This Review is the introduction of a new thematic series on Wnts in Cardiovascular Development and Disease, which includes the following articles:

- An Updated Overview on Wnt Signaling Pathways: A Prelude for More [2010;106:1798–1806]
- The Multiple Phases and Faces of Wnt Signaling During Cardiac Differentiation and Development
- Wnt Signaling in Vascular Progenitor Cells and Angiogenesis
- Wnt Signaling in Cardiac Hypertrophy and Remodelling
- Wnt Signaling in Heart Failure and Aging
- Wnt Signaling and Stem Cells

Michael Kühl, Guest Editor

The Multiple Phases and Faces of Wnt Signaling During Cardiac Differentiation and Development

Susanne Gessert, Michael Kühl

**Abstract:** Understanding heart development on a molecular level is a prerequisite for uncovering the causes of congenital heart diseases. Therapeutic approaches that try to enhance cardiac regeneration or that involve the differentiation of resident cardiac progenitor cells or patient-specific induced pluripotent stem cells will also benefit tremendously from this knowledge. Wnt proteins have been shown to play multiple roles during cardiac differentiation and development. They are extracellular growth factors that activate different intracellular signaling branches. Here, we summarize our current understanding of how these factors affect different aspects of cardiogenesis, starting from early specification of cardiac progenitors and continuing on to later developmental steps, such as morphogenetic processes, valve formation, and establishment of the conduction system. (Circ Res. 2010;107:186-199.)

**Key Words:** Wnt ■ cardiac differentiation ■ cardiac development

Wnt proteins are growth factors that function during embryonic development and in the adult organism by regulating diverse cellular processes such as gene transcription and cell proliferation, migration, polarity, or division.¹ Not surprisingly, Wnt proteins are also involved in cardiac development and differentiation. To understand the function of Wnts during cardiogenesis, a basic knowledge of how Wnt proteins transduce their signal is a prerequisite. Consistently, we start with a short introduction into Wnt signaling; for a more in-depth discussion of Wnt signal transduction pathways, more sophisticated recent reviews have been published.²⁻⁴

**Wnt Signaling Pathways: A Short Synopsis**

On a molecular level, members of the Wnt family can, in principle, activate different intracellular signaling pathways. Recent data suggest that these pathways are highly interconnected and, in fact, represent one complex signaling network.²⁻³ The classic canonical Wnt signaling pathway involves the multifunctional protein β-catenin. β-Catenin is well known for its function in cell adhesion through interaction with transmembrane proteins of the cadherin family but functions here as a signaling mediator. In absence of Wnt, cytosolic β-catenin is interacting with the destruction complex composed of axin, APC, and GSK3β, and thereby is phosphorylated and subsequently degraded by the proteasome. In the presence of a Wnt ligand, the destruction complex is disassembled, and β-catenin is stabilized, enters the nucleus, and regulates target gene transcription through interaction with TCF/LEF transcription factors. Through particular target genes such as c-myc and cyclinD1, the
canonical Wnt/β-catenin pathway is also linked to cell proliferation control. Well-known extracellular inhibitors of the Wnt/β-catenin signaling branch are the secreted proteins Dkk1 and Crescent. In contrast, noncanonical Wnt signaling branches are independent of β-catenin and involve diverse intracellular mediators. In the Wnt/JNK pathway, GTPases of the rho family are upstream of jun-N-terminal kinase (JNK), whereas in the Wnt/Ca^{2+} pathway, an intracellular release of calcium ions results in the activation of calcium-sensitive enzymes such as protein kinase (PK)C, calcium/calmodulin-independent kinase (CaMK)II, or calcineurin (CaCN). Noncanonical Wnt pathways have often been linked to cell polarity and cell migration caused by cytoskeletal rearrangements, although examples of an effect on gene expression are also known and discussed below. Of note, noncanonical Wnt signaling has been shown to be inhibitory for canonical Wnt signaling through multiple mechanisms.\(^5\) Figure 1 provides an overview of Wnt signaling pathways as required for this review.

In the past, individual Wnt ligands and their receptors of the Frizzled family have been linked to either canonical or noncanonical Wnt signaling pathways, eg, Wnt3a mostly couples to β-catenin signaling, whereas Wnt5a and Wnt11 mainly trigger noncanonical Wnt signaling. Recently, however, exceptions to this rule have been described.\(^2\)–\(^4\) An analysis of Wnt function in a particular context therefore should also aim to uncover the underlying molecular mechanism. Below, we focus on the role of Wnt signaling during different aspects of cardiac development. Wherever known, the intracellular downstream effectors are also highlighted.

**Early Heart Development: From a Common Progenitor to Different Lineages**

The mammalian heart is one of the first functional organs that develops during embryogenesis to already provide the growing embryo with sufficient nutrients and oxygen. Formation
of the vertebrate heart can be subdivided into distinct but partially overlapping phases such as specification of cardiac progenitors and formation of the linear heart tube by cell migration and morphogenetic movements, followed by cardiac looping, chamber formation, seption, and maturation. During all of these phases, Wnt ligands, Frizzled receptors, or extracellular Wnt inhibitors are expressed in relevant tissues (see Table 1). Note that, as a prerequisite for intracellular signal transduction, the main intracellular mediators of Wnt signaling are virtually expressed ubiquitously in all cells.

Our current view of early cardiac development is based on gene expression studies in different organisms, cell tracking in genetically modified mice, and classic lineage-labeling experiments in chicken, mice, and frogs (Figure 2). In mice, the cardiac progenitor cells that give rise to the linear heart tube derive from the cardiac crescent at embryonic day (E)7.5 of development, also called primary heart field or first heart field (FHF). These cells will mainly contribute to the left ventricle. To form a mature heart, the linear heart tube subsequently needs to expand. This is achieved by 2 mechanisms, cell proliferation and recruitment of additional cells. The latter was shown by lineage labeling experiments in avian and mouse embryos where cells are added to the arterial and venous poles of the linear heart tube.6–9 These cells originate in the second heart field (SHF), a cardiac precursor cell population distinct from the first heart field. In mice, different Cre-driver lines using regulatory elements of important cardiac genes such as Islet1, FGF10, and Mef2C were used to describe this second lineage of cardiac precursor cells.10–13 An Isl1 Cre-driver line indicated that cells of the SHF will mainly contribute to the outflow tract (OFT) and the right ventricle, as well as to both atria.10 Interestingly, by use of some regulatory elements of the Mef2C gene only cells of the OFT and the right ventricle can be labeled indicating that the SHF field can be further subdivided into diverse subpopulations.13 Furthermore, it should be noted that the use of these different Cre-driver lines to manipulate heart development might result in slightly different results thereby reflecting the timing, tissue specificity and penetrance of a given Cre driver. Also, in chicken, it has been shown that cells of the SHF populate the right ventricle.14 In frogs, that have just one ventricle, cells of the SHF exclusively end up in the OFT.15 The LIM homeodomain transcription factor Islet (Isl)1 has become a popular molecular marker demarcating the SHF. By gene expression, cells of the SHF are located more medial and posterior to the FHF at E7.5, and later are found more dorsally to the heart tube at E8.0 in the mouse embryo (Figure 2).10,16 FHF progenitors in contrast express the T-box transcription factor Tbx5. The homeobox transcription factor Nkx2.5 is found in both populations.15

Retrospective clonal analyses indicated that cells of both heart fields derive from one common cardiac progenitor cell population (CPC) and rather represent different cardiogenic lineages than unbridgeable cell populations. According to this model, both lineages differ with respect to the onset of terminal differentiation,17,18 with cells of the second heart field lineage entering terminal differentiation later than those of the first heart field lineage. Consistently, cells of the second lineage express Isl1 longer than those of the first

<table>
<thead>
<tr>
<th>Gene</th>
<th>Expression</th>
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<tr>
<td>Wnt2</td>
<td>Mouse: cardiac crescent (FHF/SHF)? Mouse EB: Flk1 subpopulation</td>
</tr>
<tr>
<td>Wnt2b</td>
<td>Mouse: cardiac crescent (FHF/SHF?), primordium of OFT, atrioventricular region of linear heart tube</td>
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<tr>
<td>Wnt3a</td>
<td>Mouse: primitive streak</td>
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<tr>
<td>Wnt4</td>
<td>Mouse: primitive streak</td>
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<tr>
<td>Wnt5a</td>
<td>Mouse: node, primitive streak, primordium of the OFT, OFT, common ventricle</td>
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<tr>
<td>Wnt6</td>
<td>Chicken: atrioventricular region</td>
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<tr>
<td>Wnt7a</td>
<td>Chicken: tubular heart, primitive conduction system</td>
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<tr>
<td>Wnt8, Wnt8a</td>
<td>Mouse: primitive streak, tubular heart</td>
</tr>
<tr>
<td>Wnt9, Wnt9a</td>
<td>chicken: tubular heart, OFT, atrioventricular canal</td>
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<tr>
<td>Wnt11</td>
<td>Mouse: node, cardiac crescent (FHF/SHF?), linear heart tube, OFT myocardium, ventricle</td>
</tr>
<tr>
<td>Dkk1</td>
<td>Chicken: Hensen’s Node, primitive streak</td>
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<tr>
<td>Fz2</td>
<td>Mouse: CNCC</td>
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<tr>
<td>Fz3</td>
<td>Xenopus: CNCC</td>
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<tr>
<td>Fz7</td>
<td>Xenopus: common cardiac progenitor</td>
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<td>Fz8</td>
<td>Xenopus: first heart field</td>
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<td>Fz9</td>
<td>Mouse: adult heart</td>
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<tr>
<td>Crescent</td>
<td>Xenopus: Spemann Organizer</td>
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<td>Sfrp1</td>
<td>Mouse EB: Flk1 subpopulation</td>
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<tr>
<td>Sfrp2</td>
<td>Mouse: Flk1 subpopulation</td>
</tr>
<tr>
<td>Sfrp3 (FrzB)</td>
<td>Chicken: precardiac mesoderm, OFT, atrioventricular region</td>
</tr>
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</table>

For detailed references and more information concerning expression domains, see also the recent reviews.126–128 This list is not exclusive because, for some genes, a potential cardiac expression has not been analyzed. The main intracellular mediators of Wnt signaling are present in virtually all cells and therefore are not listed here. EB indicates embryoid body.
The cardiogenic progenitor cells migrate after their ingress through the primitive streak toward the anterior side of the embryo, where they form 2 populations of cardiogenic progenitor cells (blue). The heart-forming cell populations can be subdivided into 2 different heart field lineages, a first heart field (FHF) (also called cardiac crescent at this embryonic day; red) and a second heart field (SHF) (blue). Cells of the FHF form the primary heart tube (PHT) (blue). The arterious pole is at the top; the venous pole at the bottom. D, Lateral view of a mouse embryo at E8.0. Cells of the FHF form the primary heart tube (PHT), starts of the SHF migrate into the PHT from both sides of the heart to allow a ballooning of the heart. E, Looping process of the linear heart tube at E8.5. The heart tube loops to the right (black arrows). The outflow tract (OFT) arises at the arterious pole; the inflow tract (IFT) at the venous pole. F and G, Sections through hearts at E9.5 (F) and E10.5 (G). The heart is looped to the right and consists of the epicard (blue), the myocard (green), and endocard, as well as cardiac jelly (orange). Cells of the cardiac jelly are produced by the endocard undergoing epithelial–mesenchymal transition (EMT) and represent a thick extracellular layer between the endocard and the myocard. The ventricular chambers start to develop trabeculae. The endocard and the cardiac jelly extend into the heart lumen forming the cardiac cushions and later on the cardiac valves (see I). The separations of the atrial, as well as ventricular chambers, start by forming the primary atrial septum (PAS) and the interventricular septum (IVS). H, Function of the cardiac neural crest during heart development shown at E12.5. Dorsal is at the top; anterior to the left. The cardiac neural crest originates at the dorsal neural tube between the middle portion of the otic vesicle (O) and the third somite (S3) as part of the cranial neural crest. Afterward these cells migrate to the ventral side through the pharyngeal arches 3, 4, and 6 toward the OFT. In the heart, the cardiac neural crest cells are responsible for the OFT septation in pulmonary trunk (P) and aorta (A). I, Development of the cardiac pacemaking and conduction system. Transverse sections through a heart at E14.5. The 4 heart chambers are completely separated by the atria, as well as ventricular septum (AS and VS) and the valves (TV and MV). The contraction impulse originates in the sinusatrial node (SA node) and is then transmitted to the atrioventricular (AV) node, both located in the right atrium. Afterward, the impulse is sent to the ventricular myocardium by the His bundles, bundle branches and Purkinje fibers with a slight delay to allow a coordinate beating of the different cardiac chambers. A indicates aorta; AA, aortic arch arteries; AS, atrial septum; APS, aorticopulmonary septum; LA, left atrium; LV, left ventricle; MV, mitral valve; NT, neural tube; O, otic vesicle; P, pulmonary trunk; RA, right atrium; RV, right ventricle; S, somite; SA, sinusatrial; TV, tricuspid valve; VS, ventricular septum.

Figure 2. Schematic overview of murine cardiac development. A through E, Anterior is at the top; posterior at the bottom. A, 7.0. The cardiogenic progenitor cells migrate after their ingress through the primitive streak toward the anterior side of the embryo, where they form 2 populations of cardiogenic progenitor cells (blue). B, E7.5. The heart-forming cell populations can be subdivided into 2 different heart field lineages, a first heart field (FHF) (also called cardiac crescent at this embryonic day; blue) and a second heart field (SHF) (red). Cells of the FHF form the primary heart tube (PHT) (blue). The arterious pole is at the top; the venous pole at the bottom. D, Lateral view of a mouse embryo at E8.0. Cells of the FHF form the primary heart tube (PHT), starts of the SHF migrate into the PHT from both sides of the heart to allow a ballooning of the heart. E, Looping process of the linear heart tube at E8.5. The heart tube loops to the right (black arrows). The outflow tract (OFT) arises at the arterious pole; the inflow tract (IFT) at the venous pole. F and G, Sections through hearts at E9.5 (F) and E10.5 (G). The heart is looped to the right and consists of the epicard (blue), the myocard (green), and endocard, as well as cardiac jelly (orange). Cells of the cardiac jelly are produced by the endocard undergoing epithelial–mesenchymal transition (EMT) and represent a thick extracellular layer between the endocard and the myocard. The ventricular chambers start to develop trabeculae. The endocard and the cardiac jelly extend into the heart lumen forming the cardiac cushions and later on the cardiac valves (see I). The separations of the atrial, as well as ventricular chambers, start by forming the primary atrial septum (PAS) and the interventricular septum (IVS). H, Function of the cardiac neural crest during heart development shown at E12.5. Dorsal is at the top; anterior to the left. The cardiac neural crest originates at the dorsal neural tube between the middle portion of the otic vesicle (O) and the third somite (S3) as part of the cranial neural crest. Afterward these cells migrate to the ventral side through the pharyngeal arches 3, 4, and 6 toward the OFT. In the heart, the cardiac neural crest cells are responsible for the OFT septation in pulmonary trunk (P) and aorta (A). I, Development of the cardiac pacemaking and conduction system. Transverse sections through a heart at E14.5. The 4 heart chambers are completely separated by the atria, as well as ventricular septum (AS and VS) and the valves (TV and MV). The contraction impulse originates in the sinusatrial node (SA node) and is then transmitted to the atrioventricular (AV) node, both located in the right atrium. Afterward, the impulse is sent to the ventricular myocardium by the His bundles, bundle branches and Purkinje fibers with a slight delay to allow a coordinate beating of the different cardiac chambers. A indicates aorta; AA, aortic arch arteries; AS, atrial septum; APS, aorticopulmonary septum; LA, left atrium; LV, left ventricle; MV, mitral valve; NT, neural tube; O, otic vesicle; P, pulmonary trunk; RA, right atrium; RV, right ventricle; S, somite; SA, sinusatrial; TV, tricuspid valve; VS, ventricular septum.

Wnt Signaling During Specification of the Common Cardiac Progenitor

Initial Experiments, Contradicting Results

The first data concerning the function of Wnt/β-catenin signaling during cardiac development in vertebrates were contradictory and confusing. The positive involvement of Wnt/β-catenin signaling in cardiogenesis was known for
some time in Drosophila. It was therefore quite surprising to find that activation of canonical Wnt signaling in anterior mesoderm suppresses cardiac differentiation in chicken and Xenopus embryos whereas inhibition of the pathway through inhibitors such as Crescent or Dkk1 favors cardiac development. Note that Crescent, a member of the Sfrp family of Wnt inhibitors, is not present in mammals. The endoderm specific knockout of β-catenin in the mouse even results in the formation of multiple, ectopic hearts. In contrast, cell culture–based experiments suggested a positive role for canonical Wnt signaling in early cardiac specification. In P19 teratocarcinoma stem cells interference with canonical Wnt signaling leads to a decrease in cardiac specific gene expression.

This apparent conflict of diverse roles of Wnt/β-catenin signaling has been resolved by the observation that Wnt signaling can have different effects depending on the time of action. An example is a recently published study in heat shock inducible transgenic zebrafish embryos indicating that an application of Wnt8 before gastrulation results in more, whereas an application after gastrulation results in less cardiomyocytes. A reverse effect could be achieved by the timed delivery of the Wnt inhibitor Dkk1. Recent publications confirmed these observations in mouse ES cells. In this system, the expression of some canonical Wnt ligands starts slightly earlier than the expression of cardiac genes. Inhibition of canonical Wnts at this early time point leads to an inhibition of cardiac differentiation and a reduction in contractile areas within embryoid bodies at later time points. These findings raised a model in which Wnt/β-catenin signaling was suggested to have a biphasic function during cardiogenesis. In a variation, a Wnt/β-catenin signaling gradient in time was recently suggested with the highest requirement for β-catenin signaling early during development.

More novel findings indicated that even this biphasic model is still too simple. A more advanced model has to integrate different cardiac progenitor cell populations as described by their molecular signature of expressed genes and the time of their appearance during development as well as the different cellular effects of Wnt signaling such as differentiation, proliferation, and migration. Indeed, specification of the common cardiac progenitor, which is characterized by the expression of Isl1, Nkx2.5 and Flk1, is a multistep process that involves generation of mesodermal progenitor cell and subsequent intermediate cell populations (Figure 3). Furthermore, cell-autonomous and nonautonomous effects have to be considered as many experiments were done in ES cell cultures harboring different but communicating cell types or by non–tissue-specific gain or loss-of-function experiments in frogs or chicken. Here, isolation and analysis of defined progenitor cell populations as defined by specific markers or tissue specific modulation of Wnt signaling by use of trans-
Induction of the Mesodermal Germlayer: Requirement for Wnt/β-Catenin Signaling
Cardiac progenitor cells are specified in the mesodermal germ layer early during development. Many studies have shown that the formation of mesoderm is dependent on canonical Wnt signaling. The knockout mouse of β-catenin, the central player of canonical Wnt signaling, fails to generate mesodermal tissue.36 Also the loss of Wnt3a function is accompanied by an absence of mesoderm-specific marker genes.37 In murine ES cells, the early inhibition of the canonical Wnt pathway blocks the expression of mesoderm-specific marker genes.38 The T-box transcription factor Brachyury (Bry) is a pannemesodermal marker gene and required for posterior mesoderm formation. In different organisms, it has been shown that mesoderm formation depends on Wnt/β-catenin signaling using Bry expression as a marker.39,40 This regulation is likely direct because the authors could demonstrate a binding site for TCF in the Bry promotor. These data indicate a strong requirement of canonical Wnt signaling during mesoderm induction in the early embryo, a prerequisite for the formation of cardiac progenitor cells.

Cardiac Specification: Negative Influences of Canonical Wnt Signaling
The earliest marker gene for the cardiovascular lineage is the basic helix–loop–helix (bHLH) transcription factor Mesp1. Functional studies could demonstrate an important function of Mesp1 during cardiac development.41,42 An in-depth analysis of Mesp1-regulated genes indicated that Mesp1 is on top of the cardiovascular lineage upstream of other important markers for the common cardiac progenitor such as Flk1 or Isl1.43,44 At the same time, Mesp1 represses essential genes for the hematopoietic cell lineage. The receptor tyrosine kinase Flk1 is an important marker for early mesoderm development. Two phases of Flk1 expression have been defined in ES cell culture experiments.21,45 Those cells expressing Flk1 early (Bry+/Flk+/Mesp1+) are fated to become the hemangioblast that further develops into endothelial cells and the blood lineage. A second wave of Flk1 expression slightly later results in a progenitor cell for the cardiac lineage (Bry+/Flk1+/Mesp1+) (see also Figure 3). Consistent with the above-mentioned data concerning the function of Wnt/β-catenin signaling during mesoderm formation, depletion of Wnt/β-catenin signaling results in a loss of Mesp1 along with a downregulation of Flk1 expression.34

Recently, it was shown that Notch signaling redirects Flk1+ cells fated to become hemangioblasts toward Flk1+ cells of the cardiogenic lineage.46 An in-depth analysis of this observation indicated that Notch4 activation in Bry+/Flk+ cells results in an upregulation of molecules inhibiting Wnt/β-catenin signaling such as the secreted Frizzled-related proteins Sfrp1 or Sfrp5. Indeed, the addition of Wnt3a but not of Wnt5a completely abolished this effect of Notch4. This diverse behavior of Wnt3a and Wnt5a, together with the knowledge of Wnt3a activating β-catenin during mesoderm formation, makes it likely that Wnt5a acts through β-catenin–independent noncanonical Wnt signaling branches in this context, an argument that is further substantiated below. The interplay between Notch and β-catenin signaling was also analyzed independently by the Srivastava laboratory.47 Here, the authors overexpressed a stabilized variant of β-catenin in Isl1+ CPCs, resulting in a downregulation of several important cardiac genes such as Isl1, myocardin, shh, or Smyd1 at E9.0 of mouse development. Similarly, a cardiac-specific loss of Notch1 resulted in severely affected right ventricular structures. It should be kept in mind that this requirement for Notch signaling and the negative influence of β-catenin signaling on differentiation is significantly later during development than described in the study of the Keller laboratory.46 Both studies, however, indicate that Notch mediated inhibition of canonical Wnt signaling is a recurrent motive during cardiomyocyte differentiation. In line with these data is the observation that Flk1+/CXCR4+/VE-cadherin+ cells isolated from an OP9 coculture system can give rise to cardiomyocytes under certain conditions. In this setting, inhibition of Wnt/β-catenin signaling favors the formation of cardiomyocytes. Wnt3a inhibits this process.48

In accordance with the inhibitory role of Wnt/β-catenin signaling during cardiac development, it has recently been shown that β-catenin negatively regulates the expression of GATA6, one of the major early cardiac transcription factors.49 Taken together, all of these data show that during specification of cardiac precursor cells the downregulation of Wnt/β-catenin signaling is essential.

These findings are furthermore in line with the observation that the canonical Wnt inhibitor Dkk1 can improve the differentiation of Mesp1-induced cardiomyogenesis.44 The question of whether Dkk1 is regulated directly by Mesp1 is controversial. In the search for Mesp1 target genes, Dkk1 was identified by chromatin immunoprecipitation experiments and a direct binding of Mesp1 to the Dkk1 promoter has been shown.50 In ES cells overexpressing Mesp1, Dkk1 is upregulated, whereas in Mesp1+/−/Mesp2−/− embryos, Dkk1 expression was dramatically downregulated.50 However, these findings have recently been challenged.44 Dkk1 was found to be upregulated by Mesp1 but this was not evident before 24 hours after Mesp1 activation, suggesting a more indirect effect of Mesp1 on Dkk1 expression.44 Moreover, a direct activation of Dkk1 by Mesp1 was not confirmed in a third study.43 These discrepancies might be explained by different technical approaches or levels of Mesp1 overexpression in ES cells and require further investigations. Interestingly, Dkk1 has been shown to have more functions than just inhibiting Wnt/β-catenin signaling, in particular, activating JNK signaling51,52 or a so-far unknown signaling pathway.53

Non–Cell-Autonomous Roles of Wnt/β-Catenin Signaling During Precardiac Specification: A Role for Endodermal Tissue
Expansible techniques in Xenopus suggested a role for the Spemann organizer and the underlying anterior endoderm for
Cardiac induction in vivo. This has also been analyzed recently in greater detail by the help of sophisticated combinations of anterior endoderm explants with animal caps representing a population of pluripotent progenitor cell populations. In this assay, cardiac induction by anterior endoderm was independent of β-catenin signaling. These data are in line with previous data indicating that the cardiac inducing activity of Dkk1 in Xenopus is attributable to the generation of Hex1-positive endodermal cells. In murine embryonic stem cells, β-catenin signaling has been shown to be essential for Sox17 expression. Sox17 expression itself is required for cardiogenesis in a non–cell-autonomous role, thus likely being required in endodermal tissue. Consistently, loss of Sox17 results in the downregulation of Hex1 and the endodermal marker genes Fgax and Foxa2. Thus, also in the endoderm, positive and negative roles of Wnt/β-catenin signaling have been described: a positive role for the formation of Sox17β positive progenitor cells; a negative for the formation of likely Sox17β̅/Hex1 cells (Figure 3). The endoderm then releases factors initiating cardiogenesis in the mesodermal tissue (FGF, nodal, Cerberus) that can act in parallel. Interestingly, Sox17β knockout mice do not show a severe cardiac phenotype. Note, cardiac differentiation was only examined by analyzing GATA4 expression. Sox17β expression itself is required for cardiogenesis in a non–cell-autonomous role, thus likely being required in endodermal tissue. Consistently, loss of Sox17β results in the downregulation of Hex1 and the endodermal marker genes Foxa1 and Foxa2.55 Thus, also in the endoderm, positive and negative roles of Wnt/β-catenin signaling have been described: a positive role for the formation of Sox17β positive progenitor cells; a negative for the formation of likely Sox17β̅/Hex1 cells (Figure 3). The endoderm then releases factors initiating cardiogenesis in the mesodermal tissue (FGF, nodal, Cerberus) that can act in parallel.26,56 Interestingly, Sox17β knockout mice do not show a severe cardiac phenotype.57 Of note, cardiac differentiation is impaired by inhibiting JNK or PKC. Vice versa, coactivation of JNK and PKC in embryonic explants activates cardiac marker gene expression.51 Similar in endothelial progenitor cells, mesenchymal stem cells or bone marrow stem cells, triggering a cardiogenic program by Wnt5a/Wnt11 involves activation of PKC.61,62,64 In a recent study, the type of PKC likely involved has been determined. In endothelial progenitor cells, activation of a cardiogenic program is increased on treatment with the PKC activator phorbol-12-myristate-13-acetate (PMA). This excludes the involvement of atypical PKCs that are PMA-independent. By use of isoform-specific PKC inhibitors, the involvement of PKCδ but not PKCε downstream of Wnt5a has been demonstrated.65

It might be appropriate here to discuss the requirement of Wnt2b for cardiogenesis.68 Wnt2b was initially found to be highly expressed in Flk1+ progenitor cells69 and subsequent generation of Wnt2b−/− ES cells indicated a requirement for Wnt2b for the cardiogenic but not for hematopoietic lineage.68 More detailed analyses of these cells, however, indicated that the loss of Wnt2b is accompanied by an accelerated expression of Bry and Flk1. This is an interesting observation given the positive role of Wnt/β-catenin signaling for Bry expression, as discussed above. Furthermore, activating Wnt/β-catenin signaling during early differentiation of ES cells has also been linked to accelerated differentiation of ES cells by others.70 Because, in this respect, the loss of Wnt2b function has an identical phenotype to the Wnt5a gain of function, this strongly argues for the fact that Wnt2b might activate a noncanonical Wnt pathway in this context. Of course, this awaits further analyses. Interestingly, Wnt2b and Wnt11 are both direct target genes of GATA6,69,71 and both are coexpressed in early mouse72,73 but not in Xenopus embryos.74,75 This putative functional redundancy might explain why a loss of Wnt11 results in an early cardiac phenotype in Xenopus74,75 but not in the mouse with later phenotypes.76,77 Slightly later, however, it might be Wnt2b that regulates proliferation of the common cardiac progenitor through a Wnt/β-catenin pathway.78

Cardiac Specification: Positive Influence of Noncanonical Wnt Signaling

Noncanonical Wnt signaling also contributes to correct cardiac specification in the mesodermal germ layer. The best investigated factor to this end is Wnt11. In Xenopus, Wnt11 is maternally expressed in the presumptive dorsal mesoderm and in the dorsal marginal zone during gastrulation. Loss-of-function experiments demonstrated that Wnt11 is required for normal heart development and cardiac marker gene expression.49,51 Overexpression of Wnt11 in Xenopus explants is sufficient to induce contractile tissue formation.51 This cardiac promoting activity of Wnt11 has also been shown in other model systems such as quail mesoderm,58,59 murine ES cells,32,60 endothelial progenitor cells61 and mesenchymal or bone marrow derived stem cells.62 The cardiac supporting activity of Wnt11 could be linked to noncanonical Wnt signaling branches.51,61,62 In Bry+/Flk1+ cardiogenic progenitor cells noncanonical Wnt ligands are upregulated. In particular, Wnt5a functions in synergy with BMP6 and Sfrp1 to promote formation of troponin-positive cells, likely through noncanonical Wnt signaling activities, whereas treatment with either single factor did not result in significant cardiomyocyte formation. This indicates that Wnt5a might be supportive but not required for cardiogenesis and might explain why Wnt5a knock mice do not display a cardiac differentiation phenotype. Nevertheless, this finding is of particular interest because Wnt5a is upregulated by Mesp1 and has also been suggested to function during cardiogenesis in an independent ES cell–based approach.65 Like in the ES cells system, in endothelial progenitor cells (EPCs), a cardiogenic program can also be initiated by activating Notch signaling through overexpression of the intracellular notch domain (NICD). Interestingly, this is accompanied by an upregulation of Wnt5a. Cardiogenesis in EPCs can also be triggered by cocultivation with neonatal rat cardiomyocytes and this effect was sensitive to γ-secretase inhibition thereby downregulating Notch signaling.66 Thus, upregulation of noncanonical Wnt ligands in response to either Notch signaling or Mesp1 activation might be a common theme during cardiac development.

The downstream signaling activity of these 2 Wnt ligands, Wnt5a and Wnt11, has been analyzed by different groups in more detail. The ability of Wnt11 to induce cardiac tissue in Xenopus is impaired by inhibiting JNK or PKC. Vice versa, coactivation of JNK and PKC in embryonic explants activates cardiac marker gene expression.51 Similar in endothelial progenitor cells, mesenchymal stem cells or bone marrow stem cells, triggering a cardiogenic program by Wnt5a/Wnt11 involves activation of PKC.61,62,64 In a recent study, the type of PKC likely involved has been determined. In endothelial progenitor cells, activation of a cardiogenic program is increased on treatment with the PKC activator phorbol-12-myristate-13-acetate (PMA). This excludes the involvement of atypical PKCs that are PMA-independent. By use of isoform-specific PKC inhibitors, the involvement of PKCδ but not PKCε downstream of Wnt5a has been demonstrated.65

Lineage-Specific Requirements of Wnt Signaling

SHF-Specific Manipulations of β-Catenin Signaling

At E8.0 of mouse development, cells of the SHF lineage are found dorsally to the primarily formed tubular heart and can
enter the heart tube either from the anterior site giving rise to
the right ventricle and the OFT or they can enter from the
posterior site contributing to the atriia and the inflow tract
region. Several studies could show a specific role for the
canonical Wnt signaling pathway in the second heart field
lineage and SHF-derived structures. The tissue-specific
downregulation of β-catenin in the SHF using different Cre
mouse lines leads to a reduction of Isl1-positive cells in
comparison to wild-type mice as well as a loss of the
SHF-derived OFT and right ventricle. Moreover, it has
been shown by chromatin immunoprecipitation experiments
that β-catenin is directly binding and positively regulating the
Isl1 promoter whereas others reported a negative regulation.
Also, FGF ligands were supposed to be downstream of
β-catenin signaling. The overexpression of β-catenin, how-
ever, results in an expansion of SHF-derived structures. These
phenotypes were clearly linked to an effect of Wnt/β-
catenin signaling on cell proliferation. Also in the posterior
part of the SHF Wnt signaling regulates cell proliferation.
Here Wnt2b has been identified as a ligand. Wnt2b+ embryos
display smaller and thinner atriia, a loss of the
primary atrial septum and defects in the AV cushion resembling
a human congenital condition called common atrioventricular
canal (CCAVC) or atrial septum defects. Also, the pulmonary vein is affected.

Taken together, all of these data indicate a positive effect of
the canonical Wnt signaling pathway in the development of
SHF-derived structures in the mature heart.

Nkx2.5-Cre–Specific Manipulation of β-Catenin Signaling
A specific deletion of β-catenin in the FHF lineage has not been
performed so far. The best evidence for a role of
β-catenin signaling in this lineage has been gained from mice
deleting β-catenin function with a specific Nkx2.5 enhancer
that is not active in the cardiac crescent at E7.5 but later is
expressed in both ventricular chambers. This resulted in
smaller ventricles and reduced proliferation. In contrast,
overexpressing a stabilized version of β-catenin under the
control of the same enhancer resulted in enlarged ventricular
chambers, more cells, and upregulated expression of cy-
clinD2. Because both chambers were affected, these
phenotypes strongly argue that in both cardiac lineages Wnt/β-
catenin signaling supports the expansion of committed
cardiac progenitor cells. In contrast, deleting β-catenin using
a Mesp1 driver mainly affected the SHF but not the FHF.
This would suggest that the FHF lineage is not requiring
β-catenin signaling. The differences are likely explained,
however, by the different timing and tissue specific expres-
sion in which the different Cre drivers are active.

Wnt Signaling During Terminal Differentiation
Different evidences indicate that Wnt/β-catenin signaling
inhibits terminal differentiation of cardiomyocytes. This has
been well documented in ES cell–based systems in which the
late addition of Wnt3a during embryoid body differentiation
resulted in a reduced number of cardiomyocytes. Also,
differentiation of Isl1+ cardiac progenitors was inhibited by
the addition of Wnt3a-conditioned media. Interestingly, the
above-mentioned overexpression of constitutively active
β-catenin by use of a SHF-specific enhancer of the Me2c
gene resulted in massive accumulation of Isl1-positive pro-
genitor cells attributable not only to increased proliferation
but also to a massive inhibition of terminal differentiation.
A novel extracellular inhibitor of Wnt/β-catenin signaling
was identified recently as IGFBP-4 (insulin-like growth factors binding protein). In Xenopus, it was shown that
IGFBP-4 binds to Frizzled and LRP6, thereby preventing
binding of a Wnt ligand. Interestingly, this inhibitor of
Wnt/β-catenin signaling is required for terminal differentia-
tion. In line, the intracellular Wnt/β-catenin inhibitor Dbf4
is also required for cardiogenesis. A natural in vivo ligand
for this repressive effect of Wnt/β-catenin signaling on
cardiogenesis could be Wnt6. Overexpression of Wnt6 in
transgenic Xenopus embryos using a heat shock–inducible
promoter during stages of terminal differentiation resulted in
decreased cardiac structures. In contrast, deleting Wnt6 by
use of antisense morpholino oligonucleotides resulted in
enlarged cardiac structures. Thus, Wnt6 could be required to
restrict the cardiogenic field during embryogenesis. A Wnt
ligand that shows similar properties in the mammalian sys-
tems awaits its discovery.

Also, noncanonical Wnt signaling plays important roles
during terminal differentiation. In murine ES cells, terminal
differentiation of cardiomyocytes is accompanied by a
marked increase in Wnt11 expression and adding Wnt11 to
differentiating ES cell cultures increases formation of beating
cardiomyocyte foci. In Xenopus 2 Wnt11 homologs have been identified: A
maternally expressed Wnt11 (also called Wnt11b) and the
later expressed Wnt11-R (also called Wnt11a). Deple-
tion of Wnt11-R by use of antisense morpholino oligo-
nucleotides resulted in a downregulation of marker genes for
terminal differentiation. Interestingly, the cell adhesion
molecule DM-GRASP could be identified as a potential
canonical Wnt target gene in this context. Similar to
Wnt11-R, a loss of this cell adhesion molecule also led to
defects in terminal differentiation. The phenotype on
Wnt11-R depletion could be partially reverted by adding back
DM-GRASP into the system indicating that proper cell
adhesion is required for terminal differentiation.

A Preliminary Model for Wnt Action During
Cardiac Specification and Differentiation
Taken together, these data draw a complex picture of Wnt
signaling during early cardiac specification and differentia-
tion (Figure 3A). Based on these findings, one can describe
the involvement of Wnt signaling during cardiac specification
and differentiation with 4 phases (see Figure 3B). (1) Wnt/β-
catenin signaling is required for mesoderm (and endoderm)
formation. Wnt3a is the prime candidate for this function in
mice. (2) Wnt/β-catenin signaling needs to be low for cardiac
specification and generation of multipotent cardiovascular
progenitor cells. Sfrp1 and Sfrp5 might be involved in
downregulating Wnt/β-catenin signaling. Noncanonical sig-
aling supports this step. Wnt5a and Wnt11 are good candi-
dates. (3) Wnt/β-catenin signaling is required for proliferation and expansion of several specified cardiac progenitors. Wnt2b is a good candidate to regulate proliferation of early common progenitor cells and the posterior SHF progenitor. (4) Finally, for terminal differentiation, Wnt/β-catenin signaling has to be low again. Noncanonical Wnt signaling is supportive and again Wnt11 is the prime candidate.

From the Tubular Heart Tube to the Four Chambers

Wnt Signaling Regulates Cell Adhesion of Cardiomyocytes

The process of cardiac morphogenesis includes different processes such as heart tube formation, cardiac looping, chamber formation, and maturation of the heart. For all of these processes, the adhesion of cardiac cells is an important prerequisite. In this context, N-cadherin, a calcium-dependent cell–cell adhesion molecule, plays an essential function. On loss N-cadherin, cardiomyocytes develop but cells do not form a heart tube because of defects in cell–cell contact formation.89 The function of N-cadherin depends on its intracellular interaction with α- and β-catenins. This interaction is also essential for heart development.91 Noncanonical Wnt signaling has been known for quite some time to regulate cadherin mediated cell adhesion.92 Wnt11 has been shown to regulate N-cadherin expression in the quail mesodermal progenitor cell line QCE6,59 and N-cadherin expression and function in neonatal rat cardiomyocytes is regulated by Wnt/Fz2 signaling.93 Wnt11 knockout embryos display defects in the OFT (see below)77 but also thinner ventricular walls and more trabeculae. A detailed analysis indicated defects in N-cadherin/β-catenin mediated cell adhesion as well.76 Similarly, Wnt5a promotes N-cadherin–mediated cell adhesion in cardiac myocytes.94 Besides N-cadherin, the cell adhesion molecule DM-GRASP also has been shown to be regulated by noncanonical Wnt signaling in Xenopus.89 In Xenopus, loss of Wnt11-R is accompanied by reduced cell adhesion in the heart tube.88 In addition, we could recently show that a loss of the potential Wnt11-R target gene DM-GRASP also results in reduced cell–cell contacts among the myocardial cells followed by cardiac looping defects.89 Consistently, loss of Wnt11 or Wnt11-R leads to malformed linear heart tubes or later morphogenetic defects in Xenopus.51,89 In zebrafish, the loss of Wnt11, Wnt5a, and Wnt4 results in cardiac bifida and subsequent defects in heart tube formation.95 These data highlight the functional requirement of noncanonical Wnt signaling for proper cell adhesion, tube formation, looping, and chamber maturation of the heart.

Septation and Cardiac Valve Formation

Cardiac looping is followed by the formation of the 4 chambers of the heart, which requires different morphogenetic processes: ballooning of the atria and ventricles and remodeling of the inner heart curvature, including trabeculation of the ventricles, chamber septation, and formation of valves. Chamber septation involves the formation of the primary atrial septum (PAS) segregating both atria, as well as the interventricular septum (IVS) dividing the right from the left ventricle. To our knowledge, no data are available indicating any function of Wnt signaling during intraventricular septum formation. In Wnt2b knockout embryos, however, the primary atrial septum does not form.78 During valve formation, cells of the endocardium located between the atrial and ventricular chambers, the atriocentral canal, delaminate, undergo an epithelial-mesenchymal transition (EMT), migrate into the cardiac jelly located between endocardium and myocardium, proliferate and form the so-called cardiac cushions. During further development, these cardiac cushions build the cardiac valves, the tricuspid valve (TV), separating the right atrium from the right ventricle and the mitral valve (MV) dividing the left chambers.

During valve formation, the canonical Wnt pathway possesses important functions.90 Analyses for molecular markers of endocardial cushion development identified active Wnt/β-catenin signaling as one feature.92 In zebrafish, loss of APC function, which leads to an accumulation of signaling active β-catenin, results in unlooped hearts and excessive endocardial cushions based on an increased proliferation and EMT of endocardial cells. In contrast, overexpression of APC or Dkk1 inhibits cardiac cushion formation.96,99 In line with the data in fish, Wnt9a and the Wnt inhibitor Fzrb1 are expressed in AV endocardial cushions in chicken. Whereas Wnt9a transcripts can exclusively be detected in endocardial cells, Fzrb1 expression can also be found in cells undergoing EMT. Wnt9a supports endocardial cell proliferation by activating Wnt/β-catenin signaling, whereas Fzrb1 inhibits Wnt9a function.100 Interestingly, Wnt9b has also been shown to activate Wnt/JNK signaling during kidney development.101 Thus, it is an attractive hypothesis to assume that Wnt9b might also regulate EMT and the migratory behavior of endocardial cells. Also, in Xenopus, Fzrb1 is expressed in cardiac cushions suggesting a conserved function among distinct species.102

The Neural Crest and the Development of the Outflow Tract

Cardiac neural crest cells (CNCCs) are a subpopulation of the cranial neural crest cells and originate between the otic vesicle and the third somite (Figure 2H). CNCCs are specified at the dorsal neural tube and migrate through the pharyngeal arches 3, 4 and 6 along the aortic arch arteries toward the heart. They are required for the septation of the OFT into the pulmonary trunk and the aorta by forming the aorticopulmonary septum (APS). Defects during CNCCs migration result in congenital heart malformations such as persistent truncus arteriosus (PTA), double outlet right ventricle (DORV), tetralogy of Fallot and DiGeorge syndrome. Furthermore, cell-labeling approaches indicated that CNCCs also differentiate into smooth muscle cells of the aortic arch arteries.

Wnt signaling pathways have been shown to have a role in both development of CNCC and the OFT itself. The requirement of canonical Wnt signaling in CNCC development has been shown in multiple studies. Wnt1 and Wnt3a are expressed in CNCCs of the neural tube. In mice, loss of
β-catenin in Wnt1 positive cells by use of a Wnt1-Cre line results in reduced proliferation of CNCCs. Downregulation of the Wnt coreceptor Lrp6 leads to a reduction of CNCCs in the dorsal neural tube and in the pharyngeal arches. Furthermore, the embryos display DORV, VSD (ventricular septal defect) and OFT cushion hypoplasia. Furthermore, depletion of dsh2 causes defects in OFT development such as PTA and DORV, as well as a downregulation of CNCC-specific marker genes. Tissue-specific loss of β-catenin in the CNCCs also leads to OFT malformations and in addition to an inhibited proliferation of CNCCs. As a consequence of dsh2 and β-catenin downregulation, the expression of the bicoid-related transcription factor Pitx2 is reduced. Furthermore, the authors could show cyclinD2 as a Pitx2 target gene involved in cell cycle. These results match with the observation that Pitx2 depletion in mice results in a similar phenotype, PTA and DORV. These results strongly support the hypothesis that canonical Wnt signaling is essential for cell cycle regulation of CNCC development. On the other hand, noncanonical Wnt signaling also plays an important role during neural crest development. In Xenopus, noncanonical Wnt signaling activated by Wnt4, Wnt11 or Wnt11-R are required for neural crest migration. For Wnt11-R, this has been linked to the activation of a noncanonical Wnt pathway involving calcium signaling and activation of CaMKII.

In mice, Wnt5a and Wnt11 are expressed in the myocardium of the OFT. Loss of Wnt5a results in OFT septation defects resulting in PTA and DORV. In this context Wnt5a signals to immigrated CNCC and functions via the noncanonical Wnt/Ca^{2+} pathway. This leads to a downregulation of CNCC marker genes and deficits during aorticopulmonary septum formation. Wnt11 is another Wnt ligand expressed in the OFT and activates a noncanonical Wnt pathway regulating the expression of TGFβ2. Furthermore, Wnt5a and Wnt11 are required for cytoskeletal rearrangements during morphogenesis of the OFT. Others could show a requirement for components of the planar cell polarity (PCP) pathway in OFT formation. Investigation of the surface transmembrane protein Vangl2 (also known as strabismus or Van Gogh in Drosophila), a component of PCP signaling, reveals a role for this gene in lamellipodia and filopodia formation in the OFT presumably caused by defects in cell-cell and cell–ECM interactions.

A Third Cardiac Lineage: The Epicardium

The epicardium represents the outer layer of the heart enveloping the myocardium and endocardium. The epicardium spreads over the myocardium during looping stages and originates mostly from the proepicardium. A minor part of the epicardium covering the outflow tract region originates from splanchnic mesoderm of the ventral pharynx. It has been thought for a long time that the proepicardium represents an extracardiac population of cells; however, more recent molecular data indicated that the epicardium derives from an early IsI1^+ proepicardial progenitor cell population. These proepicardial progenitors likely derive from IsI1^+/Nkx2.5^+ cardiac progenitor cells. BMP and FGF signaling pathways play an important role in the separation of epicardial and myocardial lineages. Epicardial cells are characterized by the expression of Tbx5, WTI, and Tbx18 but are finally negative for IsI1. The epicardium can further differentiate into different cell types. Based on Tbx18:GFP mice, it has been suggested that epicardial cells can contribute to the myocardium. In support of the first hypothesis, it could be demonstrated that also WTI^+ epicardial cells can give rise to heart muscles. Furthermore, it is current textbook knowledge that the epicardium forms the coronary vessels. A very recent study indicated in contrast that the coronary arteries form by developmental reprogramming of venous cells. Given this, it is tempting to assume that the epicardium either contributes to a small part of coronary arteries or supports artery formation rather by inducing the microenvironment. Besides this, the epicardium forms cardiac fibroblasts. Two studies demonstrate a function of Wnts during epicard development. Depletion of β-catenin in the proepicardium using a specific GATA5-Cre line resulted in defects in coronary artery formation that finally is lethal. A second study investigated the function of retinoic acid in epicardium development. The loss of retinoic X receptor α (RXR) in the epicardium indicated that RXR is required for EMT of epicardial cells, resulting in damaged myocardial growth and coronary artery formations. The authors indicated Wnt9b to be downstream of RXR, which in turn regulates FGF2 expression in the myocardium.

The Conduction System

A further major step in cardiac development is the establishment of the conduction system that is essential for the coordinated beating of the different cardiac chambers. This process starts by forming the so-called cardiac pacemaking region in the developing right atrium at E8.0 of mouse development (Figure 2I). Later, the sinoatrial node is formed in which the contractile impulse originates. Afterward this impulse is transduced to the other parts of the conduction system, the atrioventricular (AV) node, the His bundles, bundle branches and the Purkinje fibers. The conduction system derives from an IsI1-positive progenitor cell. The role of Wnts for this process is not well investigated. Bond and colleagues revealed a specific expression of Wnt7a as well as Wnt11 in the developing conduction system. Additionally, the treatment with endothelin-1 led to an increased expression of Wnt7a and Wnt11. However, functional data are missing.

Outlook

As outlined, Wnt signaling plays multiple roles throughout whole cardiac development (see Table 2 for a referenced summary). Future work will have to describe precisely in which cell type Wnt signals emerge and in which cell type these signals elicit their function. A detailed analysis of intracellular mediators used by these Wnt ligands will have to identify the Wnt signaling branch involved. This detailed knowledge of how Wnt proteins influence cardiac development will help to improve protocols for directed differentiation of ES cells into different cardiac lineages. Together with the establish-
ment of safe protocols to generate human, patient-specific induced pluripotent stem cells, this opens the window of opportunity to develop novel therapeutic approaches. This knowledge might also help to activate recently described resident stem cells in the heart on heart damage.122–125

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The Multiple Phases and Faces of Wnt Signaling During Cardiac Differentiation and Development

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