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The Importance of Wnt Signaling in Cardiovascular Development

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Abstract Cardiac development is comprised of a series of morphological events tightly controlled both spatially and temporally. The molecular pathways controlling early cardiac differentiation are poorly understood, but Wnt signaling is emerging as a critical pathway for multiple aspects of early cardiovascular development. The Wnt pathway plays multiple roles in regulating cellular behavior including proliferation, differentiation, cell migration, and cell polarity. Recent data have demonstrated that Wnt activity is important for early precardiac mesoderm differentiation but must be inhibited in subsequent steps for cardiomyocyte differentiation to proceed. Given the important role that Wnt signaling plays in both the differentiation of cardiomyocytes from pluripotential stem cells and tissue regeneration in general, an increased understanding of this pathway is likely to enhance our knowledge about both cardiovascular development and reparative mechanisms.

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Wnt Signaling Overview

Wnt proteins are homologs of the *Drosophila* wingless gene and have been show to play important roles in regulating cell differentiation, proliferation, and polarity (reviewed in [7, 53, 64]). Wnt proteins are cysteine-rich secreted glycoproteins that signal through several possible pathways. The best understood of these, commonly called the canonical pathway, involves binding of Wnt proteins to Frizzled cell surface receptors, which inhibits GSK-3β phosphorylation of β-catenin. Hypophosphorylated β-catenin is translocated to the nucleus, where it binds to members of the lymphoid enhancement factor/T-cell factor (LEF/TCF) family of transcription factors. Binding of β-catenin converts LEF/TCF factors from repressors to activators, thereby switching on cell-specific gene transcription.

Wnt proteins also can signal through a poorly understood network of noncanonical effectors to control subtle aspects of cell behavior such as cell polarization, adhesion, and motility [22, 35, 67, 68, 71, 87]. Activation of these effectors can inhibit canonical Wnt signaling. Moreover, it has been shown that a single Wnt ligand, such as Wnt5a, can signal through both canonical and noncanonical pathways in a cell type-dependent context [47, 77]. These issues have led some to promote the idea that Wnt signaling is more a network of interacting factors that regulate many aspects of cell biology than a simple linear signaling pathway [78]. This notion has much merit and likely will garner increased support as the diversity of cellular responses due to Wnt signaling increases (Table 1)

Table 1 Components of the Wnt pathway and their role in cardiovascular development

Gene	Species	Pathway	Phenotype	References
Wnt ligands				
Wnt2	Mouse	Canonical	Placental vascular failure, inflow tract development (E. Morrisey, unpublished observations)	[49]
Wnt5a	Mouse	Noncanonical	Outflow tract septation	[45, 59]
Wnt7a	Mouse	Canonical	Cerebral vascular development (with Wnt7b)	[14, 66]
Wnt7b	Mouse	Canonical	Pulmonary vascular smooth muscle development, cerebral vascular development (with Wnt7a)	[13, 14, 62, 66, 86]
Wnt9a	Mouse	Unknown	Epicardial development	[46]
Wnt11	Mouse, zebra fish, *Xenopus*	Noncanonical	Outflow tract development, cardiac myocyte differentiation	[15, 17, 60, 69, 91]
Wnt receptors				
Fzd4	Mouse	Unknown	Retinal vascular development (with norrin)	[70, 88]
Fzd5	Mouse	Unknown	Placental vascular development	[21]
Norrin	Mouse, human	Canonical	Retinal vascular development (with Fzd4)	[88]
Lrp5	Mouse, human	Canonical	Retinal vascular development	[24, 70, 86]
Lrp6	Mouse	Canonical	Vascular smooth muscle proliferation and survival	[85]
Signaling components				
Beta-catenin	Mouse, zebra fish	Canonical	SHF progenitor proliferation, cardiac valve development, adult hypertrophic growth	[2, 10, 12, 20, 29, 37–39, 50, 51, 76, 79, 84]
Dvl1	Mouse	Unknown	Myofibroblast proliferation	[9]
Dvl2	Mouse	Unknown	Outflow tract development	[18]
Gsk3β	Mouse	Canonical	Adult cardiac hypertrophic growth	[10, 72, 73]
Vangl2	Mouse	Noncanonical	Outflow tract development	[54]
Apc	Zebra fish	Canonical	Cardiac valve development	[20]

SHF second heart field

Noncanonical Wnt effectors are loosely organized into two pathways, the Wnt/RhoA and Wnt/Ca^{2+} pathways. In Wnt/RhoA signaling, Wnt proteins bind to Frizzled receptors, which mediate both canonical and noncanonical Wnt signaling, to activate Rho-family small GTPases and their downstream effectors such as Rho-associated kinase [22, 35, 67, 68]. This pathway is similar to the *Drosophila* planar cell polarity pathway, which is required for proper fly wing hair orientation, cell polarization during convergence and extension movements in gastrulation, and orientation of steriocilia in the cochlea [16, 19, 25, 28, 43, 55, 80, 81, 83, 92]. In Wnt/Ca^{2+} signaling, Wnt proteins induce intracellular Ca^{2+} release and activate the Ca^{2+}-dependent protein kinases PKC and CamKII via the G-protein-dependent activity of Frizzled receptors [32–34, 42, 58, 61, 63].

Wnt signaling has been shown to play an essential role in brain, limb, mammary, skin, and, more recently, cardiovascular development [3, 4, 12, 30, 37, 39, 44, 52, 56, 57, 65, 76]. There are 19 Wnt proteins, 10 Fzd receptors, and 2 LRP co-receptors in mammals. Such complexity suggests multifarious possibilities of Wnt-signaling outputs. Recent studies indicate that individual Wnt ligands can activate canonical or noncanonical Wnt signaling in a cell context-dependent manner [47, 74]. Therefore, the pathway likely is regulated in a spatial and temporal context related to the expression patterns of specific Wnt ligands and receptors.

Early Cardiovascular Development and Wnt Signaling

The heart arises from two different but overlapping sources of mesoderm called the first heart field (FHF) and the second heart field (SHF). The SHF lies peripherally in relation to the FHF in the early cardiac crescent, and both migrate to the midline to form the simple cardiac tube. SHF progenitors continue to contribute to the growing and septating heart, playing a critical role in the formation of the right ventricle, and atria, as well as the outflow and inflow tracts [26, 27]. At the molecular level, the distinctions between FHF and SHF progenitors are poorly understood, and both fields likely overlap extensively. However, recent data point to a unique sensitivity of SHF progenitor differentiation and proliferation to Wnt/β-catenin signaling [31, 76].

The FHF and SHF and their derivatives are marked by expression of specific transcription factors. Myocyte enhancer 2c (*Mef2c*), GATA-binding protein 4 (*Gata4*), and NK2 transcription factor related 5 (*Nkx2.5*) mark both the FHF and SHF [36, 40, 41, 48]. Tbx5 expression is observed in a graded pattern, with the highest levels in the left ventricle and the formation of the left ventricle is preferentially affected in loss of function mutants, supporting the notion that Tbx5 is a reasonable marker of the FHF [5, 6]. The LIM-homeodomain gene islet 1 (Isl1) is expressed primarily in the SHF and marks this group of cardiac progenitors as a distinct cell population [8].

All these factors are critical determinants of cardiomyocyte specification and development. In mice, Gata4 and Gata6 are required for specification of FHF progenitors but not SHF progenitors [90]. Loss of both Gata4 and Gata6 leads to heart agenesis, with a loss in Tbx5 expression (FHF marker) but continued Isl1 expression (SHF marker). Despite this important finding, specification of FHF and SHF cardiac progenitors is likely to rely on a complex network of interactions between multiple families of transcription factors. Moreover, the activity of several signaling networks including BMP, TGFβ, and Wnt are important in the regulation of these transcription factor interactions at the earliest stages of cardiac specification and differentiation.

The activity of the Wnt/β-catenin pathway has been explored during cardiac development using several LEF/TCF transgenic reporter lines. Although activity is observed in the pericardium, the endocardial cushions, the adjacent cardiac mesoderm, and the early outflow tract, little to no activity is observed in the developing ventricular myocardium [12, 39]. This is surprising given the high expression level of several Wnt ligands in the early heart including Wnt2, Wnt2b, Wnt11, and Wnt8a [15, 23, 49, 89]. These data could indicate that these ligands act primarily through the noncanonical portion of the Wnt network or that the LEF/TCF transgenic lines are limited in their ability to report Wnt/β-catenin signaling accurately in the developing heart.

Wnt signaling has been shown to regulate the development of the heart's anterior portion including the outflow tract and the right ventricle. Loss of β-catenin in the SHF leads to decreased numbers of anterior Isl1$^+$ progenitors, whereas stabilization of β-catenin expands the numbers of Isl1$^+$ cells [2, 12, 37, 39]. The deletion of β-catenin in the SHF causes a dramatic reduction in both the levels of Isl1 expression and the numbers of cells that express Isl1 [12, 39]. Moreover, chromatin immunoprecipitation and *in vitro* reporter assays indicate that β-catenin directly binds to and regulates the Isl1 promoter [39].

These data strongly suggest that canonical Wnt/β-catenin signaling plays a role in the initiation of Isl1 expression and in the specification of Isl1$^+$ cardiac progenitors. Isl1$^+$ progenitors also are found in the posterior region of the developing cardiac mesoderm. Our recent studies have shown that *Wnt2*, a ligand expressed specifically in the posterior pole of the developing heart, is essential for the development and differentiation of posterior structures including the atria, pulmonary veins and atrioventricular canal (Y. Tian and E. Morrisey, manuscript submitted). Wnt2 regulates the proliferation and differentiation of Isl1 + progenitors and early cardiomyocytes within the posterior pole of the heart and does so through the β-catenin-dependent canonical Wnt pathway. Thus, Wnt/β-catenin signaling is important for both outflow and inflow tract development via its regulation of Isl1$^+$ SHF progenitor cell expansion and subsequent myocyte differentiation.

Wnt Signaling During Cardiac Early Differentiation: More Than One Way of Looking at It

Studies on chick and frog embryos suggest that the initial specification of cardiac tissue is governed by the balanced expression of canonical Wnt activators and repressors both within and outside the early mesoderm. Canonical Wnt signaling, through Wnt1 and Wnt3a expression in the anterior mesoderm, inhibits the expression of early cardiac genes in the cardiac crescent, including *Nkx2.5* and *GATA4* [44, 60]. Secreted Wnt antagonists, including *crescent* and *Dikkopf* (*Dkk*), are expressed in the endoderm underlying the cardiac mesoderm [60]. Moreover, Wnt expression in the dorsal neural tube blocks cardiogenesis in the adjacent paraxial mesoderm [75]. Supporting this balance in Wnt activity are data showing that forced expression of *crescent* or *Dkk* in the noncardiac posterior mesoderm induces cardiac gene expression and the appearance of beating cardiomyoctes [44, 60].

Additional data from experiments with *Xenopus* suggest that Wnt signaling inhibits cardiogenesis in some contexts. In *Xenopus,* findings have shown that Wnt11 expression is required for heart specification [15, 17]. Wnt11 is thought to promote cardiogenesis through its ability to inhibit β-catenin-dependent canonical signaling [11, 15, 17, 52]. Blocking Wnt11 signaling in the anterior mesoderm of *Xenopus* embryos blocks the expression of early cardiac genes including *Nkx2.5*, *GATA4,* and *Tbx5,* whereas expressing *Wnt11* in the posterior mesoderm of frog and chick embryos induces ectopic expression of these markers as well as the appearance of beating cardiomyocytes [15, 60].

In *Xenopus* animal pole explants, which normally take on a neuro-ectodermal fate, Wnt11 induces cardiac tissues without inducing the expression of pan-mesodermal markers, suggesting that the effect of Wnt11 on cardiac

specification is direct and not the result of increased mesoderm induction [52]. *Wnt11* expression similarly coincides with the onset of cardiac gene expression in differentiating embryonic stem cells, and treating these cells with recombinant Wnt11 increases the specification of cardiac progenitors, indicating that *Wnt11* also plays an essential role in murine heart induction [76].

Recent data have demonstrated a link between the important cardiac transcription factors Gata4, Gata6, and the Wnt-signaling pathway. These data show that Gata4 and Gata6 are required for specification of FHF progenitors in mice [90]. Studies in *Xenopus* have shown that the cardiac-promoting abilities of Wnt11 require Gata4 and Gata6 function, whereas β-catenin simultaneously inhibits Gata4 and Gata6 expression in early frog development [1].

Recently, our lab found that Wnt2 works collaboratively with Gata6 in a positive feed-forward loop to promote the proper differentiation and proliferation of posterior pole Isl1 + cardiac progenitors and early cardiac development (Y. Tian and E. Morrisey, manuscript submitted). The discrepant results highlight the differences in Wnt-signaling activity in FHF versus SHF progenitors and their derivatives. Moreover, these data highlight the differences in Wnt-signaling activity during cardiovascular development obtained with different model organisms, which is a critical point to consider in the interpretation of such data. Together, these data suggest that Wnt/β-catenin signaling plays an important role in the expansion and early differentiation of SHF Isl1 + progenitors but inhibits further differentiation of either SHF or FHF progenitors.

As described earlier, much of the discrepant roles for Wnt signaling in cardiogenesis could be reconciled by imposing a biphasic role for the pathway in which Wnt is procardiogenic in early precardiac mesoderm and inhibitory to cardiogenesis during the later stages of cardiac differentiation. This model is supported by data from multiple systems including embryonic stem cells and the developing zebra fish. The expression of canonical Wnt ligands and the activity of Wnt reporters are transiently increased in differentiating embryonic stem cells just before the expression of cardiac genes such as *Nkx2.5* and *GATA4* [50, 76]. Blocking canonical Wnt signaling during this early period of differentiation inhibits the expression of early cardiac markers and the appearance of beating cardiomyocytes [50]. In contrast, slightly later activation of Wnt/β-catenin leads to inhibition of cardiac differentiation [50].

In zebra fish, heat- and shock-inducible expression of both activators (*wnt8*) and inhibitors (*dkk1*) show that the temporal difference between Wnt activity promoting cardiogenesis and inhibiting it is as little as 1 h [76]. The possibility that canonical Wnt signaling plays an early positive role in cardiac induction is especially interesting in light of recent data that *Wnt2* is required for cardiac differentiation in embryonic stem cells. Wnt2 has been shown to activate canonical Wnt signaling in several contexts and is expressed in the early cardiac crescent [49]. *Wnt2*-deficient embryonic stem cells exhibit enhanced hematopoietic differentiation but decreased cardiac and endothelial cell differentiation [82]. Thus, *Wnt2* and possibly its homolog *Wnt2b* may play an important role in the specification of cardiac cell types from the early mesoderm [23, 49, 89].

These data suggest that canonical Wnt signaling plays a biphasic role in mouse cardiac induction, positively regulating cardiac gene expression early and inhibiting cardiac differentiation later. Such temporal specificity also helps to explain the discrepant activities of Wnt in cardiogenesis in different model systems. Thus, the precise temporal activity of Wnt/β-catenin activity would have to be controlled carefully if this pathway is ever to be harnessed for cardiovascular regenerative therapies.

Noncanonical Wnt Signaling in Cardiac Development

Growing evidence shows that noncanonical or β-catenin-independent Wnt signaling plays a role in SHF development. Two noncanonical Wnt ligands, Wnt5a and Wnt11, are expressed at the anterior pole of the heart as SHF cells migrate through on their way to the right ventricle, and mice homozygous for mutations in Wnt5a or Wnt11 have outflow tract defects consistent with those caused by disruption of the SHF. Both Wnt5a and Wnt11 promote cardiac differentiation in embryonic and adult stem cells through noncanonical pathways and may be necessary to balance β-catenin-dependent SHF proliferation in the outflow tract. However, our current understanding of the molecular and cellular mechanisms behind the effects of Wnt5a and Wnt11 on cardiac development is far from complete. The Wnt5a and Wnt11 alleles result in global loss of function, making it difficult to conclude that the effects of these mutations on the SHF are direct and not caused by the loss of paracrine signaling to adjacent cell types. This is especially important to consider in the interpretation of Wnt5a and Wnt11 mutants as the outflow tract defects observed are similar to those observed upon disruption of proper SHF development.

Furthermore, evidence suggesting that Wnt5a and Wnt11 act noncanonically in the SHF is largely composed of data from *in vitro* experiments, and this model remains to be tested genetically. Experiments using conditional alleles of noncanonical Wnt pathway components including Wnt5a and Wnt11 in the context of Wnt transgenic reporters are required to advance our understanding of noncanonical Wnt signaling in SHF development.

Other pathways including jun N-terminal kinase (JNK) and protein kinase C (PKC) are known to act downstream

of some β-catenin-independent Wnt signaling. Inhibiting either JNK or PKC signaling blocks the ability of Wnt11 to induce cardiac specification, whereas coactivating JNK and PKC induces cardiac specification, suggesting that both the RhoA/JNK and Ca^{2+}/PKC pathways mediate Wnt11 signaling [52]. These data indicate that the activation of noncanonical Wnt signaling by Wnt11 is required for the induction of cardiac tissues through JNK and PKC signaling.

Summary

A plethora of recent data has shown that Wnt/β-catenin signaling plays an important role in many stages of cardiovascular development including progenitor proliferation and myocyte differentiation. The use of multiple model systems has resulted in contradictory data in some cases, but recent studies in the pluripotential stem cell field and with zebra fish have helped to resolve these discrepancies and have highlighted a model in which Wnt/β-catenin is procardiogenic in the early precardiac mesoderm and inhibitory later in cardiac differentiation. Given the importance of Wnt/β-catenin signaling in promoting stem/progenitor cells from various sources, it will be important to determine whether regulation of this pathway can help to generate sufficient cardiac and vascular progenitors and their derivatives for therapeutic use in the future.

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