate Tbx1 expression (*purple or white signals*) in the mesodermal core (*arrows*) and endodermal epithelium (*arrowheads*) of pharyngeal arches, head mesenchyme (*hm*), and otic vesicle (*ov*). Asterisks indicate pharyngeal arch arteries. (**e**, **f**). Transverse sections demonstrate Tbx1-lacZ expression (*blue signals*) in the myocardial layer of the cardiac outflow tract (*oft*) but not in the oft cushion (*cu*) which is mainly contributed by cardiac neural crest cells. (**g**) Tbx1-descendant cells marked by Tbx1-Cre/Rosa26R mouse system are localized in the anterior portion (*oft*) of the right ventricle (*rv*) and the main trunk of the pulmonary artery (*mpa*). *ao* Aorta, *h* head, *ht* heart, *la* left atrium, *ra* right atrium

transgenic system revealed that Tbx1-expressing descendants representing a subset of cells derived from the SHF contribute predominantly to the pulmonary infundibulum (Fig. 26.1) [30].

Although precise embryological mechanisms underlying OFT defects remain uncertain, the anatomical defects in TOF are believed to result from malrotation of the OFT that leads to misalignment of the outlet and trabecular septum and consequent overriding of the aorta above the malaligned ventricular septum [2, 3]. Contribution of CNC is thought to be essential for proper rotation and septation of the OFT. Alternatively, hypoplasia and underdevelopment of the pulmonary infundibulum may also be responsible for the infundibular obstruction and malalignment of the outlet septum [2, 3]. Accordingly, our data suggest that developmental defects of the SHF may cause hypoplasia of the pulmonary infundibulum, resulting in TOF [30]. More severe decreased number or absence of this subset of cells may affect development and/or migration of CNC, resulting in PTA. This hypothetical model is supported by the observation that the OFT defects ranging from TOF to TA are highly associated with 22q11DS (Fig. 26.2).

26.5 Mutations of GATA6

Recently, we identified and characterized mutations of GATA6 in our series of Japanese patients with OFT defects [31]. Mutations in GATA6 disrupted its transcriptional activity on downstream target genes involved in the development of the OFT. We also found that the expression of SEMA3C and PLXNA2 was directly regulated during development of the OFT through the consensus GATA binding sites well conserved across species. Mutant GATA6 proteins failed to transactivate SEMA3C and PLXNA2, and mutation of the GATA sites on enhancer elements of Sema3c and Plxna2 abolished their activity, specifically in the OFT/subpulmonary myocardium and CNC derivatives in the OFT region, respectively. These results indicate that mutations of GATA6 are implicated in genetic causes of OFT defects, as a result of the disruption of the direct regulation of semaphorin-plexin signaling (Fig. 26.2).

26.6 Future Direction: Elucidating the Interaction Between CNC and SHF

Recent studies have demonstrated that reciprocal epithelial-mesenchymal signaling is essential for proper development of the pharyngeal arches and that the primary impairment of epithelial endoderm may secondarily affect migration or differentiation of neural crest cells during the pharyngeal arch development [32–34]. As for the development of the OFT, clear roles of CNC- and SHF-derived cells have been established [35]. Future direction in this research field is to reveal how the CNC and SHF interact using complex reciprocal signaling essential for precise morphogenesis of the OFT. Importantly, mutations in genes expressed in either CNC or SHF can

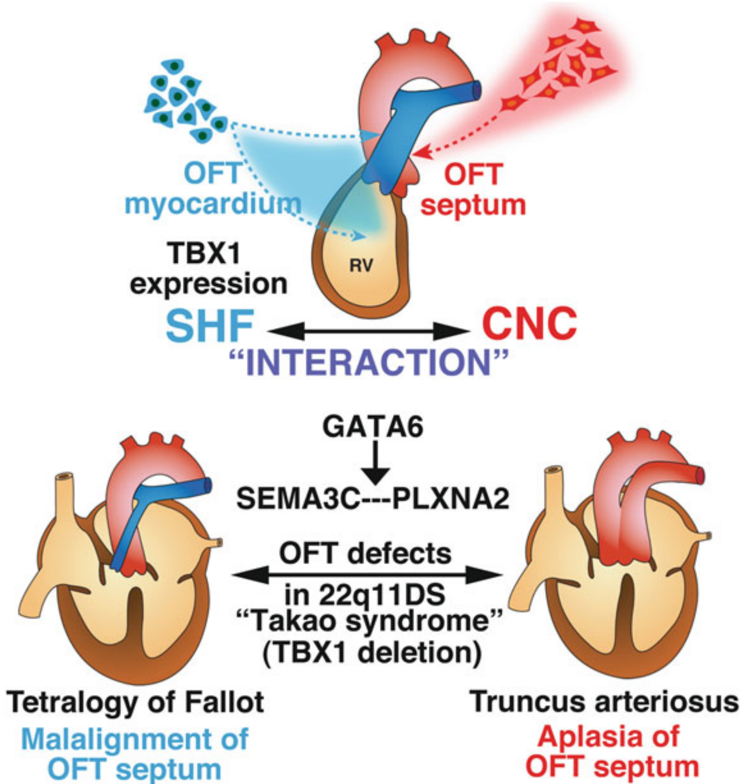

Fig. 26.2 Cellular interaction of the second heart field (*SHF*) and cardiac neural crest (*CNC*) for the outflow tract (*OFT*) development and diseases. Progenitor cells derived from the SHF and the CNC give rise to the *OFT* myocardium and septum, respectively. TBX1 is exclusively expressed in the SHF cells. TBX1 deletion in 22q11DS may affect not only the SHF cells but also the interaction between the SHF cells and CNC, resulting in OFT defects ranging from TOF, which is characterized by malalignment of the OFT septum, to PTA, which results from aplasia of the OFT septum. GATA6-SEMA3C (ligand)-PLXNA2 (receptor) pathway also plays a role in interaction between the SHF and CNC during the OFT development (Modified from [36])

result in similar OFT defects in mice. For example, Pax3 is expressed in the CNC, and Tbx1 is expressed in the SHF, and both Pax3-null mice and Tbx1-null mice show PTA. Studies are, thus, required to focus on the signals that mediate interactions between CNC and SHF in order to uncover the developmental mechanisms underlying various types of the OFT defect. Our result from the research of mutation of GATA6, described above, is an example that revealed such a molecular mechanism. Our recent preliminary data suggest that a molecular cascade involving Gata6, Foxc1/2, Tbx1, Sema3C, and Fgf8 may play roles in reciprocal signaling between SHF and CNC that are essential for the migration of CNC toward the OFT myocardium derived from the SHF (*in revision*). Further

study utilizing our model system may provide new insights into the OFT development and embryogenesis of OFT defects.

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The Loss of Foxc2 Expression in the Outflow Tract Links the Interrupted Arch in the Conditional Foxc2 Knockout Mouse

27

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Keywords
Foxc2 • Interruption of aortic arch • Conditional knockout

Congenital heart disease is the most common birth defects, affecting 1 % live births [1]. The cardiovascular system undergoes a series of morphogenetic events to form a heart and an aorta in fetuses. Formation of the heart and aorta requires migration, differentiation, and precise interactions among multiple cells from several embryonic origins [2]. Forkhead box2 (Foxc2) encodes a transcription factor and is expressed in mesodermal tissues, such as the pharyngeal artery, outflow tract endothelial/surrounding mesenchyme, bone, and kidney [3]. Simple knockout of Foxc2 in mouse causes an interrupted aortic arch, ventricular septal defect, cleft palate, and skeletal malformation [4]. The heart is made from primary and secondary heart field progenitors. The primary heart field gives rise to the left ventricle and atria, while the secondary heart field contributes mainly to the right ventricle and outflow tract [5] (Fig. 27.1).

To explore the tissue-specific roles of Foxc2 in aortic arch remodeling, we generated mice carrying a floxed allele of *Foxc2* (Foxc2^{flox}) and crossed them with several Cre mice, including the primary heart field (Nkx2.5-Cre knock-in)-specific and secondary heart field (Islet1-Cre knock-in and Tbx1-Cre transgenic)-

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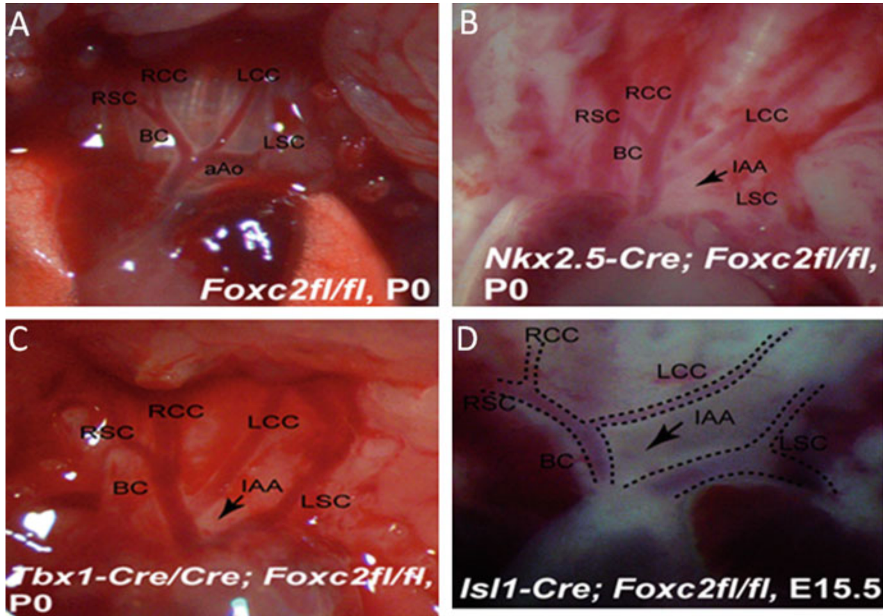

Fig. 27.1 Aortic arch abnormalities in Foxc2 conditional knockout mice. (**a**) Normal aortic arch formation in control mice (Foxc2$^{flox/flox}$). (**b**–**d**) Conditional knockout mice showed interrupted aortic arch (IAA) type B where part of the aorta between LCC and LSC is missing (*arrow*). *LCC* left common carotid artery, *LSC* left subclavian artery, *aAo* arch of the aorta, *Pt* pulmonary trunk, *RCC* right common carotid artery, *RSC* right subclavian artery, *BC* brachiocephalic artery

specific Cre lines. Surprisingly, conditional knockout (cKO) of *Foxc2* in the primary heart field (Nkx2.5-Cre;Foxc2$^{flox/flox}$) and secondary heart field (Islet1-Cre;Foxc2$^{flox/flox}$ and Tbx1-Cre;Foxc2$^{flox/flox}$) resulted in an interrupted aortic arch and perinatal lethality in mice. X-gal staining and immunostaining with anti-Foxc2 antibody confirmed that Foxc2 expression in the aortic arch was intact but deleted in the outflow tract in these cKO embryos. These results indicate that the Foxc2 expression in the outflow tract, rather than direct role in the aortic arch, is crucial for the aortic arch remodeling. It assumed that Foxc2 in the outflow tract regulates aortic arch remodeling via secreted factors such as Fgf8, Fgf10, and other genes.

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Modification of Cardiac Phenotype in Tbx1 Hypomorphic Mice

28

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Keywords
Tbx1 • Truncus arteriosus • Environmental modification

Congenital heart disease is still the leading cause of death within the first year of life. Our lab forces on understanding the morphology of congenital heart disease. Outflow tract anomalies, including abnormal alignment or septation, account for 30 % of all congenital heart disease. To solve the developmental problem of these defects, we are interested in the role of the second heart field (SHF) that gives rise to the outflow tract structure.

TBX1, a member of the T-box family of transcription factors, is a major genetic determinant of 22q11 deletion syndrome (22q11DS) in human. 22q11DS is the most frequent chromosomal microdeletion syndrome in human and characterized by abnormal development of the cardiac outflow tract, such as persistent truncus arteriosus (PTA), tetralogy of Fallot, interrupted aortic arch, and ventricular septal defects.

In the developing murine heart, Tbx1 is expressed in the SHF, but not in the cardiac neural crest cells (NCCs). Our past experiments suggested that sonic hedgehog signal was necessary for maintenance of the *Tbx1* expression in the pharyngeal mesoderm including the SHF [1]. *Tbx1* null ($Tbx1^{-/-}$) mice demonstrated PTA reminiscent of the 22q11DS heart phenotype. We generated

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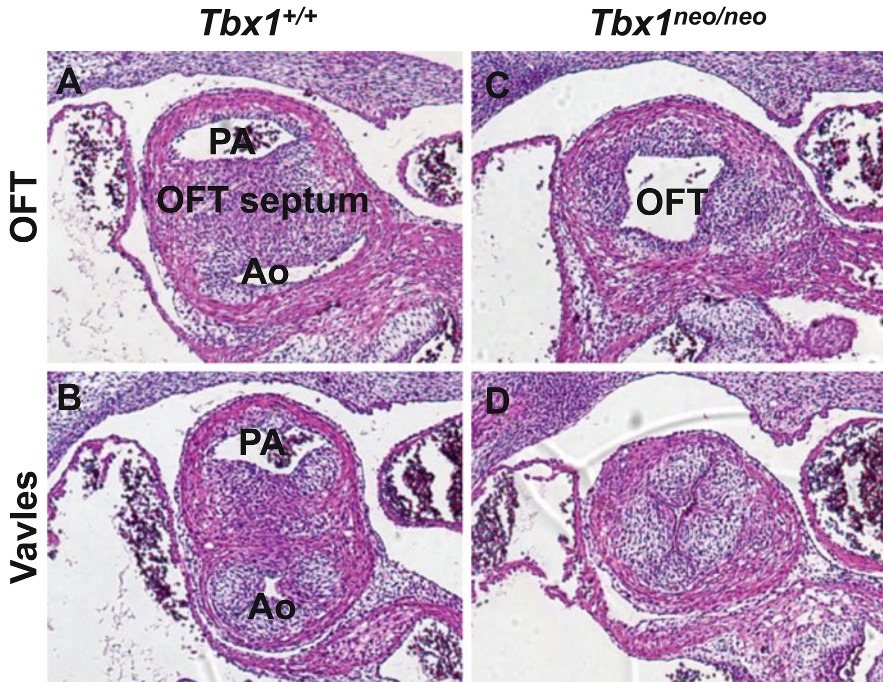

Fig. 28.1 Coronal sections of *Tbx1*$^{+/+}$ (**a**, **b**) and *Tbx1*$^{neo/neo}$ (**c**, **d**) embryos at E13.5. *Tbx1*$^{+/+}$ showed the normal outflow tract (OFT) septation, whereas *Tbx1*$^{neo/neo}$ demonstrated PTA. *Ao* Aorta, *PA* pulmonary artery

Tbx1 hypomorphic allele (*Tbx1*$^{neo/+}$) [2] for attempting to recapitulate the human genotype and phenotype correlation. Mice homozygous for this hypomorphic allele expressed around 25 % of Tbx1 mRNA compared to wild-type mice. We demonstrated that Tbx1 is a dosage-dependent gene and believe that the Tbx1 dosage can be affected by genetic and/or environmental modifiers because of highly variable phenotype of 22q11DS instead of the relatively uniform chromosomal microdeletion. We are trying to create the phenotype variability of PTA in this hypomorphic model (Fig. 28.1) by application of environmental modifiers. Through this study, we would better understand the interaction between the gene dosage and environmental factors during the development of outflow tract defects.

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Part VI

Vascular Development and Diseases

Perspective

H. Scott Baldwin

While the critical pathways that are important for normal cardiac development have focused extensively on transcriptional regulation of myocyte differentiation, critical mediators of vascular development have received much less attention. One reason for this has been the inability in the past to manipulate gene expression in a temporal and tissue-specific manner. There is no doubt that both normal vascular and normal myocardial development are essential for early embryonic survival and the two are inextricably linked; normal vascular development requires normal flow, and maturation of the myocardium requires simultaneous maturation and remodeling of the extracardiac vasculature. Ubiquitous or global gene deletions, resulting in both cardiac and extracardiac mutations, have resulted in numerous “chicken and egg” quandaries: Did the heart fail because of a primary defect in heart development, or were the defects merely secondary to upstream perturbations in extracardiac vascular defects? In this section, investigators used tissue-specific mutagenesis strategies as well as a focus on cell membrane and extracellular matrix regulation to begin to elucidate important aspects of extracardiac vascular development that are particularly relevant to human disease. Sakebe et al. generated an endothelial-specific deletion of Hrt2/Hey2, repressors of Notch signaling, to demonstrate that both Hrt1 and Hrt2 are essential for vascular development independent of their role in myocardial development. Furthermore, they suggest that the

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endothelial or vascular processes mediated by these factors, rather than the defects in myocardial development, might be the primary mechanism for embryonic demise. Exploring the role of calcium signaling in extraembryonic vascular development, Uchida and colleagues were able to document dramatic defects in placentation as early as E9.5 in the mouse as a result of combinatorial deletion of the inositol IP3 receptors. This work clearly establishes a role for calcium handling in cardiovascular viability. Changing the focus to later stages of vascular development, Dr. Imanaka-Yoshida provides a detailed description of the role of the extracellular matrix protein, tenascin-C, in smooth muscle cell recruitment of both the descending aorta and coronary arteries and provides in vitro evidence that tenascin-C promotes SMC precursor expansion and differentiation by augmenting PDG-BB/PDGFR-β signaling. Finally, Yoshikane et al. show the potential importance of delineating the role of tenascin-C in normal and abnormal coronary artery remodeling as they discuss a model of the most common acute systemic vasculitis in children, Kawasaki disease. By studying the inflammatory and abnormal vascular remodeling induced by *Candida albicans*, they demonstrate accentuation of tenascin-C expression associated with aneurysm formation. Furthermore, they document that inhibition of JNK signaling attenuated aneurysm formation potentially providing a mechanistic link between JNK signaling and tenascin-C signaling that could provide a therapeutic target for treatment of Kawasaki disease. In summary, the investigations presented in this section provide an overview of exciting work that expands the focus of cardiovascular development and disease beyond myocyte transcriptional regulation and provides new insights into extracardiac vascular development and remodeling while emphasizing the importance that the extracellular matrix is ontogeny of cardiovascular disease.

Extracellular Matrix Remodeling in Vascular Development and Disease

29

Kyoko Imanaka-Yoshida

Abstract

Blood vessels constantly subjected to mechanical stress have well-developed elastic fiber-rich frameworks, which contribute to the elasticity and distensibility of the vascular wall. Destruction of the fibrous structure due to genetic predisposition as well as acquired disorders such as Kawasaki disease often induces irreversible dilation of blood vessels, e.g., aneurysm formation. In addition to their structural role, extracellular matrix molecules also provide important biological signaling, which influences various cellular functions. Among them, increased attention has been focused on matricellular proteins, a group of non-structural extracellular matrix (ECM) proteins highly upregulated in active tissue remodeling, serving as biological mediators by interacting directly with cells or regulating the activities of growth factors, cytokines, proteases, and other ECM molecules. Tenascin-C (TNC) is a typical matricellular protein expressed during embryonic development and tissue repair/regeneration in a spatiotemporally restricted manner. Various growth factors, pro-inflammatory cytokines, and mechanical stress upregulate its expression. TNC controls cell adhesion, migration, differentiation, and synthesis of ECM molecules. Our recent results suggest that TNC may not only play a significant role in the recruitment of smooth muscle/mural cells during vascular development, but also regulate the inflammatory response during pathological remodeling. TNC may be a key molecule during vascular development, adaptation, and pathological tissue remodeling.

Keywords

Tenascin • Extracellular matrix • Coronary artery • Aorta

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29.1 Introduction

Tissue, including the cardiovascular system, is composed of diverse cells and the extracellular matrix (ECM) synthesized by those cells. Several ECM molecules form a fibrous framework and provide structural support for the tissue. Blood vessels constantly subjected to mechanical stress have a well-developed fibrous framework, which contributes to the elasticity and distensibility of the vascular wall in concert with vascular smooth muscle cells. Highly ordered structures consisting of cells and fibrous elements are formed during development and are remodeled during tissue repair/regeneration after injury. In addition to their physical role, several ECM molecules provide important biological signaling, which influences various cellular functions in physiological and pathological tissue remodeling. In particular, ECM, termed matricellular protein, has attracted increasing attention as a biological mediator. Tenascin-C (TNC) is a prototype matricellular protein expressed during embryonic development and tissue repair after injury. This chapter will focus on the role of TNC in vascular development, especially coronary arteries and the aorta.

29.2 Extracellular Matrix in Vascular Wall

Blood vessels have abundant fibrous matrix tissue: well-developed elastic fibers in the medial layer and rich collagen fibers in adventitia. It is known that several gene mutations related to these fibrous components cause vascular fragility, eventually leading to aneurysm formation or dissection. For example, the collagen gene and fibrillin-1 gene, which is important for microfibril formation, have been identified as the genes responsible for Ehlers-Danlos syndrome (reviewed in [1]) and Marfan's syndrome [2], respectively. In addition to genetic predisposition, inflammation of blood vessels in acquired disease may induce fragmentation and destruction of normal elastic fibers in the vascular wall and causes irreversible dilation of blood vessels. For example, coronary aneurysm formation is sometimes seen in patients with Kawasaki vasculitis, one of the most common acquired heart diseases in children. Evidently, the structural support by fibrous ECM is essential to maintain the proper morphology and function of blood vessels.

Besides these fibrous elements, unique ECM molecules, matricellular protein [3], have attracted considerable attention. The matricellular proteins have common unique properties: (1) do not contribute directly to structures such as fibrils or basement membranes; (2) high levels of expression during embryonic development and in response to injury; and (3) binding to many cell surface receptors, components of ECM, growth factors, cytokines, and proteases [4]. This is a growing family originally including SPARC, tenascin, and thrombospondin [3].