

# Wnt signaling: an essential regulator of cardiovascular differentiation, morphogenesis and progenitor self-renewal

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Emerging evidence indicates that Wnt signaling regulates crucial aspects of cardiovascular biology (including cardiac morphogenesis, and the self-renewal and differentiation of cardiac progenitor cells). The ability of Wnt signaling to regulate such diverse aspects of cardiovascular development rests on the multifarious downstream and tangential targets affected by this pathway. Here, we discuss the roles for Wnt signaling in cardiac and vascular development, and focus on the emerging role of Wnt signaling in cardiovascular morphogenesis and progenitor cell self-renewal.

## Introduction

Although its role in the differentiation of many cell types has been explored in detail, the role of Wnt signaling in cardiovascular morphogenesis and differentiation is less well understood. Previous studies have suggested a model in which  $\beta$ -catenin-dependent canonical Wnt signaling inhibits cardiac specification while Jun-N-terminal kinase (JNK)-dependent, non-canonical Wnt signaling promotes cardiovascular development. However, recent findings have indicated that canonical Wnt signaling has a more complex temporal role during cardiac differentiation than previously thought, with an activating role in specification and an inhibitory role in later cardiomyocyte differentiation (see Table 1).

Recent studies in mice have revealed that Wnt signaling has a complex array of functions in cardiovascular development, expanding the role for these pathways beyond cell specification and differentiation. These studies have shown that Wnt activity is a key regulator of cardiac stem/progenitor cell self-renewal and differentiation, and of the morphogenesis of the heart. In this review, we discuss how the many effectors of Wnt signaling, both  $\beta$ -catenin dependent and independent, regulate cardiovascular differentiation, morphogenesis and stem/progenitor cell self-renewal. Furthermore, similarities exist between mouse models of defective Wnt signaling and human congenital heart disease (CHD), suggesting exciting possibilities for future regenerative therapies.

## Cardiovascular development

The mouse heart develops from two domains of anterolateral mesoderm that fuse together at the anterior midline to form a crescent-shaped swathe of mesoderm that underlies the head folds and is known as the cardiac crescent (Fig. 1). The cells located at the lateral edges of the cardiac crescent migrate medially towards the ventral midline, where the opposing edges of the cardiac crescent meet and fuse together to form the initial heart tube. As the heart tube forms, cells within this primary or first heart field (FHF) differentiate into functional cardiomyocytes and the heart begins to beat.

Meanwhile, a second field of cardiogenic mesoderm, or second heart field (SHF), initially located medial to the edges of the cardiac crescent, is displaced dorsally into the pharyngeal mesoderm by the heart tube. As the heart tube undergoes rightward looping and forms distinct chambers, cells from SHF migrate into both the inflow and outflow tracts of the heart, and contribute most of the cells within the outflow tract and right ventricle, a substantial proportion of cells within the atria and some cells to the left ventricle (Kelly et al., 2001; Kelly and Buckingham, 2002).

The expression of several transcription factors, including GATA binding protein 4 (*Gata4*), NK2 transcription factor related 5 (*Nkx2.5*) and myocyte enhancer factor 2c (*Mef2c*), mark both the FHF and SHF (Kuo et al., 1997; Lin et al., 1997; Lyons et al., 1995; Molkentin et al., 1997). Additionally, the LIM-homeodomain gene islet 1 (*Isl1*) is expressed in the SHF and marks this group of cardiac progenitors as a distinct cell population (Cai et al., 2003). Although mutations in these genes cause severe heart defects, none of these factors appears to be solely required for the specification of cardiogenic mesoderm (Cai et al., 2003; Kuo et al., 1997; Lin et al., 1997; Lyons et al., 1995; Molkentin et al., 1997). Cardiac specification must therefore rely on a more complex network of interactions between these factors. These interactions are likely to be mediated by several families of secreted signaling molecules and their associated signaling pathways implicated in heart development. These include bone morphogenic proteins (BMPs), transforming growth factor  $\beta$  (TGF $\beta$ ) family members, fibroblast growth factors (FGFs) and, more recently, Wnt proteins.

## Wnt signaling: many components, multiple pathways

Wnt proteins are a large family of secreted signaling molecules that regulate crucial aspects of development, including cell-fate specification, proliferation, survival, migration and adhesion (for a review, see Nusse, 2005). Many of these effects are mediated by a canonical Wnt signaling pathway (Fig. 2A), which begins with Wnt proteins binding to a co-receptor complex that consists of frizzled (Fzd) family, seven-pass transmembrane proteins and the lipoprotein receptor related 5/6 (Lrp5/6) proteins. Wnt receptor binding activates the intracellular effector protein dishevelled (Dvl), which in turn inactivates a protein complex that includes the constitutively active serine-threonine kinase glycogen synthase kinase 3 $\beta$  (Gsk3 $\beta$ ), as well as the scaffolding proteins axin and adenomatous polyposis coli (APC). This complex normally phosphorylates  $\beta$ -catenin and targets it for degradation. Upon Wnt stimulation, however, the inhibition of the degradation complex allows high levels of  $\beta$ -catenin to accumulate in the nucleus, where it complexes with LEF/TCF family DNA binding proteins to activate the transcription of Wnt target genes.

While the canonical Wnt pathway mediates many Wnt effects, some Wnt regulated processes are  $\beta$ -catenin independent. This non-canonical Wnt signaling (Fig. 2B) is often attributed to one of two

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**Table 1. Role of various Wnt signaling components in cardiovascular development**

Gene	Phenotype	Pathway	Species	References
$\beta$ -Catenin	Required for postnatal hypertrophic growth (mouse), acts upstream of FGF signaling to regulate SHF progenitors (mouse), essential for cardiac valve development (zebrafish)	Canonical	Mouse, zebrafish	(Ai et al., 2007; Chen et al., 2006; Cohen et al., 2007; Hurlstone et al., 2003; Kioussi et al., 2002; Kwon et al., 2007; Liebner et al., 2004; Lin et al., 2007; Naito et al., 2006; Nakamura et al., 2003; Qu et al., 2007; Ueno et al., 2007; van de Schans et al., 2007; Wang et al., 2002)
<b>Wnt ligands</b>				
<i>Wnt2a</i>	Placental vascular failure	Canonical	Mouse	(Monkley et al., 1996)
<i>Wnt5a</i>	Cardiac outflow tract septation, endothelial proliferation and vessel branching in vitro	Non-canonical	Mouse	(Masckauchan et al., 2006; Schleifarth et al., 2007)
<i>Wnt7b</i>	Loss of pulmonary vascular smooth muscle integrity and hemorrhage	Canonical	Mouse	(Shu et al., 2002)
<i>Wnt9a</i>	Epicardial/coronary development	Unknown	Mouse	(Merki et al., 2005)
<i>Wnt11</i>	Cardiac specification (frogs, zebrafish), cardiac outflow tract development (mouse)	Non-canonical	Mouse, frogs, zebrafish	(Eisenberg and Eisenberg, 1999; Garriock et al., 2005; Schneider and Mercola, 2001; Terami et al., 2004; Zhou et al., 2007)
<b>Frizzled receptors</b>				
<i>Fzd4</i>	Retinal vascular development	Unknown	Mouse	(Toomes et al., 2004; Xu et al., 2004)
<i>Fzd5</i>	Placental vascular development	Unknown	Mouse	(Ishikawa et al., 2001)
<b>Dishevelled</b>				
<i>Dvl1</i>	Myofibroblast wound healing	Unknown	Mouse	(Chen et al., 2004)
<i>Dvl2</i>	OFT development	Unknown	Mouse	(Hamblet et al., 2002)
<i>Apc</i>	Cardiac valve development	Canonical	Zebrafish	(Hurlstone et al., 2003)
<i>Gsk3<math>\beta</math></i>	Positively regulates cardiomyocyte proliferation, required for postnatal hypertrophic growth	Canonical	Mouse	(Chen et al., 2006; Trivedi et al., 2007; Tseng et al., 2006)
<i>Vangl2</i>	OFT development	Non-canonical	Mouse	(Phillips et al., 2005)

Abbreviations: Apc, adenomatous polyposis coli; Gsk3 $\beta$ , glycogen synthase kinase 3 $\beta$ ; OFT, outflow tract.

pathways termed the Ca<sup>2+</sup>/protein kinase C (PKC) and RhoA/JNK pathways (for a review, see Veeman et al., 2003). In Ca<sup>2+</sup>/PKC signaling, Wnt binding activates the heterotrimeric G-protein-dependent activity of Fzd receptors, which in turn activate intracellular Ca<sup>2+</sup> signaling, as well as Ca<sup>2+</sup>-dependent protein kinases, such as protein kinase C (PKC) and calmodulin-dependent protein kinase II. In RhoA/JNK signaling, Wnt proteins activate Rho family GTPases, such as RhoA and Rac, as well as their downstream effectors, including Rho associated kinase (ROCK) and JNK through the Dvl protein. Although the Ca<sup>2+</sup>/PKC and RhoA/JNK pathways mediate most  $\beta$ -catenin-independent, non-canonical Wnt signaling, it is still unclear whether these pathways are distinct from one another, and the combinations of effectors that mediate this signaling often vary. Furthermore, activating non-canonical Wnt signaling attenuates canonical Wnt signaling in some systems (Pandur et al., 2002; Schneider and Mercola, 2001). This raises the intriguing possibility that these pathways are actually part of a larger Wnt signaling network in which unique combinations of effectors are activated in a cell type-dependent manner.

There are 19 Wnt proteins, 10 Fzd receptors and two Lrp co-receptors in mammals, suggesting that Wnt signaling specificity may be immensely complex. Wnt proteins have traditionally been separated into two classes based on their ability to induce secondary axis formation in frog embryos and to transform mammary epithelial cells, both of which rely on canonical Wnt signaling. For example, expression of *Wnt1*, *Wnt3* and *Wnt8* induce a secondary axis in *Xenopus* and transform mammary epithelial cells and are thought to signal through the canonical Wnt pathway, while expression of *Wnt5a* and *Wnt11* does not, and these ligands are thought to act

primarily through non-canonical Wnt pathways (Shimizu et al., 1997; Wong et al., 1994). However, more-recent studies suggest that individual Wnt proteins can activate either canonical or non-canonical Wnt signaling, depending on cellular context (Mikels and Nusse, 2006; Tu et al., 2007). Thus, the decision to signal through the canonical versus non-canonical pathway is likely to depend on the specific Wnt-Fzd combinations that are present at the cell surface, as well as on the intracellular factors that shunt signaling between the two pathways. This issue remains one of the most intriguing, but poorly understood, aspects of Wnt signaling, and has an enormous impact on our understanding of Wnt signaling in cardiovascular development, as discussed below.

### Wnt expression and function during cardiac specification and early differentiation

Studies on chick and frog embryos indicate that the initial specification of cardiac tissue is governed by the balanced expression of canonical Wnt activators and repressors (Fig. 3A). Activating canonical Wnt signaling in the anterior mesoderm inhibits the expression of early cardiac genes, including *Nkx2.5* and *Gata4*, in the cardiac crescent (Marvin et al., 2001; Schneider and Mercola, 2001). Canonical Wnts such as *Wnt1* and *Wnt3a*, which are expressed in the neural plate and dorsal neural tube, are therefore thought to inhibit cardiac specification in the posterior-medial mesoderm. Conversely, several secreted Wnt antagonists including *crescent*, which competes with Fzd for Wnt binding via its homology to the Fzd extracellular domain, and also *Dkkopf* (*Dkk*), which inhibits canonical Wnt signaling by causing Lrp5/6 receptor internalization, are expressed in the endoderm that underlies the cardiac mesoderm (Schneider and Mercola, 2001).

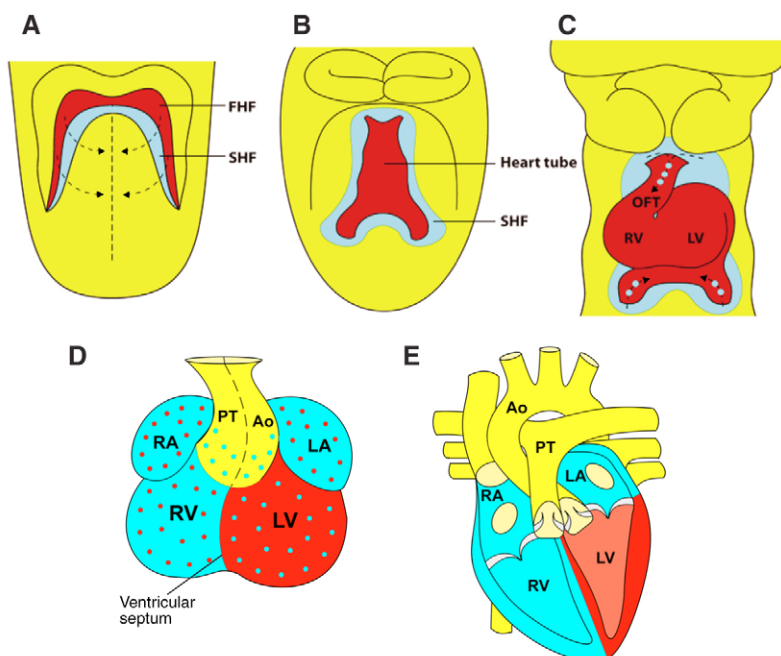
The expression of *crescent* or *Dkk* in the non-cardiac posterior mesoderm induces cardiac gene expression and the appearance of beating cardiomyocytes (Marvin et al., 2001; Schneider and Mercola, 2001). The inhibition of canonical Wnt signaling by *crescent* and *Dkk* is therefore thought to promote the specification of cardiac precursors in the anterolateral mesoderm that forms the cardiac crescent.

Experiments in mouse embryonic stem (ES) cells suggest that canonical Wnt signaling has a biphasic role in cardiac specification. The expression of canonical Wnt ligands and the activity of Wnt reporters are transiently increased in differentiating ES cells just prior to the expression of cardiac genes, such as *Nkx2.5* and *Gata4* (Naito et al., 2006; Ueno et al., 2007). Blocking canonical Wnt signaling during this early period of differentiation inhibits the expression of early cardiac markers and the appearance of beating cardiomyocytes (Naito et al., 2006). These data suggest that canonical Wnt signaling acts early to potentiate cardiac specification. Interestingly, the activation of canonical Wnt signaling slightly later during ES cell differentiation blocks cardiac induction and differentiation (Naito et al., 2006). Consistent with these data, the deletion of  $\beta$ -catenin in the definitive endoderm during mouse development leads to ectopic heart induction along the body axis (Lickert et al., 2002). These data indicate that the role for canonical Wnt signaling in early cardiogenesis is, at least in part, due to a cell autonomous role for Wnt signaling in the early endoderm. Although this early positive role for Wnt signaling in cardiac induction may be specific to mouse embryos, it is more likely that it was masked in previous studies in chick and frog embryos by the inability to temporally control the over-activation of canonical Wnt signaling in these experiments.

The possibility that canonical Wnt signaling plays an early positive role in cardiac induction is especially interesting in light of recent data that *Wnt2a* is required for cardiac differentiation in ES cells. *Wnt2a* has been shown to activate canonical Wnt signaling in several contexts and is expressed in the cardiac crescent (Monkley et al., 1996). *Wnt2a*-deficient ES cells exhibit enhanced hematopoietic differentiation at the expense of cardiac and endothelial cell types (Wang et al., 2007). Thus, *Wnt2a*, and possibly

its homologue *Wnt2b*, may play an important role in the specification of cardiac cell types from the early mesoderm. Although defects in heart development have not been reported in *Wnt2a* knockout mice, redundancy with *Wnt2b* or with other canonical Wnts, such as *Wnt8a*, which is expressed throughout the early heart tube (see Fig. 4A), may mask such effects in vivo (Jaspard et al., 2000; Monkley et al., 1996; Zakin et al., 1998). Taken together, these data show that canonical Wnt signaling plays a biphasic role in mouse cardiac induction, positively regulating cardiac gene expression early and then inhibiting cardiac differentiation at a later stage.

Non-canonical Wnt signaling by Wnt11 is also required for heart specification. *Wnt11* is expressed in the anterolateral mesoderm that is fated to become the cardiac crescent (Christiansen et al., 1996; Eisenberg and Eisenberg, 1999; Garriock et al., 2005; Pandur et al., 2002). Blocking Wnt11 signaling in the anterior mesoderm of *Xenopus* embryos blocks the expression of early cardiac genes, including *Nkx2.5*, *Gata4* and *Tbx5*, while 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 (Eisenberg and Eisenberg, 1999; Schneider and Mercola, 2001). 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 (Pandur et al., 2002). *Wnt11* expression similarly coincides with the onset of cardiac gene expression in differentiating mouse ES cells and treating these cells with Wnt11 increases the specification of cardiac progenitors, indicating that *Wnt11* also plays an essential role in murine heart induction (Ueno et al., 2007). Inhibiting either JNK or PKC signaling blocks the ability of Wnt11 to induce cardiac specification, while co-activating JNK and PKC induces cardiac specification, indicating that both the RhoA/JNK and Ca<sup>2+</sup>/PKC pathways mediate Wnt11 signaling (Pandur et al., 2002). Taken together, these data indicate that the activation of non-canonical Wnt signaling by Wnt11 is required for the induction of cardiac tissues through JNK and PKC signaling.



**Fig. 1. Cardiac development.** Mouse heart development at embryonic day (E) (A) 7.75–8.0, (B) E8.0–8.5, (C) E9.5 and (D) E12.5, and at (E) late embryonic/postnatal stages. Anterior is towards the top. (A–C) The myocardium of the heart develops from two populations of cells called the first heart field (FHF) (red) and a more medial region called the second heart field (SHF) (blue) that lie adjacent to each other at the cardiac crescent stage of development (E7.75–8.0, A). Broken line indicates the midline. (B) Lateral regions of the FHF migrate towards the ventral midline to fuse and form the primitive heart tube, while the SHF remains concentrated in the dorsal pharyngeal mesoderm. (C) Later, SHF cells (blue) migrate into the heart from both anterior and posterior regions, as noted by *Isl1* immunostaining and fate mapping with *Isl1*-cre mice (arrows). (D) By E12.5, the four chambers of the heart are well delineated and septation of the outflow tract is observed so that (E) by late postnatal stages, the outflow tract (OFT) is completely septated and both ventricular and atrial septation are complete in preparation for postnatal life. In D, broken line indicates the OFT septum/cardiac neural crest cells. Ao, aorta; LA, left atrium; LV, left ventricle; PT, pulmonary tract; RA, right atrium; RV, right ventricle.



### Wnt signaling in the specification and expansion of secondary heart field progenitors

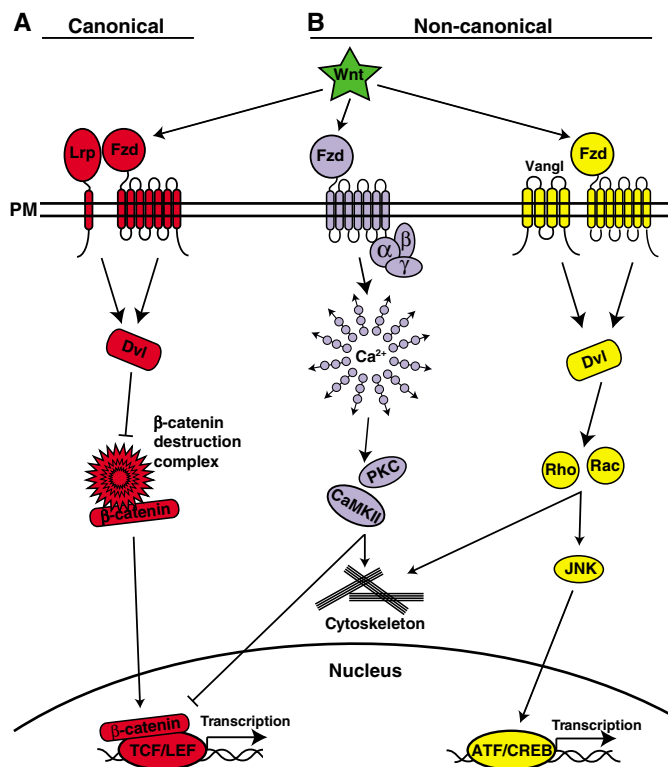
Several well-characterized mouse strains, TOPGAL, BAT-GAL and TCF/Lef-lacZ, contain transgenic reporters that express  $\beta$ -galactosidase from multimerized LEF/TCF DNA-binding sites and allow canonical Wnt signaling to be visualized in vivo (DasGupta and Fuchs, 1999; Maretto et al., 2003; Mohamed et al., 2004). These reporters are active during the development of multiple cardiac structures, including the pericardium, which surrounds the heart, the endocardial cushions, which differentiate into the atrio-ventricular (AV) valves, and the outflow tract (OFT) (Gitler et al., 2003; Maretto et al., 2003). Remarkably, none of these reporters displays high levels of activity in the developing myocardium, despite the expression of *Wnt8a*, a strong activator of the canonical Wnt pathway, and  $\beta$ -catenin throughout the developing heart tube (Cohen et al., 2007; DasGupta and Fuchs, 1999; Lin et al., 2007; Maretto et al., 2003).

Fate mapping experiments using the recently generated TOP-Cre mice, which contain a cre recombinase transgene under the control of LEF/TCF DNA-binding sites and a tamoxifen inducible

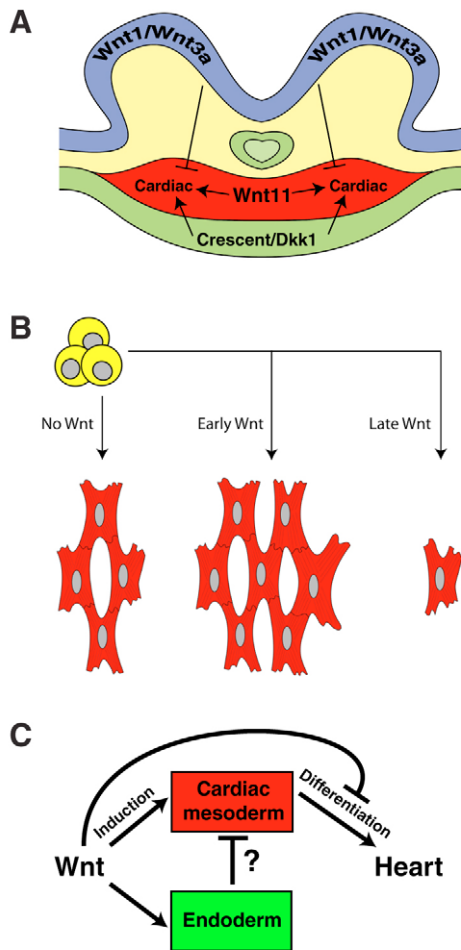
promoter, indicate that a significant proportion of the cells in the right ventricle (RV), OFT and atria receive canonical Wnt signaling prior to differentiating (Cohen et al., 2007). Consistent with these data, *Isl1* positive (*Isl1*<sup>+</sup>) cardiac progenitor cells from the SHF display high levels of BAT-GAL activity in both the pharyngeal mesenchyme and OFT as they migrate into the heart tube (Cohen et al., 2007). Moreover, loss of  $\beta$ -catenin expression in the SHF leads to a dramatic reduction in the numbers of *Isl1*<sup>+</sup> cardiac progenitor cells and the subsequent loss of SHF derived structures, while activating  $\beta$ -catenin signaling in the SHF leads to an increase in the numbers of *Isl1*<sup>+</sup> cells (Ai et al., 2007; Cohen et al., 2007; Kwon et al., 2007; Lin et al., 2007). Taken together, these data indicate that canonical Wnt/ $\beta$ -catenin signaling in the SHF and OFT plays an essential role in *Isl1*<sup>+</sup> cardiac progenitor cell expansion (Fig. 5).

Canonical Wnt/ $\beta$ -catenin signaling appears to regulate both the specification and the proliferation of *Isl1*<sup>+</sup> cardiac progenitor cells (reviewed by Laugwitz et al., 2008). The deletion of  $\beta$ -catenin in *Isl1*-cre mice, in which cre-recombinase is inserted into the *Isl1* locus, causes a dramatic reduction both in the levels of *Isl1* expression and the numbers of cells that express *Isl1* (Cohen et al., 2007; Lin et al., 2007). Consistent with this result, chromatin immunoprecipitation and in vitro reporter assays indicate that  $\beta$ -catenin directly binds to and regulates the *Isl1* promoter (Lin et al., 2007). These data strongly suggest that canonical Wnt/ $\beta$ -catenin signaling plays a role in the initiation of *Isl1* expression and in the specification of *Isl1*<sup>+</sup> cardiac progenitors. Additionally, deleting  $\beta$ -catenin in the heart with either the SM22 $\alpha$ -cre mouse line, in which cre is expressed in both myocardial and vascular smooth muscle cells, or the Nkx2.5-cre line, in which cre is expressed in myocardial cells, causes a reduction in the overall numbers of *Isl1*<sup>+</sup> cells without affecting the levels of *Isl1* expression (Cohen et al., 2007; Kwon et al., 2007). This effect appears to result from a dramatic reduction in the proliferation of *Isl1*<sup>+</sup> cells in the OFT, preventing the expansion of these progenitors (Ai et al., 2007; Cohen et al., 2007; Kwon et al., 2007; Lin et al., 2007). Canonical Wnt/ $\beta$ -catenin signaling therefore regulates both the specification and the proliferation of the *Isl1*<sup>+</sup> cardiac progenitors of the SHF.

The effect of  $\beta$ -catenin on the proliferation of *Isl1*<sup>+</sup> SHF cells is mediated, at least in part, by a reduction in FGF signaling. SHF cells express high levels of *Fgf10* when in the pharyngeal mesenchyme, as well as when in the OFT and right ventricle (Cohen et al., 2007). Although mutations in *Fgf10* do not cause defects in right heart structures, mutations in *Fgfr2*, the *Fgf10* receptor, cause severe OFT and right ventricle phenotypes, consistent with FGF10 acting redundantly with other FGF ligands (Marguerie et al., 2006). In support of this hypothesis, *Fgf3*, *Fgf16* and *Fgf20* are also induced by Wnt signaling in SHF explants (Cohen et al., 2007). Moreover, treating SHF explants with an inhibitor of FGF signaling blocks the expansion of *Isl1*<sup>+</sup> cells caused by the addition of purified Wnt3a to the culture media, suggesting that increased FGF signaling mediates the effects of Wnt signaling on SHF cells (Cohen et al., 2007). Thus, Wnt signaling acts upstream of a complex FGF-mediated pathway that includes the *Fgf3*, *Fgf10*, *Fgf16* and *Fgf20* genes. Moreover, several observations point to both *Fgf10* and *Fgf20* as being direct targets of Wnt/ $\beta$ -catenin signaling. In the case of *Fgf10*, its expression is strongly reduced in  $\beta$ -catenin mutant hearts, as are levels of ERK1/2 phosphorylation. Reporter constructs that contain the *Fgf10* promoter are responsive to  $\beta$ -catenin signaling, and chromatin immunoprecipitation assays indicate that  $\beta$ -catenin binds to the *Fgf10* promoter in vivo (Cohen et al., 2007). In the case of *Fgf20*, it has been shown to be a direct target of Wnt/ $\beta$ -catenin



**Fig. 2. Wnt signaling pathways.** (A) Canonical Wnt signaling (red) is mediated by secreted Wnt ligands (green) binding to a co-receptor complex that consists of Fzd and Lrp5/6 receptors. This activates the intracellular effector protein dishevelled (Dvl), which results in the stabilization and nuclear accumulation of  $\beta$ -catenin, leading to the activation of LEF/TCF-dependent transcription. (B) Non-canonical Wnt signaling involves at least two pathways: the Ca<sup>2+</sup>/protein kinase C (PKC) and RhoA/JNK pathways. In Ca<sup>2+</sup>/PKC signaling (purple), Wnt binding activates Fzd receptors causing G protein (G $\alpha$ , G $\beta$ , G $\gamma$ )-dependent Ca<sup>2+</sup> release. This activates PKC and calmodulin-dependent protein kinase II (CaMKII). In RhoA/JNK signaling (yellow), Wnt proteins activate Rho signaling, including Rho/Rac, and JNK through Dvl, leading to ATF/CREB activation. Non-canonical Wnt signaling often antagonizes  $\beta$ -catenin-dependent canonical signaling through mechanisms that remain poorly understood. PM, plasma membrane.



**Fig. 3. Wnt signaling in early cardiac specification and differentiation.** (A) Cross-section of the trunk of a post-gastrulation mouse embryo depicting how various Wnt ligands and Wnt antagonists impinge upon early cardiac mesoderm specification. Dorsal is uppermost. Green tube represents the notochord, the green bottom layer represents the early definitive endoderm, blue indicates the neuroectoderm, red indicates precardiac mesoderm and yellow represents splanchnic mesoderm. Wnt1 and Wnt3a are expressed in neuroectoderm, while Wnt11 and others, including Wnt2a and Wnt2b (not shown), are expressed in precardiac mesoderm. Wnt antagonists crescent and Dkk1 are expressed in the underlying definitive endoderm and inhibit canonical Wnt signaling in the precardiac mesoderm. (B) Studies in ES cells (yellow) show that activation of Wnt signaling prior to, or around the same time as, cardiac mesoderm specification results in increased cardiac differentiation of cardiac myocytes (red), whereas later Wnt signaling activation inhibits cardiac differentiation significantly (Naito et al., 2006). (C) A model showing how Wnt signaling is important for cardiac induction but is inhibitory to later cardiac differentiation. This control could be partly due to the effects of Wnt antagonists secreted from the underlying endoderm.

signaling by similar criteria, including its induction by exogenous Wnts, reduction in its expression through inhibition of Wnt signaling, and by the fact that  $\beta$ -catenin forms a complex on the *Fgf20* promoter (Chamorro et al., 2005). Wnt-FGF signaling is also required for zebrafish fin regeneration, indicating a wider use of such a signaling axis beyond that observed in the heart, in tissue regeneration and in the activation of resident stem/progenitor cells (Stoick-Cooper et al., 2007).

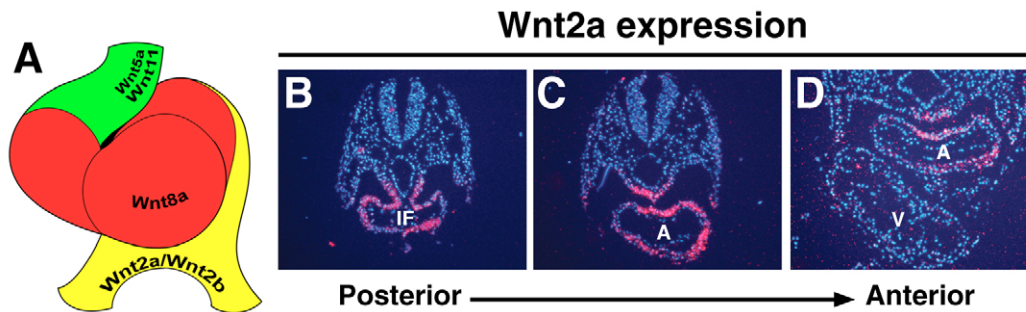
In addition to Wnt signaling promoting the expansion of *Isl1*<sup>+</sup> cardiac progenitors in vivo, Qyang et al. have shown that the activation of canonical Wnt signaling can dramatically expand *Isl1*<sup>+</sup> cardiac progenitors in vitro (Qyang et al., 2007a) (reviewed by Laugwitz et al., 2008). A small molecule screen was performed to identify compounds that could drive the expansion of *Isl1*<sup>+</sup> progenitors in vitro, which resulted in the identification of 6-bromoindirubin-3'-oxime, also known as BIO, a known Gsk3 $\beta$  inhibitor. Addition of BIO or of recombinant Wnt3a to the medium of purified *Isl1*<sup>+</sup> cardiac progenitors leads to their dramatic expansion. This finding suggests that it may be possible to harness canonical Wnt signaling to expand such progenitor cells either in vivo or ex vivo for tissue replacement, although the ability of expanded progenitors to fully differentiate into cardiomyocytes has not been tested. Along with a recent report in zebrafish that show that TOPGAL activity is activated at high levels in zebrafish heart regeneration (Stoick-Cooper et al., 2007), these studies provide some of the first clues as to how Wnt signaling may be harnessed as a potent regeneration pathway for the heart. However, whether Wnt signaling can activate cardiac regeneration in higher vertebrates is unknown and will require direct testing, most importantly in mouse models, as mammals do not exhibit the same ability for cardiac regeneration after injury as lower vertebrates.

### Non-canonical Wnt signaling in cardiac morphogenesis

In addition to its potential role in cardiac specification and progenitor expansion, non-canonical signaling by *Wnt11* plays a crucial role in cardiac morphogenesis. During *Xenopus* development *Wnt11R*, a second *Wnt11* gene found in lower vertebrates, is expressed in the precardiac mesoderm just prior to the fusion of the cardiac primordia at the ventral midline (Garriock et al., 2005). Inhibiting *Wnt11R* signaling by morpholino injection disrupts the fusion of the cardiac primordia at ventral midline resulting in cardia bifida. The overexpression of *Wnt11R* activates JNK in *Xenopus* embryos and the pharmacological inhibition of JNK results in a phenotype that is similar to the loss of *Wnt11R*. These data suggest that *Wnt11R* regulates cell movements that are important for the proper migration of early cardiac precursors to fuse and form the primitive heart tube through a JNK-mediated pathway.

Two non-canonical Wnt genes, *Wnt5a* and *Wnt11*, are expressed in the OFT of the developing mouse heart, and mutations in these genes cause OFT defects, including double outlet-right ventricle (DORV) and persistent truncus arteriosus (PTA), which are identical to some of the most common forms of human CHD (Schleiffarth et al., 2007; Zhou et al., 2007). The *Wnt5a* and *Wnt11* mutant mouse phenotypes are associated with disrupted cytoskeletal architecture in both the myocardial and smooth muscle components of the OFT, as well as reductions in matrix deposition and in the expression of matrix adhesion receptors. Interestingly, *Wnt11* positively regulates TGF $\beta$ 2 expression in the OFT through the JNK-dependent activation of activating transcription factor/cAMP responsive element binding protein (ATF/CREB) family transcription factors (Zhou et al., 2007). Mutations in *Tgfb2* cause OFT phenotypes similar to those caused by the loss of *Wnt5a* and *Wnt11* function, suggesting that the effects of non-canonical Wnt signaling on cardiac morphogenesis are mediated, at least in part, by Tgf $\beta$ 2 signaling (Bartram et al., 2001).

Other components of non-canonical Wnt signaling have demonstrated roles in cardiovascular morphogenesis. Van Gogh/strabismus is a cell surface transmembrane protein that has a key role in the planar cell polarity (PCP) pathway in *Drosophila*



**Fig. 4. Expression of Wnt ligands during cardiac morphogenesis.** (A) Several Wnt ligands are expressed during early cardiac morphogenesis, as depicted at E9.5. This includes Wnt8a, which is expressed throughout the heart (red) (Jaspard et al., 2000; Kwon et al., 2007), Wnt2a and Wnt2b, which are expressed in the inflow tract and atria during development (yellow) (Monkley et al., 1996; Zakin et al., 1998), and Wnt5a and Wnt11, which are expressed in the outflow tract (green) (Schleifarth et al., 2007; Zhou et al., 2007). (B-D) In situ hybridization shows the expression of Wnt2a (red) in a posterior-to-anterior gradient in an E9.5 mouse heart, with highest expression in the developing inflow tract (IF) and atria (A). V, ventricle.

(Wolff and Rubin, 1998). Mice have two Van Gogh/strabismus genes (*Vangl1* and *Vangl2*), and a naturally occurring *Vangl2* mutant, called the *loop-tail* (*Lp*) mouse, exhibits defects in OFT development that leads to DORV (Henderson et al., 2006; Phillips et al., 2005). In *Lp* mice, this defect is associated with aortic arch abnormalities, including persistent right-sided aorta. Phillips et al. showed that the myocardial cells in the OFT of *Lp* embryos have less extensive lamellipodia, indicating that these cells have defective cell-cell and cell-ECM interactions (Phillips et al., 2005). Given the importance of the PCP pathway in cell migration and polarity, these data suggest that *Vangl2* plays an important role in cardiac OFT development through the regulation of cell migration and cell-ECM interactions. Whether the OFT defects observed in *Wnt5a*, *Wnt11* and *Vangl2* mutants are due to the expression of these genes in the neural crest or in the SHF-derived myocardium is unknown and will require the conditional inactivation of these genes in mice for clarification. However, the defects observed in mice that carry mutations in these genes strongly implicate non-canonical Wnt signaling, and possibly the PCP pathway, in OFT development.

### Wnt signaling in cardiac neural crest

In addition to the myocardium, Wnt signaling has been implicated in the development of cardiac neural crest cells (CNCCs). CNCCs delaminate from the dorsal neural tube and migrate first into the pharyngeal arches and, second, along the arch arteries into the OFT. These cells form the initial OFT septum, become the smooth muscle layer of the ascending aortic arch and contribute to the OFT cushion mesenchyme. Two canonical Wnt genes, *Wnt1* and *Wnt3a*, are expressed in the dorsal region of the neural tube from which the CNCCs arise and have been implicated in CNCC specification and delamination. Furthermore, mutations in *Dvl2* cause OFT defects that resemble those caused by the ablation of the CNCCs, such as DORV and PTA, and the loss of CNCC-specific markers (Hamblet et al., 2002). Consistent with these results, the tissue-specific deletion of  $\beta$ -catenin from CNCCs results in OFT defects similar to those seen in *Dvl2* mouse mutants, as well as a severe decrease in CNCC proliferation (Kioussi et al., 2002).

The effects of canonical Wnt/ $\beta$ -catenin signaling on CNCCs may be mediated by both the transcriptional upregulation and functional activation of paired-like homeodomain 2 (*Pitx2*). *Pitx2* expression is lost in both *Dvl2* mutant mouse embryos and in embryos in which  $\beta$ -catenin has been conditionally deleted in the CNCCs with Wnt1-

cre. The complete knockout of *Pitx2* results in OFT defects that are similar to those seen in these mutants (Kioussi et al., 2002). Furthermore,  $\beta$ -catenin complexes with *Pitx2* and activates *Pitx2* target gene transcription in a manner that is analogous to its activation of LEF/TCF. Deleting a floxed-allele of *Pitx2* with Wnt1-cre does not result in OFT phenotypes, whereas deleting *Pitx2* in the SHF with either *Isl1*-cre or *Mef2c*-cre, both of which drive the expression of cre in the SHF, results in OFT defects that are identical to those reported in the *Dvl2* knockout mouse or following the CNCC-specific deletion of  $\beta$ -catenin (Ai et al., 2006). These data indicate that *Pitx2* has an autonomous role in SHF development and that its role in the CNCCs is mediated by paracrine signaling between these two closely apposed cell populations. How this signaling occurs is unclear but recent evidence shows that *Pitx2* is required for *Wnt11* expression in the OFT, indicating that the effects of *Pitx2* deletion are mediated by paracrine Wnt11 signaling from the SHF cells to the CNCCs (Zhou et al., 2007). Alternatively, the cell-cell adhesion function of  $\beta$ -catenin might be responsible for some aspect of these defects (Luo et al., 2006). This is supported by the phenotype of mice in which N-cadherin has been deleted in the CNCCs, which results in similar OFT defects.

### Wnt and $\beta$ -catenin in adult myocardium

As mentioned above, there is little evidence that active canonical Wnt signaling occurs in the primary developing or adult myocardium. This view is based primarily on studies that have used the TOPGAL and BAT-GAL reporter mice, and it contrasts with several reports of Wnt ligand expression being present in the adult mammalian heart (Garriock et al., 2005; Jaspard et al., 2000; Monkley et al., 1996; Zakin et al., 1998). Other studies have suggested an important role for  $\beta$ -catenin and Gsk3 $\beta$  in postnatal myocardial growth (Hardt and Sadoshima, 2002; Masuelli et al., 2003; Tseng et al., 2006). These studies have, for the most part, not invoked a Wnt ligand and are based on the role of Gsk3 $\beta$  in stabilizing  $\beta$ -catenin, leading to increased LEF/TCF activation. Stabilized  $\beta$ -catenin in isolated cardiac myocytes or in vivo results in increased myocyte growth with or without the presence of hypertrophic stimuli (Haq et al., 2003; Tseng et al., 2006). This stabilization of  $\beta$ -catenin is thought to occur in the heart through a protein kinase B/Akt-dependent pathway and not through Wnt activation. Additional studies have demonstrated that the inhibition of LEF/TCF-dependent transcription through the expression of a dominant-negative LEF protein or through the



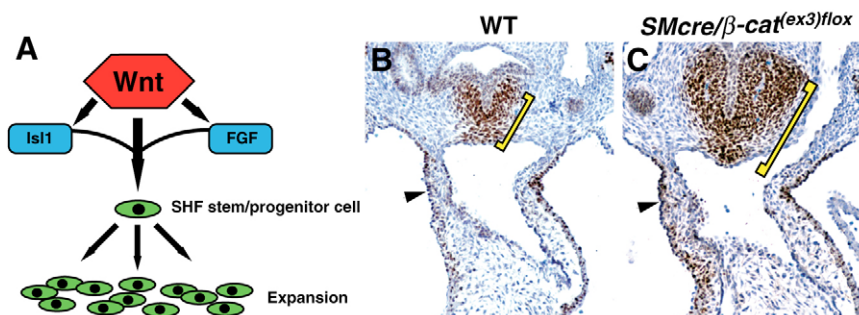
postnatal deletion of  $\beta$ -catenin results in decreased cardiac growth and in the loss of the hypertrophic response that usually occurs after the surgical constriction of the aorta (a technique called aortic banding) (Chen et al., 2006). These studies are supported by recent findings that the treatment of mammalian cardiomyocytes with the Gsk3 $\beta$  inhibitor BIO leads to their increased proliferation in culture (Tseng et al., 2006). From these studies, Gsk3 $\beta$  is implicated as being an integration point for cardiomyocyte growth and hypertrophy in response to stress. The question remains: do any of the other pathways known to regulate Gsk3 $\beta$  activity stabilize  $\beta$ -catenin in the heart? Recent evidence suggests that histone deacetylase 2 (Hdac2) can inhibit Gsk3 $\beta$  through the regulation of an inositol polyphosphate-5-phosphatase and Akt dependent pathway (Trivedi et al., 2007). Although this study did not look at  $\beta$ -catenin stabilization, it would be worth determining whether changes in cardiac growth and whether stress-induced hypertrophy can be correlated with altered  $\beta$ -catenin signaling. Together, these studies indicate that the activation of Gsk3 $\beta$ / $\beta$ -catenin signaling is important for normal cardiomyocyte growth and proliferation, but they do not directly implicate Wnt signaling per se. The failure to implicate a Wnt ligand directly in this physiological process could be the result of the significant redundancy that exists between the Wnt ligands that are expressed in both the developing and postnatal heart, the limitations of the TOPGAL and BAT-GAL Wnt reporter mice, or the fact that  $\beta$ -catenin/LEF-TCF signaling is affected by other non-Wnt signals, such as Akt. More extensive genetic experiments using Wnt-ligand or frizzled-receptor loss-of-function models, particularly in mice, will be required to determine whether Wnt signaling is directly involved in these processes.

The presence of multiple Wnt ligands and frizzled receptors expressed in the heart, the absence of active canonical Wnt signaling, and the importance of non-canonical Wnt signaling in the developing myocardium raises the intriguing possibility that the non-canonical pathway antagonizes canonical Wnt signaling during cardiac development. This would explain, at least in part, the absence of active canonical signaling in the developing (and postnatal) heart, while at the same time several Wnt ligands are being expressed. Future studies where non-canonical pathways have been inactivated in genetic backgrounds that contain Wnt reporter transgenes will be very useful in determining whether this hypothesis is correct.

### Wnt signaling in endothelial and vascular smooth muscle cells

Evidence for an *in vivo* role for Wnt/ $\beta$ -catenin signaling in vascular development and remodeling has been reported. Deletion of  $\beta$ -catenin in developing endothelial cells (EC) leads to embryonic death and loss of ECs integrity (Cattellino et al., 2003). Although the authors of this report implicated  $\beta$ -catenin in cell-cell adhesion, rather than in Wnt signaling in developing EC in the peripheral vasculature, part of the phenotype could be attributed to a loss of canonical Wnt signaling, even though TOPGAL activity is not apparent in these cells. EC-specific loss of  $\beta$ -catenin also leads to defective endocardial cushion/cardiac valve development through defective endothelial-mesenchymal transformation, and this process coincides with a decrease in TOPGAL reporter activity in the developing endocardial cushions (Liebner et al., 2004). Several Wnt ligands have been implicated in regulating EC development. *In vivo* loss of *Wnt2a* in mice leads to defective placental vascular development, with a reduced number of fetal-derived placental capillaries (Monkley et al., 1996). Moreover, *Wnt2a* mutant ES cell lines show impaired endothelial maturation and vascular plexus formation (Wang et al., 2007). Interestingly, loss of *Fzd5* also leads to defective placental vascular development and embryonic lethality (Ishikawa et al., 2001). Synergy between *Wnt2a* and *Fzd5* in ectopic axis induction assays in *Xenopus* embryos supports the notion that this ligand-receptor pair cooperatively regulate EC development (Ishikawa et al., 2001). *Wnt7b* has been implicated in apoptosis-mediated retinal EC regression associated with postnatal development (Lobov et al., 2005). Interestingly, the source of *Wnt7b* in this system is circulating macrophages, indicating the existence of a precise short-range paracrine mechanism for Wnt-mediated regulation of retinal EC development. *Wnt5a* has also been shown to regulate angiogenesis and to induce endothelial cell proliferation via the non-canonical pathway (Masckauchan et al., 2006). In the same report, the authors showed that inhibition of *Wnt5a* expression leads to reduced capillary branching in matrigel assays. Together, these data support an important role for Wnt signaling in endothelial cell development with the functional role dictated by the endothelial bed and by the Wnt ligands that are expressed.

A role for Wnt signaling in the developing epicardium, where progenitors for the coronary vasculature reside, has also been demonstrated. The contribution of the epicardium to the developing coronaries has been substantiated using fate-mapping analysis with a promoter fragment from the chicken *GATA5* gene driving cre



**Fig. 5. Wnt signaling regulates expansion of Isl1<sup>+</sup> SHF progenitors through regulation of Isl1 and FGF signaling.** (A) Multiple reports have demonstrated that Wnt/ $\beta$ -catenin signaling (red) is required upstream of Isl1 and FGF signaling to promote expansion of SHF stem/progenitor cells (green) (Ai et al., 2007; Cohen et al., 2007; Kwon et al., 2007; Qyang et al., 2007b). (B, C) Expression of an activated  $\beta$ -catenin protein in the secondary heart field (SHF) using the SM22 $\alpha$ -cre transgenic line leads to a dramatic increase in the number of Isl1<sup>+</sup> SHF progenitors in the anterior pharyngeal mesoderm (bracket) and the outflow tract of the mouse embryonic heart (arrowheads). (B, C) Reproduced, with permission, from Cohen et al. (Cohen et al., 2007).

expression (Merki et al., 2005). Merki et al. showed that loss of retinoid X receptor  $\alpha$  specifically in the developing epicardium resulted in decreased  $\beta$ -catenin protein stability and correlates with decreased *Wnt9b* expression (Merki et al., 2005). This leads to a failure in epicardial development and to the loss of ventricular compaction and to defective coronary vascular branching. Whether Wnt9a is the critical ligand in this process or acts redundantly with other Wnts awaits in vivo confirmation. This study reiterates the importance of Wnt signaling in epithelial-mesenchymal transformation, as in the developing heart valves mentioned above, and supports the notion that epicardial-derived paracrine signals, including Wnt, are of crucial importance for myocardial development.

As mentioned above, active Wnt signaling can be observed in VSMCs of the OFT during early development. However, what the roles, if any, of Wnt signaling are in VSMC development in other tissues remains relatively unknown. Some clues come from a study that shows that loss of paracrine Wnt7b signaling leads to defective VSMC development in the lung (Shu et al., 2002). *Wnt7b* is expressed at high levels in the developing airway epithelium during development but is absent from mesenchymal cell types in the lung, including the vasculature. *Wnt7b<sup>lacZ-/-</sup>* mutants develop abnormally large blood vessels during embryogenesis, and at birth, when pulmonary vascular pressure increases dramatically, these mutants display profound vessel rupture and hemorrhage around these abnormal vessels (Shu et al., 2002). Subsequent studies to determine the mechanism of *Wnt7b* signaling revealed that Wnt7b signals via the canonical pathway preferentially through Fzd1 and Fzd10 and the co-receptor Lrp5 (Wang et al., 2005). Wnt7b did not activate non-canonical JNK signaling in these experiments, although this ligand has been reported to activate other aspects of non-canonical Wnt signaling, including protein kinase C  $\delta$  (PKC $\delta$ )-dependent pathways (Tu et al., 2007). This again reiterates the importance of experimentally determining the mechanism and downstream effectors of a given Wnt ligand in the specific cell type of interest.

In VSMCs, Wnt signaling appears to regulate cell proliferation. Quasichka et al. found that inhibition of canonical Wnt signaling resulted in decreased VSMC proliferation and *cyclin D1* expression, while increasing the expression of the cyclin dependent kinase inhibitor *p21* (Quasichka et al., 2006). *Myc*, a proliferation-associated Wnt target gene, is overexpressed in proliferating medial and intimal smooth muscle cells in human stenotic bypass grafts (Hilker et al., 2001). Given that *cyclin D1* and *Myc* are well known targets of canonical Wnt signaling, these studies suggest that Wnt signaling acts to regulate cell proliferation in VSMCs during development and possibly after injury. Other studies that show canonical Wnt signaling is activated after rat carotid vessels are injured support this hypothesis and suggests that Wnt signaling could become a target of anti-angiogenic or anti-restenotic therapies (Wang et al., 2002).

## Conclusion

Wnt signaling has been known for many years to be one of the primary pathways that is involved in directing embryonic development. Given its crucial role in controlling proliferation and differentiation in other tissue systems, the finding that Wnt signaling regulates similar processes in the various cell types of the vertebrate cardiovascular system is at first glance not surprising. However, it is becoming increasingly clear that Wnts do not simply promote these processes but rather regulate and fine tune them in a cell-type- and temporally-specific manner.

One of the most important unanswered questions that remain concerning Wnt signaling in cardiac development is what function does canonical Wnt signaling serve in the primary myocardium during development? Given the high-level expression of *Wnt2a*, *Wnt2b* and *Wnt8a* in the developing mouse heart, it will be important to determine through genetic loss-of-function techniques whether any of these individually or in combination regulate cardiac differentiation or morphogenesis. Much has been gained from the genetic ablation of  $\beta$ -catenin but interpreting the findings that arise from this approach is complicated by the multiple roles that  $\beta$ -catenin has in cell-cell adhesion and Wnt signaling. The role of non-canonical versus canonical Wnt signaling and whether non-canonical signaling antagonizes canonical signaling in the developing myocardium are also concepts that need to be addressed directly using in vivo loss-of-function mouse models. Given the expression of multiple components of non-canonical Wnt signaling in the myocardium (such as RhoA, Daam1, etc.), one reason why active canonical Wnt signaling is not observed in the developing myocardium could be the presence of high levels of non-canonical Wnt signaling.

Recent evidence that Wnt/ $\beta$ -catenin signaling drives the expansion of Isl1<sup>+</sup> cardiac progenitors and is activated during zebrafish heart regeneration also underline the importance of this pathway in stem cell biology and tissue regeneration (Stoick-Cooper et al., 2007). It is no longer speculative to assert that Wnt signaling is a key pathway in the regenerative process in many tissues, including the heart. An important challenge in the future will be to determine when the activation of Wnt signaling could promote cardiac regeneration and which cardiac stem/progenitor cells of the many types reported in the literature are responsive to Wnt signaling. To advance the field significantly, it is essential that such studies include the further analysis of potential cardiac stem/progenitor cell populations using in vivo fate-mapping techniques to determine whether these cells can indeed generate new myocardium.

The re-activation of  $\beta$ -catenin signaling after cardiac stress and vascular injury also suggests that this pathway may provide an important new therapeutic area for intervention in human cardiovascular diseases as diverse as heart failure and re-stenosis after vascular injury. Wnt signaling is clearly a major cause of certain colon and intestinal tumors and probably plays a role in tumorigenesis in other tissues, including the skin (Chan et al., 1999; Korinek et al., 1997; Morin et al., 1997). Along with this attention has come increasing interest in identifying pharmacological antagonists and agonists of Wnt signaling. These compounds, which are being developed as anti-cancer therapies, could provide unique opportunities to address the enormous clinical impact of cardiovascular disease.

Studies into the paradigm that the response to injury in adult tissues leads to the reactivation of embryonic gene expression programs required for tissue repair, has revealed that some of these important signaling pathways, including the Wnt pathway, are valuable targets for future medicines. It should come as no surprise if in the next decade we see novel therapies directed towards both cancer and cardiovascular disease based on modulating Wnt signaling. However, these studies need to include the best of in vivo genetic work in the mouse coupled with exacting biochemical analysis of how Wnt signaling promotes important developmental processes required for tissue repair. Only then will the fruits of basic research be translated into the discovery of treatments for human ailments.



The authors thank members of the Morrissey laboratory for discussion and input during the process of writing this review. E.E.M. is supported by funding from the National Institutes for Health and the American Heart Association. E.D.C. is supported by a postdoctoral fellowship grant from the American Heart Association.

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