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REVIEW

Wnt Signaling in the Heart Fields: Variations on a Common Theme

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Wnt signaling plays an essential role in development and differentiation. Heart development is initiated with the induction of precardiac mesoderm requiring the tightly and spatially controlled regulation of canonical and noncanonical Wnt signaling pathways. The role of Wnt signaling in subsequent development of the heart fields is to a large extent unclear. We will discuss the role of Wnt signaling in the development of the arterial and venous pole of the heart, highlighting the dual roles of Wnt signaling with respect to its time- and dosage-dependent effects and the balance between the canonical and noncanonical signaling. Canonical signaling appears to be involved in retaining the cardiac precursors in a proliferative and precursor state, whereas noncanonical signaling promotes their differentiation. Thereafter, both canonical and noncanonical signaling regulate specific steps in differentiation of the cardiac compartments. Because heart development is a contiguous, rather than a sequential, process, analyses tend only to show a single timeframe of development. The repetitive alternating and reciprocal effect of canonical and noncanonical signaling is lost when studied in homogenates. Without the simultaneous in vivo visualization of the different Wnt signaling pathways, the mechanism of Wnt signaling in heart development remains elusive. *Developmental Dynamics 245:294–306, 2016*. © 2015 Wiley Periodicals, Inc.

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WNT Signaling Pathway Mechanisms

The Wingless-related integration site molecule (Wnt) signaling pathway is an evolutionary ancient and conserved pathway that is implicated in cellular signaling during development of both vertebrates and invertebrates (for overview see: Hoppler and Moon, 2014). Wnt signals are required in diverse crucial processes, like gastrulation (Haegel et al., 1995; Hardy et al., 2008), and in cell fate specification in various tissues, like the central nervous system and limbs (Haegel et al., 1995; Lescher et al., 1998; Galceran et al., 1999). Wnt signal proteins constitute a conserved family of secreted lipid-modified glycoproteins. Most of the secreted Wnt proteins are bound to extracellular matrix glycosaminoglicans, suggesting that they act as short-range autocrine and paracrine signaling molecules (Reichsman et al., 1996; Lescher et al., 1998). The principal Wnt receptors in the cell membrane are the Frizzled receptors (Fzd) (Nusse, 2005). Fzd receptors belong to the superfamily of G-protein-coupled receptors. The Fzd receptor proteins encompass a N-terminal signal sequence, an extracellular domain of 120 conserved amino acids with 10 invariable spaced cysteine residues (the CRD domain), a seven-pass transmembrane region, and a cytoplasmic tail (Dann et al., 2001). Binding of Wnt ligands to the extracellular CRD domain of FZD receptors triggers signaling by activating intracellular signal transduction pathways (recently reviewed by Hoppler and Nakamura, 2015). Wnt signals have been shown to activate several different pathways; the Wnt/β-catenin or canonical pathway and several β-catenin independent or noncanonical pathways, among which the Wnt/planar cell polarity (PCP) pathway and the Wnt/Ca^{2+} pathway (Komiya and Habas, 2008; Cadigan and Peifer, 2009) (Fig. 1).

To activate the Wnt/β-catenin pathway, the signaling receptor complex not only requires the Wnt-Fzd complex but also the co-receptor low density lipoprotein receptor-related protein 5 or 6 (Lrp5/6). When Wnts are not activating the receptor complex, β-catenin is phosphorylated in the multiprotein destruction complex, which comprises Axin, Adenomatosis Polyposis Coli (Apc), casein kinase 1 (Ck1), and glycogen synthase kinase 3β (Gsk3β), and is degraded (Fig. 1A). When Wnt activates the receptor complex, Dishevelled (Dvl) is recruited to the intracellular domain of the Fzd receptor, which in turn recruits Axin and other components of the destruction complex. As a result of inter-protein phosphorylation events within this multiprotein complex, the function of the negative regulators changes, allowing the accumulation of hypo-phosphorylated β-catenin in the cytoplasm. Hypo-phosphorylated β-catenin translocates into the nucleus to

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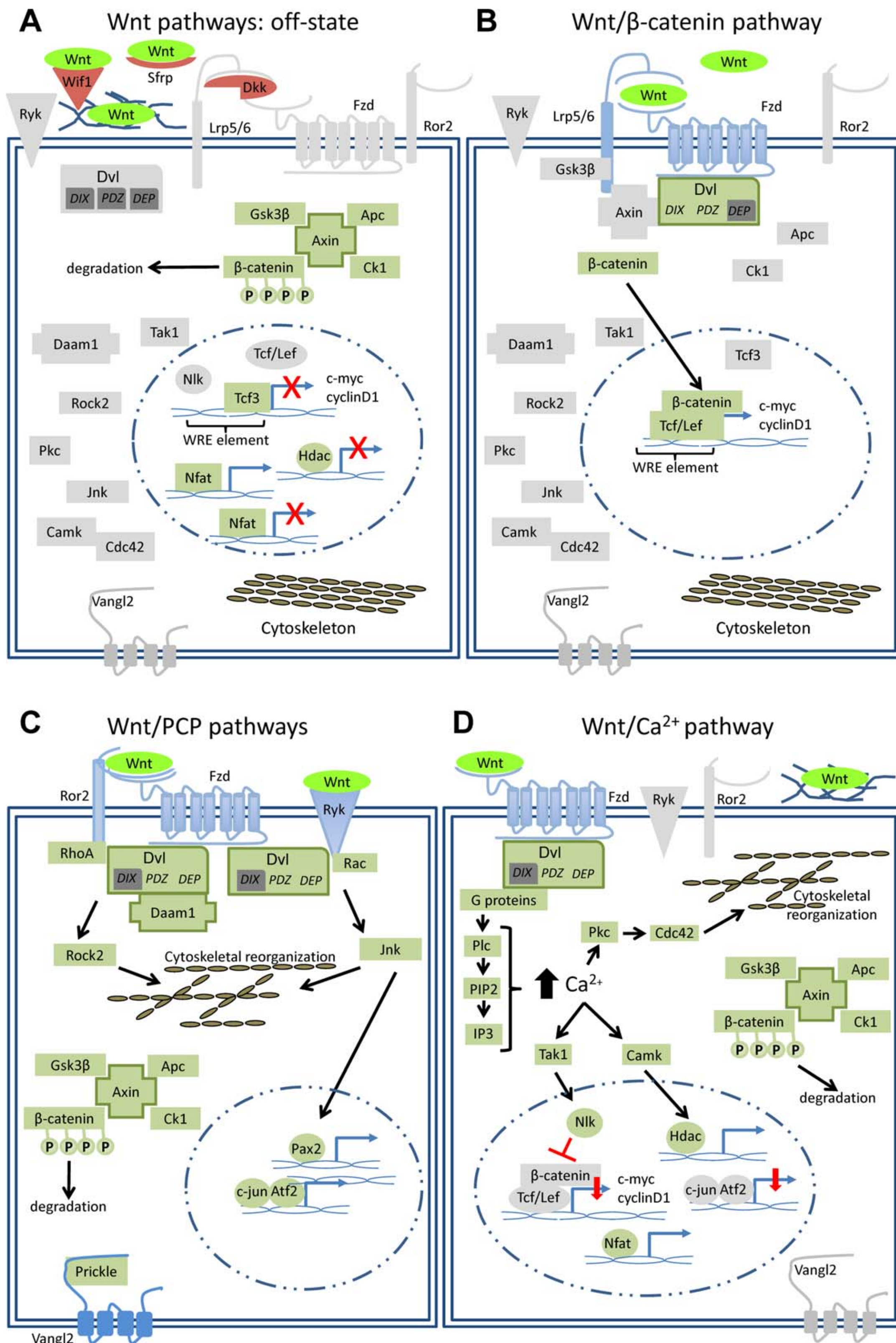

Fig. 1. Schematic representation of Wnt signaling pathways. **A**: All Wnt signaling pathways are in the off-state. Wnt ligands (lime) are bound by the extracellular matrix or inhibitors (red) preventing receptor activation. The components that are inactive are indicated in light grey and the intracellular components that are active in light green. As a result the destruction complex, comprising Axin, Gsk3β, Apc, Ck1, and β-catenin, is active and phosphorylated β-catenin is degraded. The active domains of Dvl1 are indicated in dark grey to indicate that they are inactive. **B**: The active Wnt//β-catenin signaling pathway. The interaction between Wnt ligands and the receptor complex of Fzd and LRP5/6 (light blue) activates canonical Wnt/β-catenin signaling pathway by means of the activation of the DIX and PDZ domains of Dvl. Axin is removed from the destruction complex, β-catenin translocates to the nucleus and affects downstream transcription. **C**: The Wnt/PCP pathway. The interaction between Wnt ligands and the receptor complex of Fzd and Ror2 or Ryk activates by means of the PDZ and DEP domains of Dvl down-stream signaling. The complex of Wnt, Fzd, Ror2, Dvl, and RhoA induces cytoskeletal changes by means of activation of Rock2 in cooperation with Daam1. The complex of Wnt, Fzd, Ryk, Dvl1, and Rac also induces cytoskeletal changes but in this case by means of Jnk. Jnk, furthermore, activates transcription of downstream targets by means of Pax2, cJun and Atf2. **D**: The active Wnt/Ca^{2+} pathway. The interaction between Wnt ligands and Fzd receptors results in an intracellular accumulation of calcium (Ca^{2+}) mediated by means of Dvl1. The PDZ and DEP domains of Dvl convey the signal by means of G proteins and the Plc signaling cascade. The increased intracellular calcium induces cytoskeletal change through Pkc and Cdc42, as well as through Tak1 and Camk, affecting transcription levels of downstream targets.

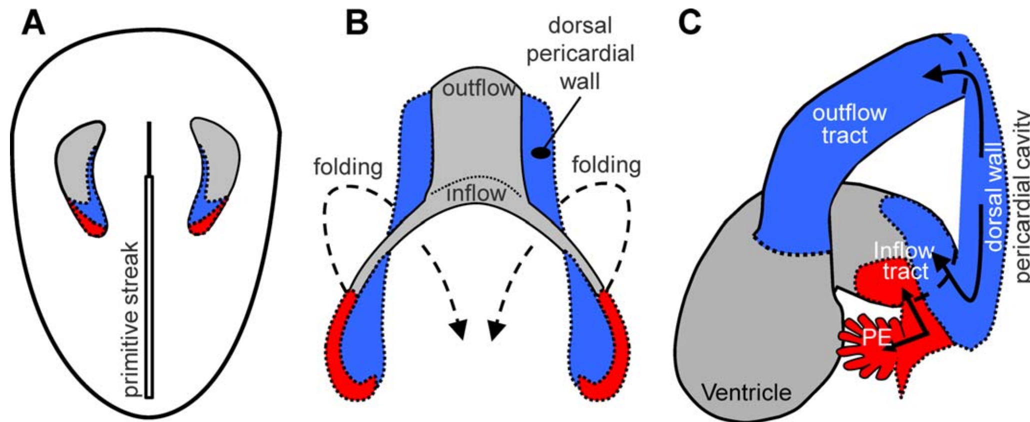

Fig. 2. Contribution of the heart fields to the forming heart in chicken. **A**: A schematic representation of the location of the heart fields within the heart forming region based on lineage analyses (approximately 20 hr of incubation, HH stage 5). The first (grey), second (blue) and third (red) heart field are indicated. The borders of the second and third heart fields are indicated with a dotted line as they have not yet been clearly defined. **B**: Due to the process of folding of the embryo the bilateral heart forming regions are brought together at the ventral midline of the embryo (approximately 40 hr of incubation; HH stage 10). **C**: After folding of the embryo and cardiac looping the second heart field cells are located within the dorsal wall of the pericardial cavity and the third heart field cells are located posterior of the forming heart. The second heart field cells contribute cardiomyocytes to the outflow and inflow of the heart. The third heart field cells contribute cardiomyocytes to the inflow of the heart and to the proepicardium (approx. 60 hours of incubation, HH stage 16).

activate transcription of downstream targets together with the transcription factors T cell factor (Tcf) and lymphoid enhancer factor (Lef) (Arnold et al., 2000; Logan and Nusse, 2004; Nusse, 2005; Cadigan and Peifer, 2009) (Fig. 1B). The DNA-binding element found in the genome that binds this transcription complex is referred to as the Wnt-response element (WRE). The conserved sequence comprises two sites, the CTTTGA core site and the GC-rich helper sequence (reviewed in Hoppler and Nakamura, 2015). An extensive array of downstream targets has been identified (reviewed in Hoppler and Nakamura, 2015), among which regulators of proliferation (e.g., cMyc, cyclinD1) (He et al., 1998; von Gise et al., 2011). The Tcf/Lef family is composed of three transcription activators (Lef1, Tcf1 and Tcf4) and one transcription inhibitor (Tcf3) (Galceran et al., 1999; Liu et al., 2005). The inhibition of transcription of target genes by Tcf3 is relieved upon phosphorylation mediated by β-catenin (Hikasa et al., 2010). Because Lef1 is also one of the downstream targets of Tcf3 (Wu et al., 2012), the net effect could be an activation of Wnt/β-catenin downstream targets.

In the Wnt/PCP pathway, the Wnt signal is transduced by Fzd together with the co-receptor receptor tyrosine kinase-like orphan receptor 2 (Ror2) or receptor-like tyrosine kinase (Ryk) (Fig. 1C). In this case, Dvl transduces the signal through either Rho or Rac. The Dvl-dependent Rho pathway requires the Dishevelled associated activator of morphogenesis-1 (Daam1) to activate Rho-associated kinase 2 (ROCK2) (Marlow et al., 2002). The Dvl-dependent Rac pathway, on the other hand, is Daam1-independent and transduces the signal through Jun N-terminal kinase (Jnk) (Rosso et al., 2005). These two options affect the cytoskeleton and migration behavior of cells, hence its name Wnt/PCP pathway (Fig. 1C). This pathway is especially relevant in the biology of epithelial tissues: Fzd and Dvl are localized to one side of an epithelial cell and Vangl2 and Prickle are localized in the facing membrane of the adjacent cell. In the presence of Wnt, an intracellular complex is formed among Frd, Wnt, and Vangle2, resulting in transduction of the signal in the Fzd expressing cell (Hoppler and Nakamura, 2015). The components comprising this signaling pathway are shown to be involved in several developmental processes, among which are cardiac outflow tract (OFT) (Henderson and Chaudhry, 2011; Ramsbottom et al., 2014), epicardial (Wu et al., 2010), and neural tube development (De Marco et al., 2014).

In the Wnt/Ca^{2+} pathway, the Wnt molecules signal through pertussis toxin-sensitive G proteins and activate a PLC-PIP2-IP3 transduction cascade that results in a rise in intracellular Ca^{2+} concentration (Fig. 1D). The increased Ca^{2+} concentration affects an extensive array of down-stream targets of which many affect cardiac development, regeneration and homeostasis. Examples are (i) the calcineurin-Nuclear factor of activated T cells (Nfatc) pathway (Roth et al., 2011), which has not only a role in development but also in cardiac hypertrophy (Bushdid et al., 2003; Mass et al., 2014); (ii) the PKC-Cdc42 pathway, which is involved in cardiac differentiation (Rottinger et al., 2006); (iii) the transforming growth factor-β-activated kinase 1 (Tak1/Map3K7)-Nemo-like kinase (Nlk) pathway, which inhibits the Wnt/β-catenin pathway by means of the Notch/Delta signaling pathway (Koyanagi et al., 2009) and has been found to be critical in myocardial survival; and (iv) the activation of Calmodulin-dependent kinase (CaMK) which modifies histone deacetylases (Hdac) and as such influences gene expression (Karamboulas et al., 2006; Kuhl and Pandur, 2008) (Fig. 1D).

Vertebrate Heart Development

Cardiac development is a highly dynamic and interactive process in which a variety of tissues provide signals to each other in a manner that is tightly regulated in space and time. Heart development is dependent on both cell-autonomous and non–cell-autonomous regulation, such that alterations in cell signaling or regulatory gene expression affect cardiac development. In short, mesodermal cardiac progenitor cells, comprising the heart forming region, are laterally positioned in the embryo and are often subdivided into three different populations; the first, second, and third heart fields (Buckingham et al., 2005; Moorman et al., 2007; van Wijk and van den Hoff, 2010; Bressan et al., 2013) (Fig. 2A).

The border between the first and second heart field is not yet fully set as this border seems to differ in space and time between different studies (for review see Moorman et al., 2007). The division between the first/second and the third heart field is less debated because of the different molecular signature of the third heart field; in contrast to the first and second heart field the cells of the third heart field are Tbx18-positive and Nkx2.5-negative.

The lateral heart forming regions are brought together anteriorly by the process of embryonic folding, giving rise to the cardiac crescent. This horseshoe-shaped sheet of mesodermal cells expresses Nkx2.5 and Isl1 and is referred to as the first heart field (FHF). With ongoing embryonic folding the cells of the FHF form the linear heart tube at the ventral midline (at Hamburger and Hamilton stage [HH] 9–HH10 in chicken, at embryonic day (E) 8 in mouse, and Carnegie stages (CS) 9–10 in human) (Hamburger and Hamilton, 1951; Theiler, 1972; O'Rahilly and Müller, 1987) (Fig. 2B). This linear heart comprises two concentric layers of cells separated by an extensive layer of extracellular matrix (ECM). The external layer of cells comprise the cardiomyocytes and the internal layer the endocardial cells. With ongoing development the linear heart loops and the future left ventricle starts to balloon out from the outer curvature. At the inflow of the heart, the left and right atrium are formed. The ventricles and atria balloon out of the primary heart tube as a result of local differentiation and proliferation of cardiomyocytes (de Jong et al., 1997; Christoffels et al., 2000, 2004).

With development, the heart tube increases in length by the addition of cardiomyocytes at both the arterial and venous poles of the heart tube rather than by proliferation of the cardiomyocytes of the primary heart tube (Soufan et al., 2006; van den Berg et al., 2009; de Boer et al., 2012) (Fig. 2C). The cardiomyocytes that are added to the heart tube are cardiac progenitor cells derived from the second heart field (SHF). The mesodermal cells of the SHF have a high proliferation rate but upon addition to the heart tube they cease to proliferate and rapidly differentiate into cardiomyocytes (van den Berg et al., 2009; de Boer et al., 2012). Differentiation of cardiomyocytes is only observed immediately adjacent to the heart tube myocardium. At the inflow, the SHF cells not only contribute to the cardiomyocytes but also enter into the heart through the dorsal mesocardium forming the dorsal mesocardial protrusion (DMP). The DMP is part of the mesenchymal complex, formed together with the mesenchymal cap of the atrial primary septum and both major atrioventricular cushions. The DMP is an essential component in the formation of the atrioventricular septum and due to muscularization of the DMP its remnant is found in the formed heart as the myocardial cap, positioned in between the atrial septum and the fibrous continuity (Briggs et al., 2012).

At the inflow of the future heart an additional pool of mesodermal cells, being the third heart field (THF), contributes cardiomyocytes to the sinus node, the myocardium covering the portion of the caval veins within the pericardial cavity, and the myocardium in between the entrance of the superior and inferior caval veins (the sinus venarum) (Kruithof et al., 2003; van den Hoff et al., 2004; Mommersteeg et al., 2010). Apart from its contribution to this myocardium, the THF also contributes cells to the pro-epicardium (Fig. 2C). The pro-epicardium is a transient structure that provides the cells of the epicardium, the outer non-myocardial layer covering the formed heart (Perez-Pomares et al., 1997). A subset of the cells of the embryonic epicardium undergoes epithelial mesenchymal transition and forms epicardial derived cells (EPDC) that populate the ECM in between epicardium and myocardium (Mikawa and Gourdie, 1996; Perez-Pomares et al., 1998). The EPDC either remain as subepicardial mesenchym, differentiate into coronary blood vessels, or migrate into the myocardial wall where they contribute coronary endothelial cells, pericytes, interstitial fibroblasts, and valvular cells (Dettman et al., 1998; Gittenberger-de Groot et al., 1998; Perez-Pomares et al., 1998; Wessels et al., 2012). It is essential for ventricular growth and compaction that EPDC populate the ventricular wall (Perez-Pomares et al., 2002, 2003; Weeke-Klimp et al., 2010)

At the arterial pole of the heart, the SHF initially contributes cardiomyocytes (Kelly et al., 2001; Mjaatvedt et al., 2001; Waldo et al., 2001) and subsequently smooth muscle cells (Waldo et al., 2005). With development also cardiac neural crest (CNC) cells invade the arterial pole (for review see Keyte et al., 2014). These CNC populate the OFT ridges, which initially are a-cellular extracellular matrix filled swellings in between the myocardium and endocardium. The CNC populate the distal part of the OFT ridges and become dispersed in the proximal part of the OFT ridges. Initially the OFT has an entire myocardial mantle, which largely disappears during development, as most of these cells contribute and differentiate into the working myocardium of the free wall of the right ventricle and a small portion disappears by apoptosis. With the absorption of the myocardial OFT, the interpericardial portions of the aorta and pulmonary trunk are formed (Rana et al., 2007; Sizarov et al., 2012). The interpericardial portions of the aorta and pulmonary trunk comprise two cell populations, with the SHF-derived smooth muscle cells primarily found in the nonfacing parts of their walls and the CNC in the facing parts of their walls (Waldo et al., 1998). The dispersed CNC in the proximal portion of the OFT ridges undergo apoptosis and are replaced by cardiomyocytes that invade from the flanking myocardium and by differentiation of mesenchymal cells in the OFT ridges. This myocardium is found back in the formed heart as the freestanding myocardial infundibulum and the outlet septum. This process is dubbed myocardialization (van den Hoff et al., 1999)

WNT Signaling During Heart Development

It has long been enigmatic how Wnt signals and Fzd receptors confer pathway specificity (van Amerongen, 2012). It has, however, become evident that Dvl, an essential component of the destruction complex, is at the heart of the regulation of the specificity (Rothbacher et al., 1995; Kafka et al., 2014). In mouse and human, three homologous genes are found: Dvl1, Dvl2, and Dvl3. Two isoforms of both Dvl1 and Dvl3 exist as a result of alternative splicing. Although all Dvl isoforms are expressed abundantly and broadly, Dvl2 is the most abundant. Knock out analysis in mice revealed that the Dvl isoforms have both complementary and unique features (Wynshaw-Boris, 2012). Of relevance for this review is that the cardiac phenotype after homozygous functional disruption of Dvl2 includes double outlet right ventricle or common trunk (Hamblet et al., 2002) and that additional removal of one copy of Dvl3 results in abnormal cardiac looping (Etheridge et al., 2008). The specificity for the different Wnt signal transduction pathways is mediated by three conserved protein domains of Dvl, being (i) the DIX (DIshevelled and aXin) domain, which is related to the canonical Wnt pathway through the DAX domain

in Axin; (ii) the PDZ (postsynaptic density 95, disc large, zonula occludens-1) domain, which interacts directly with Fzd receptors and as such appears to be involved in all Wnt signaling pathways; and (iii) the DEP (dishevelled, egl-10, and pleckstrin) domain, which interacts with G-proteins and Daam1 and as such mediates noncanonical Wnt pathway signaling. Upon receptor-mediated activation of the canonical signaling pathway, the homotypic interaction of the DAX domain in Axin is altered into a heterotypic interaction between the Axin DAX domain and the Dsh DIX domain, which turns off the activity of the destruction complex (Fiedler et al., 2011) (compare Figs. 1A–D).

Axin is not only the scaffold of the destruction complex but is also considered the rate-limiting factor, because the Axin concentration is lowest of the proteins contributing to the destruction complex (Lee et al., 2003; MacDonald et al., 2009; Tan et al., 2012). In vertebrates, two genes for Axin exist; with Axin1 being constitutively expressed and Axin2 being a direct transcriptional target of Wnt/β-catenin signaling in a regulatory negative feedback loop (Jho et al., 2002; Song et al., 2014). The pattern of expression of Axin2, therefore, can be used to monitor intracellular Wnt/β-catenin signaling activity in general. A tissue-specific effect of Axin2 on the inhibition and activation of Wnt signaling has been described in the mouse embryo (Qian et al., 2011). In the heart, Axin2 mRNA expression is found in the cardiac cushions and valves (Rudat et al., 2013). In reporter assays, the presence of Axin2 was also observed in the inflow and atrioventricular canal during cardiac development (Gillers et al., 2015). Axin1 knockout mice die before cardiac development as a result of axis formation abnormalities (Zeng et al., 1997). Axin2 knock out mice are viable, displaying craniosynostosis (McGee-Lawrence et al., 2013). Of interest, a mutation causing increased stability of Axin2 protein (canopus) shows cardiac bifida and duplication of all cardiac compartments at 9.5 days of mouse heart development (Qian et al., 2011). In this respect, it is interesting to note that in chicken the expression of Wnt3a in migrating cardiac progenitors affects their migratory trajectory by means of the Wnt/PCP-RhoA pathway and results in a high incidence of cardiac bifida (Yue et al., 2008). This finding might be consistent with the mouse phenotype found upon Axin2 stabilization. A detailed analysis of these abnormalities is required to clarify the underlying mechanism.

Another relevant level of complexity in the Wnt pathway regulation is found at the extracellular level by four groups of extracellular Wnt antagonists, being the Dickkopf (Dkk) proteins, the soluble Frizzled-proteins (Sfrp), the Shisa proteins and a heterogeneous group of proteins, among which Wnt-inhibitor protein (Wif) and Cerberus (Cruciat and Niehrs, 2013).

Dickkopf (Dkk) proteins comprise a family of four members with Dkk1, Dkk2, and Dkk4 being more similar, based on sequence identity, than Dkk3. Dkk3 does not affect Wnt-signaling, but rather TGFβ signaling (for extensive review, see Cruciat and Niehrs, 2013). Dkk1, -2, and -3 are expressed in the developing heart and the surrounding tissues, with Dkk1 and Dkk3 being expressed at higher levels than Dkk2 (Monaghan et al., 1999; Bazzi et al., 2007). Dkk1 and -2 inhibit the Wnt/β-catenin pathway by binding to Lrp5 and Lrp6. Dkk4 only binds to Lrp6 and activates the noncanonical Wnt/PCP pathway, while inhibiting the Wnt/β-catenin pathway (Hirata et al., 2011). Except for Dkk3, the Dkk proteins also bind to the Kremen1 and -2 membrane proteins. The complex of Dkk1, Lrp5/6 and Kremen is rapidly removed from the plasma membrane by endocytosis, resulting in an enhancement of the inhibitory effect on the Wnt/β-catenin pathway (Mao and Niehrs, 2003). Dkk2 in the presence of Kremen2 turns from an inhibitor into an activator of Wnt/β-catenin signaling (Mao and Niehrs, 2003). *The soluble Frizzled-proteins (Sfrp)* form an extensive and complex group. In mouse and human five Sfrps have been identified, with Sfrp3 being the orthologue of the founding member FrzB (Jones and Jomary, 2002). In chicken, Xenopus, and zebrafish, but not in mammals, an additional group has been identified, comprising Crescent, Sizzled, and Sizzled2 (Lee et al., 2006). In chicken and Xenopus Crescent and Sizzled have been shown to be regulators of early cardiogenesis in the FHF (Marvin et al., 2001; Schneider and Mercola, 2001; Wittler et al., 2008). Sizzled is also implicated in OFT development as its expression is found in the SHF (Wittler et al., 2008).

The Sfrp proteins contain a Wnt binding domain, that allows interaction with different affinities and scavenging of Wnts. Based on interaction studies, it has been proposed that Sfrp can also dimerize with Fzd, creating nonfunctional receptor complexes (Bovolenta et al., 2014). These modes of action are in principle not specific for a particular Wnt signaling pathway. Because the different Wnt signaling pathways are interlinked an Sfrp induced inhibition of the Wnt/PCP pathway, can antagonize the Wnt/β-catenin pathway in a stage- and tissue-specific manner, which might result in activation of the Wnt/β-catenin pathway (Swain et al., 2005). Sfrp proteins have been found to play important roles in cardiac disease (Zeisberg et al., 2007) but knockout analysis of each individual Sfrp does not reveal cardiac abnormalities (Satoh et al., 2006). In contrast, morpholino-mediated knock down of Sfrp1 in Xenopus revealed aberrant cardiac differentiation resulting in reduced myocardial tissue and heart size (Gibb et al., 2013). The expression pattern of Sfrp1 in the anterior site of the gastrulating mouse embryo led to the, untested, suggestion that its knock-out would result in a cardiac phenotype (Esteve et al., 2000).

The *Shisa proteins* comprise four family members in mouse, Shisa1, -3, -4, and -5, which have a transmembrane spanning region. Shisa2 is not found in mice. In Xenopus, Shisa1 and Shisa2 (Silva et al., 2006) were described and their pattern of expression resembles mouse Shisa1 (Filipe et al., 2006). Shisas inhibit Wnt signaling by trapping Fzd in the endoplasmic reticulum, preventing exposure of mature Fzd on the plasma membrane. Knockout analysis of the individual Shisa members and double knockouts of Shisa1 with either Shisa3, -4, or -5 did result in phenotypic abnormalities (Yamamoto et al., 2005; Furushima et al., 2007). In chicken, the expression of Shisa2 in the anterior portion of the gastrulating embryo and during subsequent development immediately posterior of the inflow of the heart suggests a role in the differentiation of cardiomyocytes (Hedge and Mason, 2008).

There is a further group of proteins that are not structurally related but have in common that they inhibit Wnt signaling. *Wnt inhibitory factor 1 (Wif1)* is a highly conserved secreted protein that acts as an inhibitor of the action of Wnt ligands. Wif1 not only binds Wnts through five EGF-like repeats but also interacts with ECM proteoglycans (Avanesov et al., 2012). Wif1 does not seem to be able to affect all Wnt signaling pathways. Wif-1 knockout mice develop and reproduce normally and do not show obvious defects (Kansara et al., 2009), suggesting redundancy. In avian embryos, ectopic delivery of Wif1 in ovo induces precocious cardiomyocyte differentiation of the THF (Tbx18-positive)

progenitor pool at the inflow tract of the embryonic heart (Buermans et al., 2010). It has, however, not been evaluated which Wnt signaling pathway is operational. It can be anticipated that Wif-1 affects in this case the Wnt/β-catenin pathway (Bovolenta et al., 2014) because β-catenin mediated signaling was found to be essential for cardiac inflow formation (Norden et al., 2011).

Cerberus (Cer1) is a founding member of the Dan family of secreted proteins. Although Dan family proteins were found to antagonize bone morphogenetic protein (BMP) signaling, Cer1 was also found to inhibit Wnt signaling (Piccolo et al., 1999; Bell et al., 2003; Katoh and Katoh, 2006). In chicken, Cer1 is expressed in the left flank of the embryo and ectopic delivery of Cer1 at the right side, results in left–right asymmetry with the heart looping to the right instead of to the left (Zhu et al., 1999). Studies in Xenopus showed that Nodal-mediated induction of Cer1 in the endoderm flanking the cardiogenic mesoderm is essential for cardiogenesis (Foley et al., 2007). Deletion of Cer1 from the mouse genome does not affect normal mouse morphogenesis (Simpson et al., 1999). Moreover, targeted disruption of Cerberus-like 2 (Cerl2), a close family member of Cer1, leads to randomization of the left right axis (Marques et al., 2004) and lethality in the first postnatal week because of ventricular hyperplasia and systolic dysfunction (Araujo et al., 2014).

A further level of complexity in the regulation of Wnt signaling is found at the level of posttranscriptional modifications and secretion of Wnts (Cheng and Xu, 2014). An array of proteins has been described to be involved in the transport of Wnts from the endoplasmic reticulum by means of the Golgi to the membrane for secretion as well as in the lipid-modification and glycosylation of Wnt proteins (for review, see Maurice and Korswagen, 2014). In this review, we highlight two proteins, Porcupine (Porcn) and Wntless (Wls), because of their potential role in heart development.

Porcupine (Porcn) is an endoplasmic reticulum membrane spanning O-acyltransferase, which is responsible for the post-translation modification and secretion of Wnt ligands (Zhai et al., 2004). Porcn is required for the lipid modification of Wnts (Tang et al., 2012). The region responsible for lipid modification is highly conserved in evolution. Deletion of Porcn (Barrott et al., 2011; Liu et al., 2013) in mouse or Drosophila (Zhai et al., 2004) revealed embryonic lethality at gastrulation as a result of ER accumulation and, therefore, absence of secretion of Wnts. These findings suggest that Porcn can be considered a “bottleneck” in Wnt signaling (Barrott et al., 2011). A conditional knockout of Porcn (Barrott et al., 2011) exists, but a heart-specific knock out has not been evaluated.

The traffic between the Golgi and the membrane is mediated by *Wntless (Wls)*. Wls functions downstream of Porcn, is also highly conserved in evolution (Banziger et al., 2006; Bartscherer et al., 2006) and loss of function assays unveiled specificity for transport of Wnt ligands to the plasma membrane (Banziger et al., 2006). Like other molecules involved in vesicular trafficking, Wls is recycled (Maurice and Korswagen, 2014). In mouse, but not in Drosophila, Wls was found to be a direct target of the Wnt/β-catenin pathway suggesting that Wnt proteins can stimulate their own secretion by upregulating Wls expression (Fu et al., 2009; Herr and Basler, 2012). Knocking out Wls in mice results in embryonic death before gastrulation at the egg cylinder stage (Carpenter et al., 2010). A conditional knock out of Wls (Carpenter et al., 2010) does exist, but a heart-specific deletion has not been evaluated.

WNT Signaling Pathways Regulate the Fate Of Cardiac Progenitor Cells in all Three Heart Fields

First Heart Field (FHF)

Studies in Xenopus, chicken, and mouse have shown that induction of visceral mesodermal cells that give rise to the FHF require the tight temporal and spatial control of Wnt signaling pathways (Wagner and Siddiqui, 2007). Expression of Wnt3a and Wnt8c in the posterior part of the embryo inhibits by means of the β-catenin signaling pathway the specification of the gastrulating mesodermal cells into precardiac progenitors. Knocking out β-catenin (Haegel et al., 1995) or Wnt3a (Liu et al., 1999) results in embryonic defects at gastrulation, not allowing the analysis of a possible function during cardiogenesis. In chicken, the Wnt3a/PCP-RhoA pathway cooperates with the BMP-Smad1 pathway in the regulation of the migration of cardiac progenitors toward their anteriolateral position (Yue et al., 2008; Song et al., 2014). During their migration, the precardiac progenitors enter anteriorly into the expression domain of the Wnt inhibitors Dkk1 and Crescent. The expression of Dkk1 and Crescent inhibit the Wnt/β-catenin pathway, which relieves the inhibition of the Wnt/β-catenin pathway on the initiation of the cardiac gene expression program (Marvin et al., 2001; Schneider and Mercola, 2001). The cardiac gene expression program is initiated by the cooperative action of BMP- and FGF-signaling (reviewed in van den Hoff et al., 2004). The initiation of the cardiac gene program in the cardiac progenitors becomes evident with the expression of the cardiac transcription factors such as Nkx2.5 and cardiogenic Gata genes. Wnt11, which is expressed in the region of the FHF, cooperates in the induction of the cardiac gene program, by means of the Wnt/Ca^{2+} and Wnt/Jnk pathways (Eisenberg and Eisenberg, 1999; Marvin et al., 2001; Schneider and Mercola, 2001; Pandur et al., 2002; Sheldahl et al., 2003; Munsterberg and Yue, 2008; Klaus et al., 2012). In Xenopus, it was shown that transcription factors Nkx2.5 and Gata4 and 6 functionally interact with Wnt signaling in two different stages. At stage 8, Wnt/β-catenin negatively regulates the expression of Gata4 and 6 in the precardiac mesoderm and at stage 18, after cardiac specification but before migration of the cardiac progenitors to the ventral midline of the embryo, the expression of these factors is maintained partially by means of noncanonical Wnt11b (Afouda et al., 2008).

β-catenin functions beyond Wnt signaling in cadherin-mediated cell adhesion (Pettitt, 2014). Indeed, with the differentiation of the cardiac progenitor cells into cardiomyocytes, β-catenin accumulates at the cytoplasmic side of the sarcolemma interacting with N-cadherin. With the maturation of the cardiomyocytes into their adult rod-shape, the N-cadherin-β-catenin complexes become displaced to the intercalated discs, the contacts between cardiomyocytes. However, conditional removal of β-catenin revealed that β-catenin at the intercalated discs is not essential for cardiac function, likely due to the presence of plakoglobin/γ-catenin (Zhou et al., 2007). Immunofluorescent staining of β-catenin reveals a very intense staining at the plasma membrane, which obscures low level expression of β-catenin in the cytoplasm or hypophosphorylated β-catenin in the nucleus regulating the expression level of genes downstream of Wnt/β-catenin signaling (Fig. 3). As a consequence, it is very difficult to visualize the activity of Wnt/β-catenin signaling using this

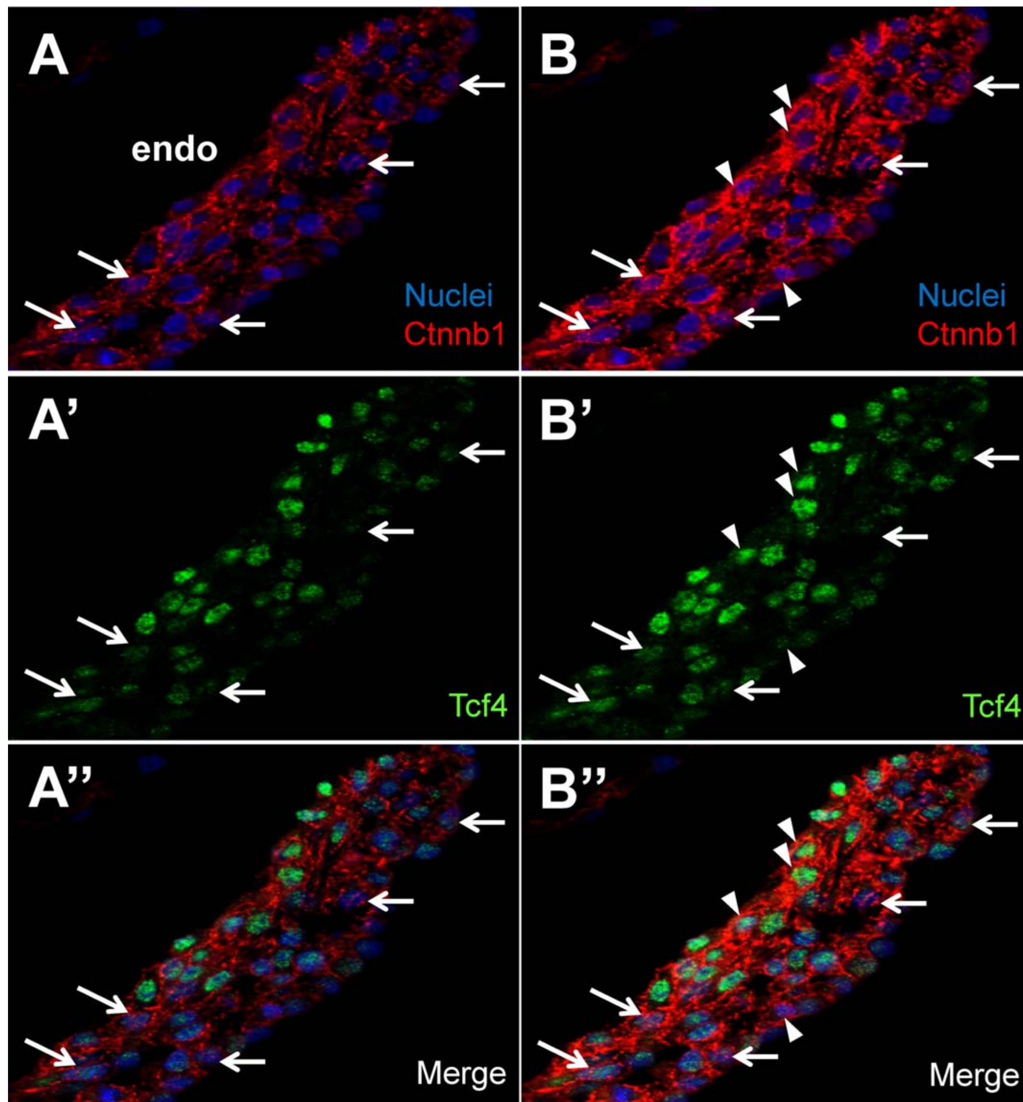

Fig. 3. Confocal microscopy images showing the same section of the ventricular wall of a HH14 chicken embryonic heart. **A**: Confocal laser scanning microscopy analysis of immunofluorescently stained β-catenin (red) shows staining in the cytoplasm and at the membrane of cardiomyocytes. **A′**: Immunostaining of Tcf4 (green) on the same section shows a larger number of positive nuclei. The arrows indicate the nuclei identified in A, as well. Because Tcf4 is considered an effector of the canonical Wnt pathway, the patterns seem to be contradictory. In panel **A″** the merge of panel A and A′ is shown. **B–B″**: Increasing the power of the laser reveals additional nuclei in which β-catenin is found, i.e. the canonical Wnt pathway is active (arrowheads in B). B″: Also the staining pattern of Tcf4 reveals more positive nuclei at the endocardial site of the myocardium. B″: Despite the enhancement of the visualization a complete overlap between β-catenin and Tcf4 staining is not found. Endo, endocardial side

technique. So-called Bat-Gal mice (Maretto et al., 2003), which are considered Wnt/β-catenin reporter mice, showed that the Wnt/β-catenin pathway is not active in the adult heart (Liebner et al., 2004).

Second Heart Field (SHF)

Once the linear heart tube is formed, SHF cells, expressing Isl1 and Nkx2.5, are located in the dorsal pericardial wall, and are added to both the arterial and venous pole of the heart with ongoing development (Buckingham et al., 2005). The SHF cells are relatively small cells with a high proliferative rate (Soufan et al., 2006; van den Berg et al., 2009). Proliferation of the SHF is regulated by Wnt2 by means of the canonical β-catenin pathway (Tian et al., 2010; Norden et al., 2011; Norden and Kispert, 2012). The Wnt/β-catenin pathway was found to maintain the SHF cells not only in a proliferative state but also undifferentiated (Klaus et al., 2007; Kwon et al., 2009). Conditional removal of β-catenin from the SHF using Isl1-Cre results in a decrease of the number of cells within the SHF and as a consequence impaired development of the OFT and RV (Klaus et al., 2007). When the SHF cells are added to the heart tube, the cardiac progenitors enter into an area, in which canonical Wnt signaling is gradually down regulated and BMP signaling is highly active. In this zone the SHF cells are arrested in proliferation and initiate the myocardial gene program (van den Berg et al., 2009; van Wijk and van den Hoff, 2010; Jain et al., 2015).

At the arterial pole of the heart, Wnt11 and Wnt5a signaling through the Wnt/PCP pathway were found to be essential (Pandur et al., 2002; Eisenberg and Eisenberg, 2006; Cohen et al.,

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2012). Knocking out both Wnt11 and Wnt5a results in an absence of SHF cells added to the arterial pole of the heart (Cohen et al., 2012). During normal development the PCP pathway includes the membrane protein Vangl1 as an essential component. When Vangl protein expression is disrupted, the alignment of the OFT is abnormal resulting in double OFT right ventricle (DORV) and absence of muscularization of the outlet septum (Phillips et al., 2005). DORV has a complex etiology in which different cell types and signaling pathways are operational. The mechanism underlying the development of DORV can be due to abnormal development of components derived from the SHF or due to aberrant contribution of CNC cells to the OFT (van den Hoff and Moorman, 2000).

At the venous pole of the heart, lineage tracing of SHF progenitors has revealed that SHF cells contribute to the smooth walled atrial myocardium, whereas the trabeculated atrial appendages are derived from the FHF (Hoogaars et al., 2007). The knock out of Wnt2 results in abnormal development of the inflow of the heart, affecting the atrioventricular canal, atria, and pulmonary veins (Tian et al., 2010). In these Wnt2 knockout embryos, Axin2, Lef1, and the SHF marker Isl1 were found to be down-regulated and the number of SHF cells was greatly reduced underlying the observed inflow tract abnormalities. Isl1-Cre mediated conditional deletion of β-catenin revealed a phenotype at the IFT (Klaus et al., 2012) similar to the Wnt2 knockout phenotype, suggesting that Wnt2 signaling by means of the canonical pathway is essential for IFT development. In line with this idea, administering the classic and broad Gskβ inhibitor LiCl to pregnant mice rescued the phenotype due to activation of the canonical Wnt signaling pathway (Tian et al., 2010). Recently, an Axin2-lacZ reporter mouse was evaluated, which also showed expression within the myocardium upstream of the ventricle, including the AV node and AV valve endocardium and mesenchyme (Gillers et al., 2015). This further supports the idea that Wnt/β-catenin signaling is operational in the forming myocardium of the inflow tract.

A similar mechanism seems to be operational in the subpopulation of SHF cells that are added to the heart by means of the dorsal mesenchymal protrusion (DMP). Lineage tracing experiments have shown that compared with the other SHF cells that are added to the venous pole of the heart, the DMP-derived cells are subjected to Sonic Hedgehog (Shh) signaling (Goddeeris et al., 2008; Briggs et al., 2015). Within the cells of the DMP, hypophosphorylation of β-catenin and elevated expression of Lef1 are found, suggesting that also in this population Wnt/β-catenin maintains the progenitor cells in an undifferentiated and proliferative state (Briggs et al., 2015). A similar mechanism was identified in neuroblastoma cells. In these cells, the physical interaction of β-catenin with Gli1 results in their degradation and inhibition of cell cycle progression (Zinke et al., 2015).

Third Heart Field (THF)

Finally, the progenitors in the THF, which are in contrast to the FHF and SHF Nkx2.5-negative and Tbx18-positive, contribute cardiomyocytes that form the sinus node and the caval myocardium (Mommersteeg et al., 2010). With respect to Wnt signaling a dichotomy is observed in this THF population. Those THF cells that differentiate into the myocardium overlaying the sinus horns and thus form the caval myocardium are characterized by high levels of active Wnt/β-catenin signaling (Norden et al., 2011) and expression of Wnt2 (Tian et al., 2010). The THF cells that contribute to the sinus node, on the other hand, are characterized by low, if any, Wnt/β-catenin signaling (Norden et al., 2011). Overexpression of constitutive active β-catenin within the sinus node progenitors results in an inhibition of sinus node differentiation (Norden et al., 2011).

In chicken, Wif1 is expressed in THF cells. Application of Wif1 to chicken embryos results in an expansion of the myocardium beyond the border of the pericardial cavity (Buermans et al., 2010). We hypothesized that the inhibition of the Wnt/β-catenin pathway by Wif-1 leads to inhibition of proliferation of the progenitor cells, allowing their precocious differentiation into cardiomyocytes. In Xenopus, a comparable mechanism seems to be operational at the inflow, although apparently using different molecules. In Xenopus, the Wnt inhibitor Sfrp1 may control heart muscle cell formation through the inhibition of Wnt6 (Lavery et al., 2008; Gibb et al., 2013). The THF not only contributes cardiomyocytes to the heart but also to the nonmyocardial component by means of the pro-epicardium. In the above mentioned publications on the role of Sfrp1 and Wnt6 in the differentiation of IFT cardiomyocytes, the role of these factors in epicardial formation was not considered (Lavery et al., 2008; Gibb et al., 2013).

On the other hand, the analysis of ectopic delivery of Wif1, revealed that the covering of the myocardium with epicardium was inhibited, (Buermans et al., 2010) suggesting that Wnt signaling is involved in epicardial cell migration. In line with this idea, double mutants of Dkk1 and Dkk2 show a hypercellular proepicardium that gives rise to a multilayered epicardium and an increased myocardial proliferation rate (Phillips et al., 2011). Gata5-Cre mediated deletion of β-catenin had revealed that the formation of the embryonic epicardium was not affected, but that the subsequent development of the derived lineages was severely impaired, resulting in embryonic death which is most probably due to aberrant coronary vessel formation (Zamora et al., 2007). The role of Wnt signaling in the development of the embryonic epicardium was further substantiated (Wu et al., 2010). These analyses showed a disruption of the adherens junctions as well as of mitotic spindle formation in embryonic epicardial cells. The resulting randomization of epicardial cell division leads to a multi-layered epicardium and fewer EPDC within the extracellular matrix in between the epicardium and myocardium The use of different epicardium-specific Cre lines, Tcf21-Cre or Wt1-Cre, to delete β-catenin resulted in normal epicardial mesenchymal transition, but impaired migration of the EPDC into the myocardium (von Gise et al., 2011).

The confusion further increased, when β-catenin expression was altered using another epicardium-specific Cre line, Tbx18-Cre (Rudat et al., 2013). This study showed that epicardial β-catenin loss-of-function had no effect on epicardial formation, epicardial mesenchymal transition and EPDC differentiation. Moreover, expression of a gain-of-function mutation of β-catenin in the epicardium resulted in a severe, lethal (E12.5) cardiac phenotype, including reduced overall heart size, large ballooning atria, thin ventricular myocardium, and hyperplasia of the epicardial cells. Analyzing the migratory capacity of a mouse epicardial derived cell line, dubbed EPIC cells, in fibrin gels revealed that Wnt3a as well as Wnt5a addition abrogated the degradation of the fibrin matrix and severely reduced the migration of cells into the matrix (Ruiz-Villalba et al., 2013). Expression of the mediator of Wnt/PCP signaling pathway Vangl2 in

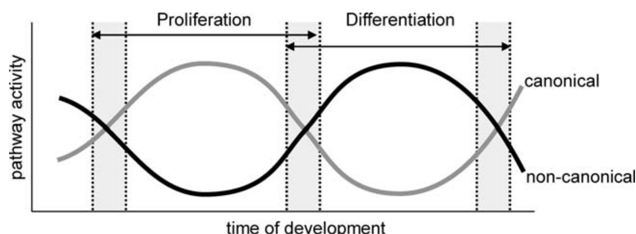

Fig. 4. Schematic model illustrating the periodic and reciprocal activity of canonical and noncanonical Wnt signaling. Considering individual cells, a periodicity in the balance between canonical and noncanonical Wnt signaling activity is found; canonical signaling correlates with proliferation and stemness of a progenitor cell and noncanonical signaling with differentiation of a progenitor cell into a cardiomyocytes. The gray areas in between the dotted lines indicate the transition from the proliferation into differentiation and vice versa. Analyses performed in homogenates, which comprise many different cells will lose specificity and show both signaling pathways, due to the repetitive alternating and reciprocal effects of canonical and noncanonical signaling.

the myocardium was found to be required for EPDC differentiation into coronary smooth muscle cells (Phillips et al., 2008).

Taken together, these data point to a delicate balance between canonical and noncanonical Wnt signaling in epicardium formation and differentiation; with Wnt/β-catenin signaling being essential in pro-epicardial and epicardial cell expansion, and Wnt/PCP signaling in epicardial-derived cell differentiation.

CANONICAL AND NONCANONICAL WNT PATHWAYS: A BALANCING ACT?

Although canonical and noncanonical Wnt signal transduction pathways are generally described separately, evidence accumulates that supports the suggestion that the subdivision into canonical and noncanoncial Wnt signaling pathways is not appropriate anymore (van Amerongen and Nusse, 2009; Rao and Kuhl, 2010). Different transduction signals downstream of activated Fzd receptors give rise to different Wnt signaling branches that regulate each other in a network of interactions (Kestler and Kuhl, 2008). Moreover, in vitro analysis of the role of Wnt signaling in the migration of EPDC demonstrated that Wnt3a and Wnt5a, which are considered the archetypes of canonical and noncanonical Wnt signaling, respectively, both show the same effect (Ruiz-Villalba et al., 2013). These findings suggest that segregation between canonical and noncanonical Wnt signal transduction is less strict than proposed, resulting in a net activation of the canonical Wnt signaling pathway by Wnt5a.

Wnt3a and Wnt8c inhibit cardiomyocyte formation by means of the canonical Wnt/β-catenin pathway and Wnt11 stimulates cardiomyocyte formation by means of the noncanonical Wnt/PCP pathway as well as by inhibiting the canonical Wnt/β-catenin pathway. In Xenopus, inhibition of Wnt11b, which is expressed in the region of the heart fields, results in a loss of cardiomyocytes formation (Pandur et al., 2002). More recent studies show that Wnt11b stimulates Nkx2.5 expression and cardiomyogenesis (Afouda et al., 2008). These observations help to comprehend the observation that Wnt11 stimulates heart muscle cell formation in P19 embryonic carcinoma cells (Pandur et al., 2002) and cardiomyocytes formation in posterior noncardiac mesoderm (Eisenberg and Eisenberg, 1999). The further differentiation of these cardiomyocytes requires high levels of Smad-mediated BMP activity that, in turn, is essential for maintaining Nkx2.5 and Gata4 expression in these differentiating cardiomyocytes. In line with the observations on noncanonical Wnt/PCP signaling, BMP signals by means of Jnk and thus enhances the noncanonical pathway (Monzen et al., 2002).

Conclusions

Analysis of Wnt signaling in the developing heart is complex. This complexity is not only due the existence of several different Wnt signaling pathways but also due to the complex nature of the development of the heart, a three-dimensional structure changing in time and cellular composition. Heart formation does not comprise a sequence of individual sequential events, but a contiguous process in which the morphogenetic processes run together or partly overlap. Therefore, each analysis conducted on homogenates to evaluate heart development, only shows a snapshot of a developmental moment and as such a blend of overlapping and interacting process active at the analyzed stage of development. With respect to Wnt signaling, one will find canonical signaling involved in retaining cardiac precursors in a proliferative and precursor state, noncanonical signaling involved in inducing cardiac differentiation and both canonical and noncanonical signaling in regulating specific steps in the differentiation of cardiac components (modeled in Fig. 4). The repetitive alternating and reciprocal effects of canonical and noncanonical Wnt signaling on the maintenance of the progenitor population and the differentiation of these cells, respectively, is lost when Wnt signaling is studied in homogenates. To obtain this developmental information, the activity of the different Wnt signaling pathways should be visualized within the morphological context of the developing heart in vivo. Such an analysis approach requires the identification of specific unique reporters for each Wnt signaling pathway. Without the simultaneous in vivo visualization of such specific reporters, the mechanism of Wnt signaling in heart development will remain elusive.

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