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# WNT Signaling in Cardiac and Vascular Disease

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**ABBREVIATIONS:** AC, arrhythmogenic cardiomyopathy; AGE, advanced glycation end product; AngII, angiotensin II; APC, adenomatous polyposis coli; AR, androgen receptor; BBB, blood-brain barrier; BIO, 6-bromoindirubin-3'-oxime; BMP, bone morphogenetic protein; BNP, brain natriuretic peptide; BRB, blood-retina barrier; CBP, CREB binding protein; CK1, casein kinase 1; CNS, central nervous system; COX-2, cyclooxygenase-2; CRD, cysteine-rich domain; Cx, connexin; DEP, disheveled-EGL10-plekstrin; DIX, disheveled-axin; DKK, dickkopf; DVL, disheveled; E, embryonic day; EC, endothelial cell; EMT, epithelial-mesenchymal transformation; FZD, frizzled; GSK3, glycogen synthase kinase-3; HUVEC, human umbilical vein endothelial cells; IL, interleukin; IWR, inhibitors of WNT response; JNK, c-Jun NH<sub>2</sub>-terminal kinase; KO, knockout; LDL, low-density lipoprotein; LGR, leucine-rich repeat-containing G protein-coupled receptor; LiCl, lithium chloride; LRP, lipoprotein-related receptor protein; MI, myocardial infarction; MMP, matrix metalloproteinase; MSC, mesenchymal stem cells; NFAT, nuclear factor of activated T-cells; ox, oxidized; PAH, pulmonary arterial hypertension; PASMC, pulmonary artery smooth muscle cells; PCP, planar cell polarity; PKC, protein kinase C; PLVAP, plasmalemma vesicle-associated protein expression; PORCN, porcupine; PRR, (pro)renin receptor; RAGE, receptor for advanced glycation end products; RAS, renin angiotensin system; ROR, receptor tyrosine kinase-like orphan receptor; sFRP, secreted frizzled-related protein; SHF, second heart failure; SHHF, spontaneously hypertensive rat heart failure; TCF/LEF, T-cell factor/lymphoid enhancer factor; TM, transmembrane; TNF, tumor necrosis factor; TNK, tankyrases; VEGF, vascular endothelial growth factor; WD, windorphen; WIF, WNT inhibitory factor; WLS, Wntless; WT, wild type.

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**Abstract**—WNT signaling is an elaborate and complex collection of signal transduction pathways mediated by multiple signaling molecules. WNT signaling is critically important for developmental processes, including cell proliferation, differentiation and tissue patterning. Little WNT signaling activity is present in the cardiovascular system of healthy adults, but reactivation of the pathway is observed in many pathologies of heart and blood vessels. The high prevalence of these pathologies and their significant contribution to human disease burden has raised interest in WNT signaling as a potential target for therapeutic intervention. In this review, we first will focus on the constituents of the pathway and their regulation and the different signaling routes. Subsequently, the role of WNT signaling in cardiovascular development is addressed, followed by a detailed discussion of its

involvement in vascular and cardiac disease. After highlighting the crosstalk between WNT, transforming growth factor- $\beta$  and angiotensin II signaling, and the emerging role of WNT signaling in the regulation of stem cells, we provide an overview of drugs targeting the pathway at different levels. From the combined studies we conclude that, despite the sometimes conflicting experimental data, a general picture is emerging that excessive stimulation of WNT signaling adversely affects cardiovascular pathology. The rapidly increasing collection of drugs interfering at different levels of WNT signaling will allow the evaluation of therapeutic interventions in the pathway in relevant animal models of cardiovascular diseases and eventually in patients in the near future, translating the outcomes of the many preclinical studies into a clinically relevant context.

## I. Introduction

Cardiovascular diseases include a wide variety of conditions, such as atherosclerosis leading to tissue ischemia, cardiac hypertrophy, valvular defects, and cardiac arrhythmias. At a first glance, these conditions may seem to have only few similarities, but the underlying disease mechanisms such as inflammation, fibrosis, and endothelial dysfunction are common denominators in cardiovascular diseases. This is also illustrated by the fact that, despite the apparent differences in the symptoms, the current pharmacotherapeutic arsenal for the treatment of these conditions is remarkably similar. Inhibitors of the renin-angiotensin system, Ca<sup>2+</sup>-antagonists, inhibitors of the sympathetic nervous system, diuretics, and statins form the cornerstones of the therapy, supplemented with anticoagulants where needed. Although these drugs have been proven to be useful in treating the symptoms of many cardiovascular diseases, their effect on disease progression is limited. Moreover, recent efforts to develop new drugs

for cardiovascular diseases, particularly for heart failure, have not been very successful (Vaduganathan et al., 2013).

Obviously, the ideal cardiovascular therapy would induce regression of the disease process and eventually cure the patient. This illustrates that there is a need for a better understanding of the underlying signaling mechanisms involved in the disease process. In this review we will discuss the role of the WNT signal transduction pathway in cardiovascular diseases. WNT signaling is well known for its role in developmental biology (van Amerongen and Nusse, 2009), but there are many indications that the pathway is reactivated in disease (Clevers and Nusse, 2012). In this review, we will first provide an overview of the components of the cascade and their regulation, followed by their interactions in the different signaling pathways and a description of their role in the development of the cardiovascular system. Subsequently, we will provide an overview of the experimental evidence for involvement of WNT signaling in vascular and cardiac pathology.

Next, we will discuss the role of WNT signaling in stem cell maintenance and differentiation, a relatively new field that may hold promise for the regeneration of defective or malfunctioning tissue. Finally, we will provide an overview of the drugs developed for pharmacological intervention at different levels of the signaling cascade. In this review, we will use the term “WNT signaling” to refer to the pathway as a whole in all its complexity. Specific signaling routes will be indicated by “ $\beta$ -catenin-mediated WNT signaling” or “WNT/ $\beta$ -catenin signaling” and “non- $\beta$ -catenin-mediated WNT signaling,” respectively.

## II. The History of WNT Signaling

It is now more than 30 years ago that Nusse and Varmus (1982) published the first report on a gene they discovered in a search for preferential integration sites for mouse mammary tumor virus. Although the sequence of the gene, which they named *int1*, did not share any homology with other genes known at that time, overexpression studies established that *int1* behaved as a bona fide protooncogene (Tsukamoto et al., 1988). The *int1* gene displayed a high degree of conservation across species, which appeared to be key in identifying the role of this gene (Nusse and Varmus, 2012). Around the same time, Nusslein-Volhard and Wieschaus (1980) investigated the molecular mechanisms leading to developmental mutants of *Drosophila*. They identified a class of segment-polarity genes, showing similar patterning defects when mutated. The names of these genes were derived from the description of their phenotype, such as *Wingless*, *Armadillo*, and *Arrow*. Cloning and sequencing of both the *int1* and *Wingless* genes revealed that they were in fact homologs, making them one of the first examples of a gene that is active in both normal development and in malignancies (Rijsewijk et al., 1987).

Expression of WNT proteins has been observed during the development of multicellular organisms throughout the animal kingdom, but not in plants. Multiple orthologs of *WNT* genes are found in most animals, with a staggering total of 19 *WNT* genes in mouse and man subdivided into 12 conserved subfamilies (Kusserow et al., 2005). WNT proteins have the capacity to induce polarization of cells by activating the planar cell polarity (PCP) pathway and to activate their lineage-specifying signaling, two prerequisites for the development of an asymmetric shape and cellular diversity of metazoans (Loh et al., 2016). Over time, activation of more pathways, including the WNT/ $\beta$ -catenin (frequently referred to as “canonical” WNT signaling) and WNT/ $\text{Ca}^{2+}$  signaling, have been identified.

Over the years, research on WNT signaling has been hampered by several experimental difficulties. First, there was the problem of the purification of WNT proteins. The transportation of WNT proteins to the extracellular compartment appeared to be a complicated

process, involving the covalent linking of a palmitoleate moiety, which is essential for the biologic activity of the protein. It took until 2003 before a protocol was developed that allowed the purification of active WNT protein (Willert et al., 2003). The development of specific antibodies for WNT proteins has also been a time-consuming and frustrating endeavor (Nusse and Varmus, 2012). Moreover, it took more than a decade to identify proteins from the frizzled (FZD) family to act as the principal receptors for WNT proteins (Wang et al., 2016a). The identification of coreceptors, multiple signaling pathways, and endogenous antagonists further have contributed to our knowledge on this intriguing yet complex regulatory network.

The role of WNT signaling in disease has been an area of intense research in the last decades. Some 20 years ago, mutations in the human adenomatous polyposis coli (APC) gene were found to lead to multiple polyps in the colon, significantly increasing the risk of developing colon carcinoma (Kinzler and Vogelstein, 1996). This observation led to the discovery of  $\beta$ -catenin as an intracellular signaling protein in the WNT/ $\beta$ -catenin signaling pathway (Korinek et al., 1997). In the meantime, WNT signaling has been linked to many diseases, including osteoporosis, neurodegenerative diseases, and cardiovascular diseases (Hermans and Blankesteijn, 2015). It is important to note, however, that in most of these diseases an aberrant activation of the pathway rather than a mutation of one of its components is contributing to the disease process. This may have important implications for the choice of the therapeutic target, as will be discussed in *section XI* of this review.

## III. Components of the Receptor Complex

### A. Synthesis and Posttranslational Modifications of WNT

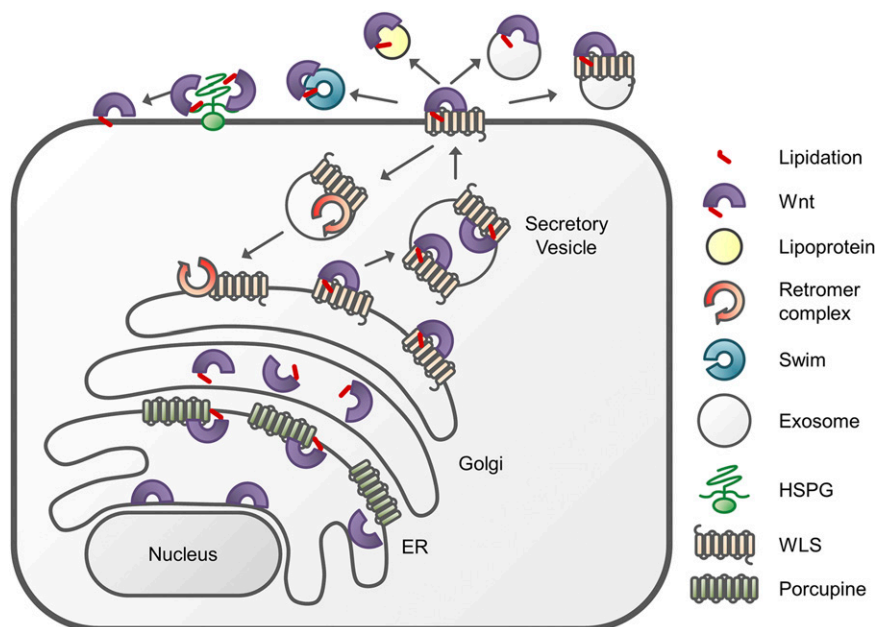
WNTs are secreted proteins for which homologs have been detected in multicellular organisms ranging from sponges to vertebrates. They contain 22–24 conserved cysteine residues, responsible for the maintenance of the spatial structure by the formation of disulfide bonds. The 19 *WNT* genes identified in mammals range in molecular weight from 39–46 kDa and belong to 12 subfamilies: many *WNT* genes (including *WNT2*, -3, -5, -7, -8, -9, and -10) have shown duplications, giving rise to two closely related family members (e.g., *WNT 3a* and *WNT 3b*). Wnts can also be classified according to their function. A large group of Wnts is capable of inducing oncogenic transformation and duplication of the body axis in developing *Xenopus* embryos (*WNT1*, -3a, -7a, -7b, -8), whereas another group does not (e.g., *WNT4*, -5a, -6, and -11). This distinction is related to different signal transduction pathways that are activated by these WNTs, as will be further discussed in *section V* of this review (Croce and McClay, 2008). However, it has

to be noted that not only the subtype of WNT protein, but also the FZD family member and probably also the coreceptors, are important in determining the choice of the signaling pathway (Schulte, 2015).

WNT proteins are subjected to extensive posttranslational modification to become secreted in a biologically active form. All WNT proteins carry a signal sequence that is required for their secretion (Langton et al., 2016). All WNT proteins except *Drosophila* WntD carry a palmitate group at a conserved cysteine residue located near the N terminus (Cys<sup>77</sup> in WNT3A) and a palmitoleate group at a conserved serine residue (Ser<sup>209</sup> in WNT3A). Originally, the palmitate was thought to be necessary for receptor binding, internalization, and signaling, whereas the palmitoleate group was thought to be required for intracellular transport (Willert et al., 2003; Schulte, 2010; Harterink and Korswagen, 2012). However, after the resolution of the crystal structure of the xWnt8/Fzd<sub>8</sub> complex, WNT was shown to have a three-dimensional structure resembling a hand, with two extension resembling the thumb and index finger grasping the CRD of FZD. The palmitoleated Ser<sup>209</sup> residue was found to be located at the tip of the so-called “thumb” region, which, together with the index finger, has been shown to be involved in the interaction of WNT proteins with receptors from the FZD family (Janda et al., 2012; Langton et al., 2016). Since the palmitated Cys<sup>77</sup> residue is not located at a distinctive region involved in receptor interaction, the role of its lipidation has become less clear. However, the addition of a hydrophobic group may help to concentrate active

WNT proteins at the cell membrane, which may assist in activation of signaling (Kaemmerer and Gassler, 2016). The addition of the palmitoleate group to WNT proteins takes place in the endoplasmic reticulum by an enzyme called porcupine (PORCN). PORCN is a member of the membrane-associated *O*-acyl transferase enzymes, which promote the coupling between a palmitoleate and the conserved serine residue in the thumb region. Next to lipidations, WNT proteins can also be glycosylated at two specific sites (Lorenowicz and Korswagen, 2009). For WNT3A, this glycosylation was found to precede the palmitoylation (Komekado et al., 2007). The glycosylation appears to be critical in controlling WNT folding and secretion, but is not essential for WNT signaling (Willert and Nusse, 2012).

Apart from its importance in binding to FZD, attaching a palmitoleate to the WNT protein also plays a crucial role in the secretion of WNT proteins because it stimulates the binding of WNTs to Wntless (WLS), a chaperone protein that escorts the palmitoleated WNT to the plasma membrane (Lorenowicz and Korswagen, 2009; Langton et al., 2016). As illustrated in Fig. 1, the binding of WNT to WLS occurs in the endoplasmic reticulum and is essential for the progression through the Golgi network. When reaching the plasma membrane, the WNT/WLS complex dissociates and WLS is recycled back to the Golgi apparatus by the retromer complex, which is responsible for endosome to Golgi trafficking (MacDonald et al., 2009; Harterink and Korswagen, 2012). Interestingly, WNT proteins have been shown to be released in a polarized fashion in



**Fig. 1.** Synthesis, posttranslational modification, and exporting of WNT proteins. After translation of WNT proteins, a palmitoleate group is attached by Porcupine in the endoplasmic reticulum (ER). This promotes the binding to Wntless (WLS), a chaperone protein that facilitates the migration of WNT through the Golgi complex. When WNT has reached the plasma membrane, it can either be secreted in exosomes or be attached to lipoproteins or Swim proteins; this is required to shield the large lipophilic moiety and increase the water solubility of the WNT protein. WNT can also attach to the plasma membrane of the producing cell by forming a complex with heparin sulfate proteoglycan (HSPG). Upon delivery of WNT to the plasma membrane, WLS is recycled via the retromer complex to the Golgi apparatus.

epithelial cells, with different secretion profiles on the basal and apical sides of the cell (Lorenowicz and Korswagen, 2009; Langton et al., 2016). However, there is still debate whether secretion of WNT proteins from cells is actually necessary for their biologic activity, because WNTs have been found attached to cytonemes, actin-based cellular extensions that extend distances of several hundreds of micrometers (Stanganello and Scholpp, 2016).

Due to the attachment of the lipophilic groups, the solubility of WNT proteins in an aqueous environment is poor. Therefore, four mechanisms have been proposed for the secretion of WNT proteins into the extracellular compartment: 1) association with a lipoprotein particle such as lipophorin in *Drosophila* (Panakova et al., 2005); 2) binding to the secreted WNT-interacting molecule Swim, a member of the lipocalin family (Mulligan et al., 2012); 3) spreading of WNT over the plasma membrane with the lipid group inserted in the membrane (Stanganello et al., 2015); and 4) secretion of WNT proteins in exosomes (Gross et al., 2012). The first two mechanisms are thought to increase the water solubility by shielding the palmitoleate group, whereas in the latter two mechanisms this group is inserted in the hydrophobic membrane environment (Langton et al., 2016).

### B. Frizzled Proteins

The name “frizzled” is derived from a *Drosophila* homolog of this gene, the mutants of which give rise to a distinctive derangement of the orientation of cuticular wing hairs and bristles (Gubb and Garcia-Bellido, 1982). Up until now, 10 FZD homologs have been identified in mammals. On the basis of structural homology, they can be clustered in five subfamilies: FZD<sub>1/2/7</sub>, FZD<sub>5/8</sub>, FZD<sub>9/10</sub>, FZD<sub>4</sub>, and FZD<sub>3/6</sub>; smoothened is a protein structurally related to the FZDs but no member of the FZD family. Major differences exist in the genetic structure of the different FZD genes with FZD<sub>3</sub> and FZD<sub>6</sub> having five introns in their coding region and FZD<sub>4</sub> having a single exon, whereas the other FZDs are intronless.

FZD proteins are a family of seven transmembrane (TM) receptors, meaning that there are seven helices of hydrophobic amino acids present in the protein allowing its embedding in a membranous structure. Although this feature is similar to many other members of the class of G protein-coupled receptors, the International Union of Basic and Clinical Pharmacology has categorized FZD proteins into a separate class, together with the smoothened protein, a member of the Hedgehog family (Foord et al., 2005). The reason for this classification is that FZDs have a unique extracellular domain at the N-terminal side, in which five disulfide bonds formed by 10 highly conserved Cys residues determine the three-dimensional shape. This so-called cysteine-rich domain (CRD) is thought to be the site of interaction

with WNT proteins. Interestingly, similar CRDs are present in other non-seven transmembrane (7TM) proteins such as soluble frizzled-related proteins (sFRPs), the receptor tyrosine kinase-like orphan receptor (ROR), carboxypeptidase Z and a splice variant of collagen XVIII. For several of these proteins, interaction with WNT has actually been demonstrated (Xu and Nusse, 1998). Upstream of the CRD, the N-terminal part of the FZD proteins contains a signal sequence, necessary for transport to the plasma membrane, which is cleaved off in the active receptor protein. The CRD of the receptor is connected to the 7TM domain by means of a spacer of variable length (Schulte, 2010). On the intracellular side of the receptor, three loops and the free C-terminal end of the protein are present as in all G protein-coupled receptors. Although several domains important for G protein coupling still need to be defined (Gammons et al., 2016), there is increasing evidence that, among coupling to other signal transduction pathways, G protein signaling can actually be activated by FZD proteins, as discussed in more detail in *section V.E* of this review (Dijksterhuis et al., 2014). A remarkable feature of the C-terminal part of the FZDs is the fully conserved KTxxxW domain, located at the eighth helix (Gammons et al., 2016). Mutation experiments have shown that this domain is indispensable for interaction with the PDZ-domain of disheveled (DVL), a protein involved in most WNT/FZD signal transduction pathways (Schulte, 2010).

Targeted mutagenesis experiments have revealed that FZDs can show significant functional redundancy within their subfamily, so mice lacking a single FZD gene quite frequently show no clear phenotype. In the context of this review, we focus on the (combinations of) deletions that give rise to a cardiovascular phenotype; for a comprehensive overview of the phenotypes of all FZD knockouts, we refer the reader to the recent review by Wang et al. (2016a). When focusing on the FZD<sub>1/2/7</sub> subfamily, *Fzd<sub>1</sub><sup>-/-</sup>* mice show no clear phenotype, whereas about half of the *Fzd<sub>2</sub><sup>-/-</sup>* mice show a cleft palate and ±15% of the *Fzd<sub>7</sub><sup>-/-</sup>* have a ventricular septal defect. Combining the different targeted mutants aggravates the frequency of the phenotypes: all *Fzd<sub>1</sub><sup>-/-</sup>*; *Fzd<sub>2</sub><sup>-/-</sup>* double knockouts were found to have a cleft palate and the majority of them had cardiac defects as well, ranging from a ventricular septal defect to a double outlet right ventricle (Yu et al., 2010). The combination of *Fzd<sub>2</sub><sup>-/-</sup>*; *Fzd<sub>7</sub><sup>-/-</sup>* was found to be embryonically lethal due to failure of closing its neural tube and failure to develop cardiac asymmetry, impairing cardiac function (Yu et al., 2012). These results demonstrate that adequate signaling via the FZD<sub>1/2/7</sub> subfamily is a prerequisite for a correct development of the heart.

Loss of FZD<sub>4</sub> has been shown to reduce vascular cell proliferation and migration. Moreover, tube formation by endothelial cells (ECs) is impaired, resulting in a diminished arterial network in heart and kidney

(Descamps et al., 2012). A severe defect in retinal vascularization was observed in mice lacking *Fzd4* (Wang et al., 2012). This is associated with the progressive development of hearing loss and degeneration of the cerebellum, the latter caused by leakage of the blood-brain barrier in these mice (Wang et al., 2001). The combined findings stress the importance of proper FZD<sub>4</sub> signaling for the development and maintenance of vascular function, which will be further elaborated in *section VII.C*.

### C. Lipoprotein-Related Receptor Protein 5/6

After the identification of FZD proteins as receptors for WNT, further research revealed that additional binding partners are required to activate WNT/ $\beta$ -catenin signaling. This led to the simultaneous publication by three independent research groups of two members of the low-density lipoprotein receptor family. These proteins are called low-density lipoprotein-related receptor (LRP) 5 and 6 and are homologs of *Drosophila Arrow* (Pinson et al., 2000; Tamai et al., 2000; Wehrli et al., 2000). These receptor proteins are over 1600 amino acids in size and have a single TM domain. The bulky extracellular part of LRP5 and 6 has a modular structure consisting of four six-bladed  $\beta$ -propellers connected by EGF-like domains. A flexible hinge region between the two pairs of  $\beta$ -propellers allows for some bending of the protein, giving rise to a concave top surface of the extracellular domain (Chen et al., 2011; Cheng et al., 2011b). The  $\beta$ -propeller part is connected to the transmembrane region via three LDL type A repeats. Overexpression of the LRP6 intracellular domain can activate WNT signaling in a constitutive fashion, confirming the regulatory role of the extracellular domain in the control of the signal transduction (Niehrs and Shen, 2010).

The intracellular part of LRP5/6 contains multiple phosphorylation sites, the phosphorylation of which is a key step in the initiation of the signal transduction via WNT/ $\beta$ -catenin signaling. Several kinases have been found to phosphorylate LRP6, either in a WNT-inducible (G protein receptor kinase 5/6) or WNT-independent (protein kinase A, PFTAIRES protein kinase 1) way. For an overview, we refer to a review article on this topic (Niehrs and Shen, 2010).

WNT proteins can bind to LRP5/6 at two different sites, albeit with lower affinity than to FZD proteins (Tamai et al., 2000). The most N-terminal  $\beta$ -propeller can bind most WNT members, with the exception of WNT3 and WNT3A that bind to the third  $\beta$ -propeller. Several endogenous inhibitors of LRP5 and 6, including members of the dickkopf (DKK) family, SOST and WISE, were shown to interact with the first  $\beta$ -propeller, whereas DKK proteins also bind to the third  $\beta$ -propeller. Interestingly, antibodies specific for each of the two binding sites have been generated, allowing the specific blocking of each site (Joiner et al., 2013).

Mice lacking either the *Lrp5* or *-6* gene show distinct phenotypes. Inactivating mutations of *Lrp5* led to low bone mass and abnormal retinal vascularization, combined with defects in cholesterol and glucose metabolism. Delays in mammary development and resistance to WNT1-induced mammary tumorigenesis have also been reported. Global deletion of *Lrp6* led to postnatal lethality with skeletal, neurologic, and mammary gland defects and cardiac abnormalities including a double outlet right ventricle. Double mutants show abnormal posterior patterning of the epiblast. Several mutations in *LRP5* and *-6* are linked to human diseases, including osteoporosis pseudoglioma, Alzheimer's disease and degenerative joint disease (Joiner et al., 2013). Similar to the phenotype of the *FZD4* mutant described above, mutations in *LRP5* can lead to familial exudative vitreoretinopathy (Toomes et al., 2004). The R611C mutation in *LRP6* was found to be associated with elevated serum lipids and glucose, hypertension, and premature coronary artery disease (Mani et al., 2007). Moreover, increased *LRP5* levels have been associated with calcified aortic valves, a finding closely linked to the importance of WNT signaling in bone formation, as described in more detail in *section VIII.B* (Rajamannan, 2011).

### D. Receptor Tyrosine Kinase-like Orphan Receptor 1/2 and Ryk

ROR1 and *-2* were initially identified because of their layout showing similarity to tyrosine kinase receptors. These receptor proteins contain a single TM domain and the intracellular parts display kinase activity. Unlike other tyrosine kinase receptors, however, these receptor proteins were found to have an extracellular CRD domain that closely resembles the CRDs of FZD proteins (Saldanha et al., 1998). This suggests that WNT proteins serve as ligands for ROR1 and *-2*, which was indeed shown to be the case (Oishi et al., 2003). In the meantime, it has become clear that, in contrast to LRP5/6, ROR1 and *-2* are mostly involved in  $\beta$ -catenin-independent WNT signaling (Green et al., 2014).

*Ror2* null mice show skeletal defects, abnormal orientation of inner ear hairs, and abnormalities in lungs and ventricular septal defects (Takeuchi et al., 2000). The phenotype of *Ror1*-deficient mice is less severe, but deletion of *Ror1* augments the phenotype of *Ror2* deficiency and introduces additional defects such as transposition of the great arteries. This suggests a genetic interaction between *Ror1* and *-2* in cardiac and skeletal development (Nomi et al., 2001).

Ryk proteins also have a single membrane-spanning domain, like the ROR proteins, but contain an extracellular domain with homology to WNT inhibitory factor (WIF) rather than the CRD found in FZD and ROR proteins. Moreover, Ryk also deviates from RORs in that its intracellular domain has no enzymatic activity. This suggests that Ryk most likely functions

as a coreceptor for WNTs together with FZD proteins (Green et al., 2014).

### *E. Interaction between WNT, FZD, and Their Coreceptors*

**1. Physical Interaction between WNT and FZD.** The selectivity and specificity of the WNT-FZD interaction is far from being fully understood. This obviously has to do with the large number of WNT and FZD homologs and the role of coreceptors in the binding and signal transduction. Moreover, the highly lipophilic nature of the WNT proteins forms an experimental challenge for traditional receptor-binding studies as it results in high levels of nonspecific binding (Dijksterhuis et al., 2014). Despite these technical difficulties, several authors reported experimental approaches to study the physical interaction between WNTs and the CRDs of different FZD homologs, including an enzyme-linked immunosorbent assay-based protein-protein interaction assay (Carmon and Loose, 2008, 2010) and immunoprecipitation (Sato et al., 2010a). Moreover, in many publications, functional assays such as TOPFlash/TOPGal reporter assays or receptor internalization were used as a readout for WNT/FZD interaction (Schulte, 2010). In most of these experiments, the affinity of the WNT/FZD interaction was found to be in the nanomolar range. From the available experimental evidence, of which a nice overview is provided by Dijksterhuis et al. (2014), it can be deduced that most FZDs can interact with multiple WNTs, whereas FZD<sub>9</sub> and FZD<sub>10</sub> appear to interact exclusively with WNT2 and WNT7B, respectively. Interestingly, there appears to be no such thing as “WNT/ $\beta$ -catenin” or “WNT/ $\beta$ -catenin independent” FZDs as both WNT3A (a prototype activator of WNT/ $\beta$ -catenin signaling) and WNT5A (a prototype activator of WNT/ $\beta$ -catenin independent signaling) can bind to the vast majority of FZDs. However, a word of caution is in place here: the fact that a physical interaction between certain WNTs and FZDs can be detected under artificial experimental conditions does not necessarily mean that these interactions also occur in vivo and/or can lead to signaling under physiologic conditions.

**2. Structural Data.** Despite the publication of the structure of the CRD of the FZD protein (Dann et al., 2001), little was known about the site(s) of interaction between WNT and FZD until the publication of the crystal structure of a glycosylated *Xenopus* Wnt8/mouse Fzd<sub>8</sub>-CRD complex (Janda et al., 2012). A first hint toward the mode of interaction could be derived from the observation that the water solubility of this complex was far better than for xWnt8 alone, suggesting a shielding of the hydrophobic palmitoleate group by the mFzd<sub>8</sub> CRD. This allowed the crystallization of the xWnt8/mFzd<sub>8</sub>-CRD complex in a detergent-free environment. In this complex, Wnt appears to be shaped like a hand where the two ends, conveniently named

“thumb” and “index finger,” grasp the mFzd<sub>8</sub> CRD. The thumb region, consisting of amino acids 185–195, contains two disulfide bridges stabilizing the tertiary structure of the loop. The palmitoleate group, located at the tip of the thumb region, directly fits into a groove located in the mFzd<sub>8</sub>-CRD in which multiple nonpolar amino acids are present for interaction with the lipid extension. These amino acids appear to be present in all FZD CRDs, suggesting a conserved mode of interaction between multiple WNTs and FZDs.

The site of interaction between the index finger of xWnt8 and the CRD of mFzd<sub>8</sub> is located on the opposite side of the horseshoe and consists of a loop formed by the amino acid residues Arg<sup>301</sup> to Cys<sup>338</sup>. The structure of the index finger consists of a long beta strand in which three disulfide bonds maintain the tertiary structure. The tip is characterized by an unusual disulfide tandem formed by Cys<sup>320</sup>, Cys<sup>321</sup>, and a Trp<sup>319</sup> residue, the side chain of which fits in pocket on the FZD<sub>8</sub> surface. The C-terminal part of the xWnt8 protein, containing only the index finger, could bind to several FZD CRDs with variable affinity, suggesting that this interaction may be responsible for selectivity of Wnt/FZD interaction (Janda et al., 2012).

**3. Interaction with Coreceptors.** The sites of interaction between WNTs and their coreceptors, such as LRP5/6, Ryk, and ROR2, are not yet resolved by crystallography but based on the identification of conserved regions. An analysis of the 19 WNT sequences showed a large patch of high conservation, located at the top of the central part of the WNT protein. This patch contains three short but highly conserved and solvent-exposed stretches of amino acids that localize in close proximity in the top central part of the WNT protein and was predicted to be the interaction site for the coreceptors. However, experimental evidence that this site is actually binding to the coreceptors still has to be provided (Janda et al., 2012).

**4. Formation of Multimeric Complexes.** An increasing number of G protein-coupled receptors has been shown to be able to aggregate into homologous or heterologous oligomeric complexes, affecting their ligand binding, signaling, and regulation (Milligan, 2009). Several studies have shown the formation of multimeric FZD complexes (Schulte, 2015), which is sufficient to activate signaling (Carron et al., 2003). Spontaneous dimerization was also reported for FZD<sub>3</sub> receptors in *Xenopus*, whereas the forced dimerization of the CRDs of xFZD<sub>7</sub> induced activation of signal transduction in this species (Carron et al., 2003). Although the presence of CRD dimers appears to be contradicted by the 1:1 stoichiometry observed in the crystallography data for the xWnt8/FZD<sub>8</sub> complex described above, a computational modeling study showed the docking of a dimeric FZD<sub>1</sub> CRD complex inside the U-shaped space formed by the thumb and index finger of WNT1 (Ain et al., 2013). Additional research will be



required to identify the stoichiometry of WNT/FZD interaction under physiological conditions and the potential effects on signaling of single versus multi-meric CRDs.

#### IV. Regulation of WNT Signaling

WNT signaling is not only characterized by a large number of ligands, receptors, and coreceptors, but can also be regulated at many different levels. Regulation can take place by intervention in the synthesis and excretion of WNT (e.g., by Notum), its lipidation (e.g., by PORCN), by inactivation of the WNT protein (e.g., by Tiki), by scavenging of WNT proteins (e.g., by sFRPs), and by controlling the density of FZD proteins at the plasma membrane (e.g., by LGR5). Moreover, inhibitors of the LRP5/6 coreceptors (DKK, sclerostin) have been described to control signaling. Interestingly, non-WNT-related activators of the signaling pathways, such as R-spondins and norrin, have also been identified. In this review, we will focus on those regulatory mechanisms that have been described in mammals and are likely to have a link with human disease (Fig. 2).

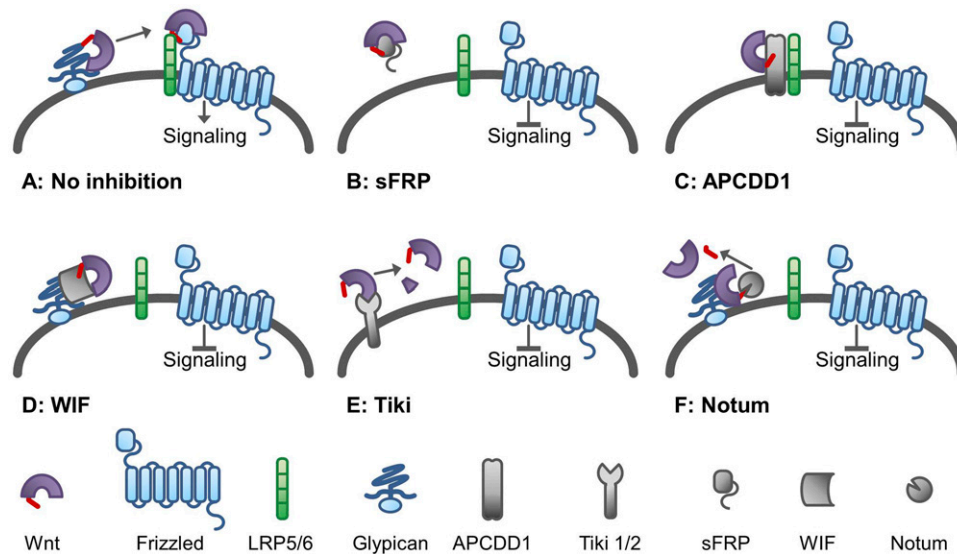
##### A. WNT/Notum/Tiki

In addition to a complex intracellular machinery for the secretion of WNT proteins, there are several extracellular mechanisms controlling the activity of secreted WNT proteins. Membrane-associated glypicans (named *Dally* in *Drosophila*) can bind WNT proteins to the cell surface, regulating their extracellular distribution and

signaling activity. Adenomatous polyposis coli down-regulated 1 (*APCDD1*) has a similar mode of action and has been shown to inhibit WNT signaling in cell culture assays (Cruciat and Niehrs, 2013). More recently, WNT-specific metalloproteinases from the Tiki family have been described that can cleave the first 9–20 amino acids from amino-terminal side of WNT2B, -3, -3A, -5A, -5B, -6, -8A, -8B, and -16, thereby rendering them inactive (Zhang et al., 2016b). Finally, a carboxylesterase named Notum has been shown to enzymatically remove the palmitoleate from WNT proteins, which results in a loss of biologic activity (Kakugawa et al., 2015). The many mechanisms involved in the secretion and (in)activation of WNT proteins highlight the complexity and tight control of WNT activity.

##### B. Secreted Frizzled-Related Proteins

As the name implies, sFRPs resemble FZD proteins in that they contain a CRD at the N-terminal side of the protein that shows 30%–50% sequence similarity with the FZD CRD. The first sFRP, originally named SFRB, which later turned out to be sFRP3, was in fact discovered based on its homology to FZD proteins (Hoang et al., 1996). In humans, five different sFRPs have been identified varying in size from 295 to 346 amino acids (Cruciat and Niehrs, 2013). The CRD contains 10 conserved Cys residues; the disulfide bridges formed by these residues contribute to the tertiary structure of the CRD. At the C-terminal side of the protein, a Netrin-related motif is located containing six conserved Cys-residues that form three disulfide



**Fig. 2.** Mechanisms of regulating WNT signaling activity. (A) In the absence of regulating mechanisms, WNT can bind to the FZD/LRP5/6 complex and activate signaling. (B) In the classic view, soluble frizzled-related proteins (sFRPs) can bind WNT and prevent its interaction with the receptor complex. However, sFRPs can modulate Wnt signaling in different ways, as described in the text (C). Adenomatous polyposis coli downregulated-1 (*APCDD1*) is a membrane-bound glycoprotein that can bind WNT and prevent its interaction with the receptor complex. (D) WNT-inhibitory factor (*WIF*) is a secreted protein shown to attenuate WNT signaling by binding to the protein that prevents the association with the receptor complex. (E) *Tiki* is a transmembrane protein with proteolytic activity, cleaving a peptide of 8–20 amino acids from the N-terminal part of WNT proteins. This causes the formation of oligomers of WNT proteins that are inactive in signal transduction. (F) *Notum* is a carboxylesterase capable of removing the palmitoleic acid residue from WNT, making the protein biologically inactive.

bridges (Chong et al., 2002). Netrin-related motifs are present in many unrelated proteins, including the axon-guidance molecule Netrin, tissue inhibitors of metalloproteinases (TIMPs), type-1 procollagen C-protein enhancer proteins, and complement proteins C3, C4, and C5 (Cruciat and Niehrs, 2013). The sFRPs can be classified into two subgroups based on phylogenetic analysis: the sFRP1, -2, and -5 subgroup and the sFRP3 and -4 subgroup (Jones and Jomary, 2002). Posttranslational modifications have been observed in sFRP1 and -5 (N-glycosylation and sulphation of two Tyr residues), but these are absent in sFRP2, -3, and -4 (Bovolenta et al., 2008).

The main mechanism of action of sFRPs is the inhibition of WNT signaling by scavenging extracellular WNT proteins, thereby preventing their interaction with the receptor complex. At first sight, the CRD region of the sFRPs appears to be responsible for this scavenging effect, as it can be anticipated to bind WNT proteins in a fashion similar to FZD proteins (Cruciat and Niehrs, 2013). However, structure-function analyses of sFRP1 has shown that the Netrin-related motif in the C-terminal region contributes to the inhibition of WNT function (Bhat et al., 2007; Bovolenta et al., 2008). The binding affinity for the interaction of sFRPs and WNT proteins has been reported to be in the nanomolar range, similar to the affinities of the WNT-FZD interaction. However, some specificity has been reported for the sFRP-WNT interaction: WNT3A binds to all sFRPs except sFRP5, whereas WNT5 only binds to sFRP1 and -2. As for the WNT-FZD interaction, so far no comprehensive studies have been published where all possible combinations were tested in a systematic way (Bovolenta et al., 2008).

In the meantime, several studies on sFRPs have offered conflicting results. Different sFRPs were shown to have opposing effects on apoptosis in breast tumors, and sFRP2 was shown to block the inhibitory effects of sFRP1 on kidney tubule formation (Bovolenta et al., 2008). Moreover, structural analysis of the CRDs of sFRPs has suggested that these CRDs might be able to form homo- or heterodimers with CRDs or either FZD or other sFRPs (Dann et al., 2001). Based on these observations, four mechanisms have been proposed by which sFRPs can modulate WNT signaling, two of which are inhibitory and two of which are stimulatory: 1) by scavenging WNT protein, which inhibits signaling because WNT is unavailable for interaction with its receptors; 2) by forming homo- or heteromeric sFRP complexes, which would favor WNT signaling; 3) by direct interaction with the CRDs of FZD, which would inhibit WNT signaling; and 4) by binding WNT to the Netrin-related motif, presenting WNT to the CRD of FZD, which would stimulate WNT signaling (Bovolenta et al., 2008). It is at present not clear how this control mechanism is directed in either of these four directions.

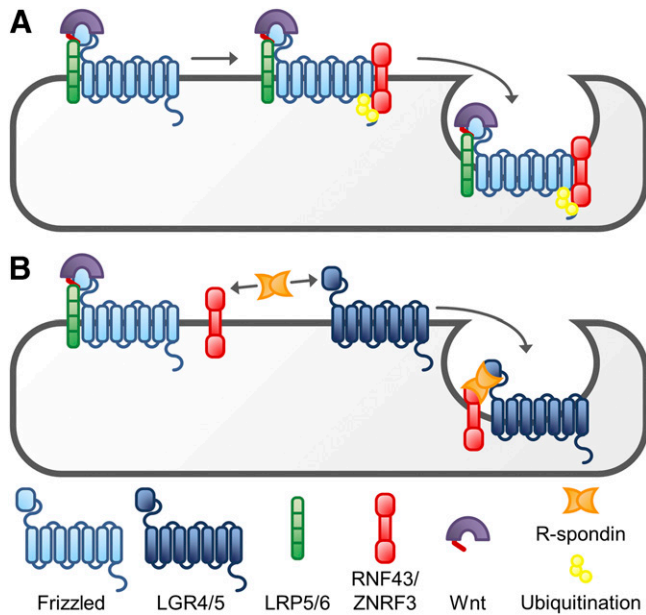
### C. WNT-inhibitory Factor

WIF1 is a 379-amino acid protein that has been shown to inhibit WNT signaling by binding WNT proteins (Hsieh et al., 1999). The protein contains a WIF domain, five EGF repeats, and a hydrophilic tail. In fact, the WIF domain is similar to that present at the extracellular end of the RYK tyrosine kinase receptor. WIF1 has been shown to interact with multiple WNTs from both the WNT1 (WNT/ $\beta$ -catenin) and WNT5A (WNT/ $\beta$ -catenin independent) subclasses. However, it is not clear what the mechanism of action is for the regulation of WNT signaling (Cruciat and Niehrs, 2013).

### D. Leucine-rich Repeat-containing G Protein-Coupled Receptor 4/5/R-Spondin

Regulation of WNT signaling does not only depend on the amount of WNT available for receptor activation, but also on the receptor density on the cell membrane. A very effective mechanism for the regulation of the density of FZD proteins has been identified by analysis of WNT target gene expression (Fig. 3). Two highly homologous single transmembrane proteins, RNF43 and ZNRF3, were found to be upregulated as result of activation of WNT signaling. Both RNF43 and ZNRF3 are E3 ligases, capable of transferring ubiquitin groups to multiple lysine residues located in the intracellular loops of FZD proteins via the RING domain that is present in their cytoplasmic tail. This ubiquitination induces the endocytosis of the FZD proteins, making them inaccessible for ligand stimulation and causing an effective downregulation of WNT signaling (Hao et al., 2012; Koo et al., 2012).

Interestingly, the localization at the plasma membrane of the E3 ligases RNF43 and ZNRF3 is under the control of another group of WNT target genes, LGR4, -5, and -6. LGR5 was originally identified as a marker for intestinal stem cells (Barker et al., 2007). Together with LGR4 and -6, these receptors form a subgroup of the leucine-rich repeat-containing G protein-coupled receptor (LGR) family. This seven-transmembrane receptor family is characterized by a large extracellular domain consisting of leucine-rich repeats, which is connected to the seven-transmembrane domain via a hinge region. Other members of this family are the receptors for follicle-stimulating hormone (LGR1), luteinizing hormone (LGR2), and thyroid-stimulating hormone (LGR3) (de Lau et al., 2014). The ligands for LGR4 and 5, members of the R-spondin family, are known as secreted enhancers of WNT signaling (de Lau et al., 2011). Binding of an R-Spondin to the leucine-rich repeat of LGR4/5 initiates the formation of a complex with RNF43 or ZNRF3, which is rapidly internalized. The loss of RNF43 and ZNRF3 from the plasma membrane leads to an attenuation of the ubiquitination of FZD, so more FZD remains at the plasma membrane to interact



**Fig. 3.** Regulation of the amount of FZD protein on the plasma membrane. (A) Association of the transmembrane E3 ligases RNF43 and/or ZNRF3 with the FZD receptor complex induces its ubiquitination and leads to internalization of the receptor complex, making it unavailable for stimulation with WNT. (B) R-spondin is capable of redirecting the E3 ligases toward LGR4 or -5, inducing the internalization of this transmembrane protein rather than the FZD/LRP5/6 complex. This effectively leaves more FZD receptors at the plasma membrane, available for stimulation with WNT proteins.

with WNT and activate the signal transduction (de Lau et al., 2014). It has to be noted, however, that an alternative mechanism has been described for the internalization of FZD4, i.e., via a DVL2-mediated recruitment of  $\beta$ -arrestin-2 (Chen et al., 2003).

### E. Dickkopf

The first DKK protein was originally identified as an inducer of the Spemann organizer, a tissue with strong head-inducing capacity, which explains the name of the protein (dickkopf is German for big head) (Glinka et al., 1998). The DKK family consists of four members in vertebrates, named DKK1-4, varying in size between 255 and 350 amino acids. DKK1, -2, and -4 have a high structural similarity with an N-terminal cysteine-rich domain (DKK\_N) and a C-terminal collipase fold (DKK\_C). DKK3 deviates from the other DKK family members in that this protein contains an additional soggy domain that is also found in dickkopf-like protein-1 (Cruciat and Niehrs, 2013).

DKK proteins -1, -2, and -4 can bind to LRP5/6 coreceptors with nanomolar affinity, thereby acting as functional antagonists of WNT/ $\beta$ -catenin signaling (Joiner et al., 2013). As described above, the extracellular part of LRP5/6 consists of two pairs of  $\beta$ -propellers connected by EGF-like domains. Crystallization studies revealed that the DKK1\_C domain binds to LRP6  $\beta$ -propeller pair located closest to the plasma membrane. Unexpectedly, however, the complex appeared to

consist of an equimolar mixture of a bound LRP6 inner propeller pair-DKK1\_C complex and an unbound LRP6 inner propeller pair, a finding that has not yet been explained. The DKK1\_N binds to the outer LRP6  $\beta$ -propeller pair at the site where interaction with most of the WNT proteins takes place (Ahn et al., 2011).

Apart from binding to LRP5/6, DKK1 and -2 have been shown to bind to Kremen receptors 1 and 2 with high affinity. The interaction between LRP6, DKK, and Kremen results in the rapid endocytosis of the complex from the plasma membrane, thereby providing an additional mechanism for the inhibition of WNT signaling (Mao et al., 2002).

### E. Micro-RNAs

Micro-RNAs (miRNAs) are short noncoding RNA molecules that can regulate the translation of many genes by controlling mRNA stability. Most miRNAs are encoded by genomic regions distant from annotated genes but some are located in introns of annotated genes. The primary transcripts are subsequently processed by Drosha and Dicer to form the typical double-stranded RNA strands with a length of 20–25 nucleotides. One of these strands associates with the RNA-induced silencing complex to interact with the target mRNA. This interaction is initiated by the seed sequence, a stretch of 6–8 nucleotides located at the 5' terminus of the miRNA and stabilized by the anchor sequence at the 3'-side of the miRNA. It is now increasingly clear that the translation of multiple genes can be regulated by a single miRNA, but on the other hand, single target mRNA can have multiple miRNA binding sites (Song et al., 2015). Over the last years an overwhelming number of studies have been published where the role of miRNA regulation in a variety of diseases and signaling pathways, including WNT signaling, is reported (Anton et al., 2011).

The expression of virtually all components of the WNT/ $\beta$ -catenin signal transduction pathway are under the control of at least one, but usually many more, miRNAs. This is also true for many of the inhibitors, including sFRPs, DKK proteins, and WIF. The result is a highly dynamic posttranscriptional regulation of the pathway, which may contribute to diseases such as cancer. A comprehensive overview of the many miRNAs regulating the different components of WNT signaling is beyond the scope of this review so we refer to a recent publication by Peng et al. (2017), where the crosstalk between miRNAs WNT/ $\beta$ -catenin signaling is extensively discussed. Here we will focus on the miRNAs that have been reported to regulate WNT components in a cardiovascular context.

Regulation of WNT signaling by miRNAs has been shown to affect cardiac physiology in several studies. As an example, overexpression of miR-19b targets the expression of *ctnnb1*, the gene encoding  $\beta$ -catenin, thereby disturbing the left-right symmetry and the

development of the heart in zebrafish embryos (Li et al., 2014a). Both miR-218 and miR-499 have been shown to affect cardiomyocyte differentiation of cardiac and mesenchymal stem cells via modulation of the expression of sFRP2 and  $\beta$ -catenin, respectively (Zhang et al., 2012; Wang et al., 2016b). miRNAs can also modulate the activation of cardiac fibroblasts. This was recently shown for miR-154, which, by suppressing the DKK2 expression, activated WNT signaling (Sun et al., 2016). Moreover, the stability of atherosclerotic lesions was found to be enhanced by miR-210, targeting the expression of the APC gene and thereby regulating smooth muscle cell differentiation and survival (Eken et al., 2017). It is highly likely that these studies just form the starting point for a multitude of investigations where the modulation of WNT signaling via miRNAs will be further explored in a cardiovascular context.

## V. Signal Transduction

### A. Disheveled

The DVL proteins form an essential component in the transduction of WNT signaling from the receptor level to the intracellular compartment. DVL acts as a scaffold for the formation of the WNT/FZD receptor complex. The protein was originally described as a segment polarity gene required for wingless signaling in *Drosophila* (Klingensmith et al., 1994; Noordermeer et al., 1994) where *disheveled* mutants show a phenotype similar to the *wingless* mutant (Nusslein-Volhard and Wieschaus, 1980). Three DVL homologs have been identified in vertebrates, but the domain structure of the protein is highly conserved. At the N-terminal side of the protein, a DIX (disheveled-axin) domain that, as the name implies, is involved in the binding of the axin protein. The DIX domain is involved in the transduction of WNT/ $\beta$ -catenin signals, where a complex of DVL, FZD, axin, and LRP5/6 is formed. In the central part of the DVL protein, there is a PDZ domain where the interaction with the KTxxxW motif of FZD proteins takes place. At the C-terminal side of the protein the DEP (disheveled-EGL10-plekstrin) domain is located. This domain can interact with a bipartite motif in the third intracellular loop of FZD and forms electrostatic interactions with membrane lipids. Recently, the DEP domain was shown to undergo a conformational change, followed by its dimerization, to trigger the formation of the WNT/FZD signalosome (Gammons et al., 2016). All DVL proteins are heavily phosphorylated and, although the mechanisms leading to this modification are still obscure, some phosphorylation sites have been shown to be necessary for DVL function (Dijksterhuis et al., 2014; Mlodzik, 2016).

Targeted mutations of the *Dvl* genes in mice give rise to relatively mild phenotypes. The *Dvl1* knockout mouse shows neuronal and social behavioral

abnormalities (Lijam et al., 1997). *Dvl2* and *Dvl3* null mutants show cardiac outflow tract defects, including double outlet right ventricle, transposition of the great arteries, or persistent truncus arteriosus (Hamblet et al., 2002; Etheridge et al., 2008). All these abnormalities can be attributed to defects in PCP signaling, whereas DVL proteins show a high level of redundancy for WNT/ $\beta$ -catenin signaling: the presence of only a single allele of one of the three *Dvl* genes is sufficient for  $\beta$ -catenin-mediated signaling in mice (Wynshaw-Boris, 2012).

Next to their role in WNT/ $\beta$ -catenin and WNT-PCP signaling, several other cellular functions have been described for DVL proteins, including nuclear functions, localization and/or anchoring of ciliary basal bodies in multiciliated cells, inhibition of Notch signaling, and a WNT-independent role in cell viability (Mlodzik, 2016). The relevance of these mechanisms, compared with the role of DVL in WNT signaling, still needs to be elucidated.

### B. WNT/ $\beta$ -Catenin Signaling

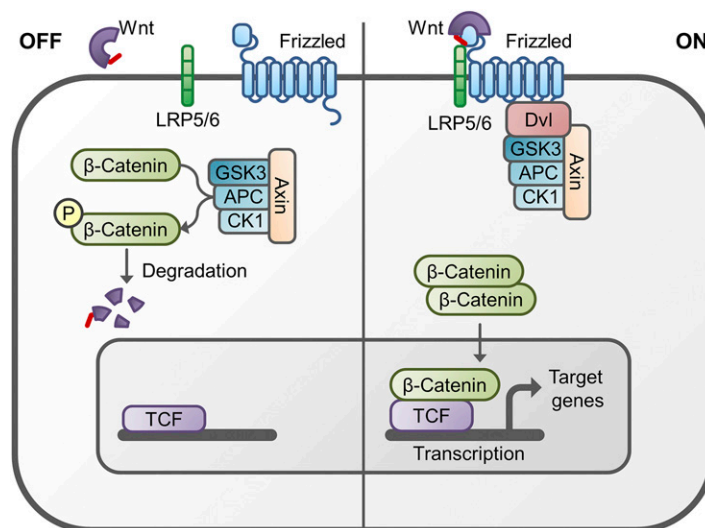
1. *Characteristics of  $\beta$ -Catenin.*  $\beta$ -Catenin is a central molecule in the WNT/ $\beta$ -catenin signaling pathway.  $\beta$ -Catenin is protein of  $\sim 80$  kDa that is present throughout the animal kingdom. The characteristics of this multifunctional protein have been extensively described in a review by Valenta et al. (2012), so we will only provide a brief summary here.  $\beta$ -Catenin has two rather diverse functions in the cell: on the one hand, it has a structural role as part of the cadherin-catenin cell adhesion complex, whereas on the other hand it acts as a modulator of gene transcription. It has a highly conserved structure with 12 so-called Armadillo repeats (Armadillo is the *Drosophila* homolog of  $\beta$ -catenin) in the middle, flanked by N-terminal and C-terminal domains. The Armadillo repeats form a positively charged groove where many partners, including cadherins, APC protein, and transcription factors from the TCF/LEF family, can bind. Extensive mutation analyses have demonstrated that the N-terminal side of the Armadillo domain supports heterodimerization with  $\alpha$ -catenin and subsequent formation of the cadherin-catenin complex, whereas the region C-terminal from the Armadillo domain is involved in the binding of transcription coactivators. There are multiple phosphorylation sites present in  $\beta$ -catenin, allowing the fine tuning of its function and degradation. (Valenta et al., 2012).

The  $\beta$ -catenin-dependent signal transduction pathway is unusual in that activation of WNT/FZD signaling inhibits the degradation of the intracellular signaling protein  $\beta$ -catenin, leading to a higher concentration of  $\beta$ -catenin inside the cell. This is in contrast to most GPCRs, the activation of which stimulates the intracellular production of a second messenger rather than its degradation. This  $\beta$ -catenin subsequently can either move to the cell adhesion complex or migrate to the

nucleus where it can activate gene transcription (Fig. 4). At present it is not clear how  $\beta$ -catenins distribute over the nucleus and cell adhesion complexes. In contrast to the nuclear  $\beta$ -catenin pool, which has a half-life of minutes,  $\beta$ -catenin that is incorporated in cell adhesion complexes appears to be very stable (Clevers and Nusse, 2012). This rapid turnover of  $\beta$ -catenin in the nucleus may create a concentration gradient with the cytoplasm, favoring the transport to the nucleus, where  $\beta$ -catenin can be bound to a transcription complex, as will be discussed in detail below.

**2. The  $\beta$ -Catenin Destruction Complex.** In the absence of WNT signaling, a  $\beta$ -catenin destruction complex is formed in the cytoplasm consisting of axin, APC protein, glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ), casein kinase 1 $\alpha$  (CK1 $\alpha$ ), and  $\beta$ -catenin. The axin protein contains distinct binding domains for these constituents of the complex and therefore acts as a scaffold protein. Upon the formation of the complex, sequential phosphorylation of  $\beta$ -catenin takes place. The first phosphorylation is at Ser<sup>45</sup> by CK1 $\alpha$ , followed by phosphorylation of Thr<sup>41</sup>, Ser<sup>37</sup>, and Ser<sup>33</sup> by GSK3 $\beta$ . Phosphorylated  $\beta$ -catenin is released from the complex and bound by the E3 ubiquitin ligase  $\beta$ -Trcp (also called TRCP-1) for ubiquitination at the N-terminal end of the protein and subsequent degradation. This results in a lowering of the cytoplasmic  $\beta$ -catenin content. axin and APC can also be phosphorylated by GSK3 $\beta$  and CK1 $\alpha$ , leading to enhanced  $\beta$ -catenin phosphorylation. The phosphorylation mechanisms are counteracted by two phosphatases, PP1 and PP2A, which can dephosphorylate axin and  $\beta$ -catenin, respectively, resulting in an increase in the amount of cytoplasmic  $\beta$ -catenin (MacDonald et al., 2009).

**3. Activation of WNT/ $\beta$ -Catenin Signaling.** Activation of WNT/ $\beta$ -catenin signaling results in the formation of a receptor complex consisting of WNT, FZD, and LRP5/6 at the plasma membrane. DVL is recruited to this receptor complex and, subsequently, axin, GSK3 $\beta$ , and CK1 migrate from the destruction complex toward the plasma membrane. The two kinases phosphorylate each of the five PPPSPxS motifs present on the intracellular tail of LRP5/6, creating a docking site for axin and thereby promoting WNT signaling (Wolf et al., 2008). Opposite to the phosphorylation of  $\beta$ -catenin, here GSK3 $\beta$  appears to be priming kinase as CK1 can only phosphorylate PPPSPxS motifs previously phosphorylated by GSK3 $\beta$  (Niehrs and Shen, 2010). The migration of axin to the receptor complex at the plasma membrane results in a disruption of the  $\beta$ -catenin destruction complex, leading to an accumulation of the intracellular signaling protein in the cytoplasm (MacDonald et al., 2009). In the meantime, several other mechanisms have been described by which activation of WNT signaling leads to cytoplasmic accumulation of  $\beta$ -catenin. These mechanisms include the WNT-induced degradation of axin, direct inhibition of GSK3 $\beta$  through the phosphorylated cytoplasmic tail of LRP6, and internalization of the signalosome into multivesicular bodies, which shields the cytoplasmic  $\beta$ -catenin from GSK3 $\beta$  by the vesicular membrane (MacDonald and He, 2012). Moreover, tankyrase is an enzyme that positively regulates WNT/ $\beta$ -catenin signaling by attaching a poly-ADPribose chain to axin, thereby targeting this scaffold molecule for degradation (Guettler, 2016). At present it is unclear what the individual contributions of these different mechanisms



**Fig. 4.** Schematic representation of the WNT/ $\beta$ -catenin signal transduction pathway. (Left) In the “Off” state,  $\beta$ -catenin is bound in a so-called  $\beta$ -catenin destruction complex containing glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ), axin, adenomatous polyposis coli (APC) and casein kinase-1 (CK-1). The kinases in this complex phosphorylate  $\beta$ -catenin, thereby targeting it for degradation by the ubiquitin proteasome system. (Right) In the “On” state, the receptor complex consisting of frizzled and LRP5/6 bind WNT, which recruits the disheveled (DVL) protein to the plasma membrane. Subsequently, several components of the  $\beta$ -catenin destruction complex are recruited to the membrane, which prevents the phosphorylation of  $\beta$ -catenin. Therefore, this protein can now accumulate in the cytoplasm and translocate to the nucleus to associate with transcription factors and stimulate the transcription of WNT target genes such as cycline-D1, c-myc, and axin2.

are to the accumulation of cytoplasmic  $\beta$ -catenin upon WNT stimulation.

4. *Nuclear Translocation of  $\beta$ -Catenin and Gene Expression.* The accumulated cytoplasmic  $\beta$ -catenin can translocate to the nucleus, where it can activate transcription of WNT-responsive genes (Clevers and Nusse, 2012). Because the nucleus is surrounded by a membrane that is poorly permeable for large proteins, it contains multiple pores formed by nuclear core complexes. Most proteins require the interaction with specific nuclear importing or exporting proteins that act as chaperones during the nuclear membrane transport. The nuclear membrane transport of  $\beta$ -catenin, however, appears to be unusual because it can migrate over the nuclear membrane by a direct interaction with the nuclear core complex (Jamieson et al., 2014). Recently, interaction of hydrophobic patches in the tail region of  $\beta$ -catenin with the nuclear pore complex has been reported, which may explain this specific interaction (Sharma et al., 2016).

The  $\beta$ -catenin protein does not contain a DNA binding domain. This implies that for regulation of gene expression,  $\beta$ -catenin needs to form a complex with transcription factors to bind to WNT-responsive elements in the promoter region of its target genes.  $\beta$ -Catenin can form a complex with members of the TCF/LEF family of transcription factors that bind to specific DNA sequences, which are conserved from *Drosophila* to man (Molenaar et al., 1996; van de Wetering et al., 1997). In the absence of  $\beta$ -catenin, TCF is bound to the transcriptional repressor Groucho, which effectively silences the TCF-mediated gene expression.  $\beta$ -Catenin is capable of replacing Groucho, which is the first step in the formation of a transcription complex. In mammals, four TCF homologs, respectively named TCF-1, -3, and -4 and LEF-1, are present. The  $\beta$ -catenin/LEF-1 complex typically activates gene expression, whereas the  $\beta$ -catenin/TCF-3 complex acts as a repressor. The binding of TCF/LEF transcription factors involves the central region of the  $\beta$ -catenin protein (Arm repeats 3–10). Complexes of  $\beta$ -catenin with TCF-1 and -4 can serve both as activators and repressors, depending on the context. Moreover, each of them have different splice variants that can affect their  $\beta$ -catenin binding potential. (Valenta et al., 2012).

Additional regulation of the  $\beta$ -catenin/TCF complex is provided by several transcriptional coactivators. These coactivators can have different functions, such as modifying the chromatin structure (e.g., CBP, p300, BCL9, and Tip60), the nucleosome arrangement (e.g., SWI and ISWI), or the association of the  $\beta$ -catenin/TCF complex with RNA polymerase II (members of the Mediator and paf1 complex) (MacDonald et al., 2009). Most of these co-activators, however, are not specific for the  $\beta$ -catenin/TCF complex. In contrast, the coactivator BCL9 interacts specifically with the first Armadillo repeat of  $\beta$ -catenin. In association with the coactivator Pygopus, BCL9 introduces specificity to the transcription

complex (Clevers and Nusse, 2012; Valenta et al., 2012). The formation of the transcription complex can activate the expression of a large number of WNT target genes that are mostly specific for certain tissues and/or developmental stages (Lustig et al., 2002). Recently, nuclear roles for other catenin family members such as  $\alpha$ -catenin and P120-catenin have been proposed that may also participate in the regulatory control of WNT signaling. This suggests the existence of a network of nuclear catenins involved in gene expression control (McCrea and Gottardi, 2016).

#### C. WNT/ $Ca^{2+}$ Signaling.

One of the most abundantly used second messengers is  $Ca^{2+}$ , leading to processes as diverse as muscle contraction, activation of enzymes such as PKC, and gene transcription.  $Ca^{2+}$ -mediated signal transduction of FZD proteins was already reported in the 90s to involve PKC and phosphatidyl inositol signaling (Cook et al., 1996; Slusarski et al., 1997). The current view on this topic is that two pathways can be employed in WNT/ $Ca^{2+}$  signaling, one involving phospholipase-C-mediated production of inositol triphosphate and diacylglycerol and the other via the cyclic GMP-selective phosphodiesterase and p38-MAPK (Ma and Wang, 2007). It is generally accepted that for both pathways the interaction between FZD proteins and heterotrimeric G proteins is needed, more specifically the  $G_{\alpha_{i/o}}$  and the  $G_{\alpha_t}$  subfamilies; for further details, refer to *section V.E* of this review. At present it is not conclusively established whether DVL is essential for the activation of WNT/ $Ca^{2+}$  signaling (Schulte, 2010).

#### D. WNT/*Planar Cell Polarity* Signaling

During development, cell proliferation and differentiation are required to make the embryo grow and develop different organs and tissues. However, another essential mechanism is the transfer of directional information to form specific structures and to allow organs and/or tissues to grow in their final shape. Planar cell polarity (PCP) was identified as a mechanism that is involved in the generation of both global and local directional information. Examples are the morphologic changes in mesenchymal cells but also the apical/basal polarization of epithelial cells (Yang and Mlodzik, 2015). PCP was first demonstrated in *Drosophila* where cuticular structures such as bristles and wing hairs show a clear polarization (Adler, 2012). In vertebrates, PCP signaling regulates anterior-posterior body axis elongation during gastrulation through convergent extension and is also involved in neural tube formation. PCP signaling is also necessary for the orientation of sensory hair cells in the inner ear, as well as the orientation of hair follicles in mouse skin and cilia in airway epithelium (Yang and Mlodzik, 2015). Although less well understood than in *Drosophila*, the importance of PCP in vertebrate development is

stressed by defects in cardiac outflow tracts and incomplete closure of the neural tube as observed in spina bifida, which are caused by inadequate PCP signaling. Mutations in WNT/PCP signaling components were reported for both conditions (Phillips et al., 2005; Kibar et al., 2007).

Six core factors have been identified that interact with each other in *Drosophila* PCP signaling: the 7TM receptor protein FZD, the 4TM protein Van Gogh/striabismus, the cadherin Flamingo, and the cytoplasmic factors DVL, Prickle, and Diego. The vertebrate homologs of these proteins are the transmembrane proteins FZD, Van Gogh-like 1 and -2 (Vang1 and -2) and Celsr, and the cytoplasmic proteins DVL, Prickle, and Inversin/Diversin. As illustrated in Fig. 5, these core PCP proteins form two complexes on the opposite sides of each cell: on one side, FZD forms a complex with DVL, Inversin/Diversin, and Celsr, whereas at the opposing side the complex consists of Vangl1/2, Prickle, and Celsr. The intracellular components of both complexes are mutually inhibitory, supporting their spatial separation at two sides of the cell. However, interaction of the complexes from two neighboring cells appears to have a stabilizing effect of the complexes where the extracellular structures of Vang1 interact with the extracellular structures of FZD. Gene dosage effects have learned that either too much or too little of a PCP core factor has detrimental effects on tissue polarity (Yang and Mlodzik, 2015).

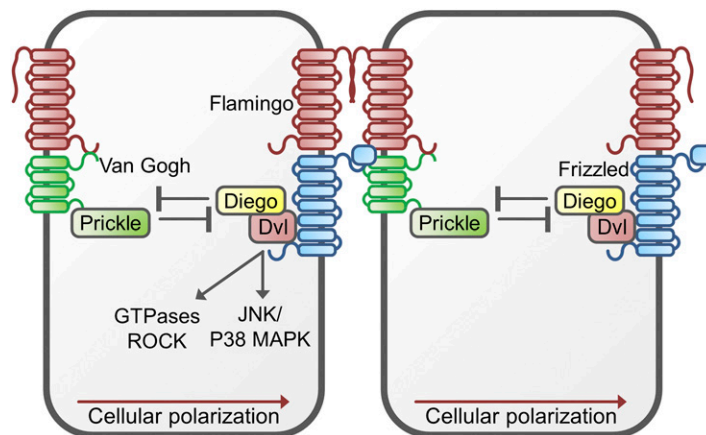
The interaction of these core PCP components can activate several signaling pathways. The FZD/DVL complex can activate the FZD/DVL PCP pathway via the PDZ and DIX domains of DVL, which can induce cytoskeletal reorganization but can also modulate gene transcription. The cytoskeletal reorganization is linked to the activation of small GTPases (such as Rho, Rac,

and Cdc42) and Rho-associated kinase. Rho-associated kinase can mediate the cytoskeletal rearrangements, which are characteristic for planar polarity signaling. On the other hand, PCP signaling can also induce a transcriptional response via JNK/p38-type MAP kinase signaling (Yang and Mlodzik, 2015).

The role for WNT in the PCP pathway is only beginning to be elucidated. In *Drosophila*, PCP axes are directed toward the prospective margins that are a source of WNT. In this model WNT proteins are thought to modulate the extracellular Vangl1-FZD interactions, thereby disturbing the balance between the signaling of the Vangl1/Prickle/Celsr complex on the one hand and the FZD/DVL/Diego/Celsr complex on the other hand. In this model, WNTs are instructive regulators of the FZD/Vangl1 polarization (Wu et al., 2013). However, it has to be noted that most of these studies have been carried out in *Drosophila*; for vertebrates additional research will be needed to fully elucidate the exact role of WNT in PCP signaling. Complicating factors are the multiple vertebrate WNTs, only two of which (WNT5A and -11) show selectivity for PCP signaling. Moreover, PCP signaling does not make use of LRP5/6; several PCP-specific coreceptors have been identified in vertebrates such as ROR1/2 and Ryk, tyrosine kinase receptors that may serve in the relay of the signal by phosphorylating DVL.

#### *E. Involvement of Heterotrimeric G Proteins in WNT/FZD Signaling*

Despite their seven-transmembrane spanning structure, FZD proteins deviate sufficiently from regular G protein-coupled receptors (GPCRs) to form a distinct class of receptors (Schulte, 2010). At present, it is not entirely clear what these structural differences mean for the interaction with components



**Fig. 5.** Schematic representation of the planar cell polarity pathway. In this pathways two receptor complexes are formed at the opposite sides of a cell: On one side, frizzled forms a complex with Flamingo/Celsr, disheveled (Dvl), and Diego/Diversin, whereas the other side the complex consists of Flamingo/Celsr, Van Gogh/Vang, and Prickle. The extracellular parts of these complexes interact, thereby controlling the cellular polarization via activation of JNK/p38 MAPK, small GTPase and Rho-associated kinase (ROCK) signaling. Although the role of Wnt in this signal transduction cascade is only partially understood, this protein may interfere with the Vangl1-FZD interaction. This could subsequently disturb the balance between the signaling of the Vangl1/Prickle/Celsr complex and the FZD/DVL/Diego/Celsr complex.

of different signal transduction pathways. Although the most prominent differences between FZD proteins and regular GPCRs are located at the extracellular side of the receptor, where FZD proteins show a bulky and highly conserved CRD, intracellular structures, particularly the third intracellular loop and the C-terminal end, also deviate. Because these intracellular domains form the point of interaction between regular GPCRs and G proteins, there is an ongoing debate whether FZD proteins can actually interact with G proteins and what the consequences of this interaction would be (Dijksterhuis et al., 2014). As mentioned before, the strongest evidence for FZD-G protein interaction is available for the WNT/Ca<sup>2+</sup> signaling route, where interactions with G $\alpha_{i/o}$  and the G $\alpha_t$  subfamilies are well established (Schulte, 2010). However, there is accumulating evidence for G protein involvement in WNT/ $\beta$ -catenin signaling, e.g., in mouse primary microglia cells where pertussis toxin (an inhibitor of G $\alpha_{i/o}$ ) was shown to block specific events such as phosphorylation of LRP6 and stabilization of  $\beta$ -catenin (Halleskog and Schulte, 2013). Moreover, interactions of the G $\alpha_o$  subunit with axin and its  $\beta\gamma$ -subunit with DVL have been demonstrated (Egger-Adam and Katanaev, 2010). An unresolved issue in this context is that there is overlap between the predicted binding sites for DVL and the G protein at the intracellular side of the FZD protein, and because interaction with DVL is generally accepted to be essential for WNT/ $\beta$ -catenin signaling, it is not quite clear how the interaction with the G protein actually will take place (Schulte, 2015).

## VI. WNT Signaling in Cardiac and Vascular Development

The cardiovascular system is one of the first functional systems to form during embryogenesis. Both  $\beta$ -catenin and non- $\beta$ -catenin-mediated WNT signaling play a critical role in cardiovascular development. Formation of the heart is a multistep process that starts with the specification of the cardiac progenitor cells, formation of a heart tube, with subsequent cardiac looping, chamber formation, septation, and maturation. WNT proteins, which regulate processes such as proliferation, migration, and differentiation during embryonic development and in adults, are intrinsically involved in cardiovascular development. This chapter is meant to provide the reader with sufficient background on this subject, needed for understanding the role of Wnt signaling in cardiovascular disease. This is not intended to be a comprehensive review of the development of the cardiovascular system and the role of Wnt signaling in it. We refer the readers to some high-quality review articles providing more detailed information on this subject (Moorman

and Christoffels, 2003; Dejana, 2010; Sylva et al., 2014).

### A. $\beta$ -Catenin-Mediated

Early during development, the mesodermal cell layer is the source of cardiac progenitor cells. The formation of the mesoderm appears to be dependent on WNT/ $\beta$ -catenin signaling. Mice devoid of  $\beta$ -catenin, a key player in the WNT/ $\beta$ -catenin pathway, lack mesoderm (Huelsken et al., 2000), and WNT3A deficiency is associated with attenuated expression of mesodermal markers (Liu et al., 1999), making it challenging to analyze their function during cardiogenesis. However, these findings suggest a necessary requirement of WNT/ $\beta$ -catenin signaling during formation of mesoderm, a source of cardiac progenitor cells. The initial data on the role of WNT signaling during cardiac development in vertebrates has been seemingly confusing and demonstrated contrasting results. Because of the known role of WNT signaling in promoting cardiogenesis in *Drosophila*, it was assumed that similar results would apply to vertebrates (Park et al., 1996). However, the activation of the WNT/ $\beta$ -catenin signaling in chicken and *Xenopus* embryos suppressed cardiac differentiation, and the opposite effect was seen by applying WNT inhibitors (Marvin et al., 2001; Schneider and Mercola, 2001). Also, similar results were observed in mice, where multiple ectopic hearts were seen by knocking out  $\beta$ -catenin in the endoderm (Lickert et al., 2002). Other studies with cell-based experiments suggest a positive role for WNT/ $\beta$ -catenin signaling on cardiac development (Nakamura et al., 2003). However, these seemingly contradicting results can be explained by the findings that the specific effects of WNT signaling are time and concentration dependent (Naito et al., 2006; Ueno et al., 2007; Wu, 2008). Once the heart tube is formed, second heart field (SHF) cells, a group of *Isl1* and *Nkx2.5* expressing cells, migrate to the developing heart from the dorsal pericardial wall (Buckingham et al., 2005). These highly proliferative cells form the second heart field and are tightly regulated by Wnt2 via the  $\beta$ -catenin pathway (Tian et al., 2010; Norden and Kispert, 2012; Ruiz-Villalba et al., 2016). Removal of the  $\beta$ -catenin resulted in a reduction of cells within SHF with subsequent aberrant development of the right ventricle and outflow tract (Klaus et al., 2007; Pahnke et al., 2016).

### B. Involvement of Non- $\beta$ -Catenin-Mediated WNT Signaling

Non- $\beta$ -catenin-mediated WNT signaling, although less well studied, is also known to play an important role in cardiac development. WNT11 appears to be required for proper heart development by displaying cardiac promoting activity, and these effects are mainly thought to be mediated by non- $\beta$ -catenin-mediated signaling



branches (Pandur et al., 2002). Overexpression of Wnt11 in *Xenopus* explants induces the formation of cells with a contractile phenotype. This Wnt11 promoting activity has been shown in other models as well, such as murine embryonic stem cells, bone marrow-derived stem cells, and endothelial progenitor cells (Terami et al., 2004; Belema Bedada et al., 2005; Koyanagi et al., 2005). Furthermore, these analyses have shown that the Wnt11 procardiogenic effects are impaired by inhibiting JNK or PKC and stimulated by their activation.

WNT5A, another member of the WNT family, promotes troponin-positive cells through non- $\beta$ -catenin-mediated signaling in synergy with BMP6 and sFRP1 (Chen et al., 2008). However, treatment with either factor did not result in cardiomyocyte formation, which suggests that WNT5A acts in a supportive role but is not required for cardiogenesis. In contrast to WNT5A, WNT2B is required for cardiogenesis. (Wang et al., 2007). WNT2B-deficient embryonic stem cells have a significantly impaired ability to form cardiogenic progenitor cells but not hematopoietic progenitor cells. The loss of WNT2B leads to an accelerated expression of Bry and Flk1. Interestingly, WNT/ $\beta$ -catenin signaling also plays a positive role in Bry expression. In addition, activation of WNT/ $\beta$ -catenin signaling during differentiation of embryonic stem cells leads to enhanced differentiation of these cells (Anton et al., 2007). Furthermore, the loss of WNT2B function is very similar to the WNT3A function; it suggests that WNT2B might activate non- $\beta$ -catenin-mediated WNT signaling. Terminal differentiation of cardiomyocytes in mouse embryonic cells is associated with a significant increase of *Wnt11* expression and adding WNT11 to cultured embryonic stem cells causes an increase of beating cells (Naito et al., 2006). In *Xenopus*, two Wnt11 homologs have been identified, and one of them, called Wnt11A (also called Wnt11R), is expressed later (Garrick et al., 2005). Reduction of Wnt11R leads to a decrease of marker genes for cardiac terminal differentiation, and it is a cell adhesion molecule DM-GRASP that could be a non- $\beta$ -catenin-mediated WNT pathway target gene (Gessert et al., 2008).

Based on the data on the role of WNT signaling during cardiac specification, the  $\beta$ -catenin-dependent WNT signaling pathway is required for mesoderm specification, and subsequently this pathway needs to be downregulated for formation of multipotent cardiovascular progenitor cells. This critical step is supported by the activation of the non- $\beta$ -catenin-mediated WNT pathway. In the next step, the  $\beta$ -catenin-mediated WNT pathway is necessary for the proliferation of these cardiac progenitor cells, and finally, for the terminal differentiation, it is the non- $\beta$ -catenin-mediated WNT signaling that is activated and the  $\beta$ -catenin-dependent WNT signaling that is downregulated (Gessert and Kuhl, 2010). After heart tube formation, the noncanonical pathway of WNT5A and Wnt11 appears to be critical

in the SHF cells migration to the arterial pole of the heart (Cohen et al., 2012).

### C. Formation of Outflow Tract, Valves, and Conduction System

Outflow tract formation and septation is intrinsically linked to cardiac neural crest cells, which are a sub-population of cranial neural crest cells. These cells originate at the dorsal neural tube and migrate along the aortic arch arteries through the pharyngeal arches 3, 4, and 6. The development of cardiac neural crest cells as well at outflow tract appears to be significantly dependent on the WNT signaling pathways. These crest cells express WNT1 and WNT3A and loss of  $\beta$ -catenin on WNT1-positive cells leads to reduction of cardiac neural crest cells as well as outflow hypoplasia in mice (Brault et al., 2001). In addition, WNT5A and WNT11 appear to be expressed in the outflow tract myocardium. Loss of WNT5A leads to septation defects of the outflow tract, resulting in congenital heart defects such as double outlet right ventricle or persistent truncus arteriosus (Schleiffarth et al., 2007). In addition, WNT11 activates a non- $\beta$ -catenin-mediated signaling pathway in the outflow tract and regulates the expression of TGF $\beta$ -2 (Zhou et al., 2007). However, the underlying mechanisms leading to double outlet right ventricle formation could be due aberrant second heart field components or abnormal contribution of cardiac neural crest cells to the outflow tract (van den Hoff and Moorman, 2000). Outflow tract formation and septation is intrinsically associated with valve formation. Hence it stands to reason that WNTs participate in valve formation.

Valve formation is a delicate and complex process where cells from the atrioventricular canal, the endocardium located between the atrial and ventricular chambers, undergo an epithelial-mesenchymal transition (EMT), migrate in the space between endocardium and myocardium, proliferate, and form the so-called cardiac cushions. Subsequently, these cardiac cushions are the precursors of the tricuspid valve and mitral valve, which separate the right and the left chambers, respectively. WNT/ $\beta$ -catenin signaling is active during endocardial cushion development (Gitler et al., 2003). Furthermore, loss of APC function in zebrafish, which in turn leads to an abundance of active  $\beta$ -catenin signaling, results in excessive endocardial cushion formation. Overexpression of APC leads to inhibition of cardiac cushion formation (Liebner et al., 2004), demonstrating that WNTs by its inhibitory effect likely exert an inhibition on cushion formation.

WNTs likely also contribute to the development of the conduction system. This system originates from the Isl1-positive progenitor cells (Sun et al., 2007). This process starts early in development, and around embryonic day 8 in mice the cardiac pacemaking region starts taking shape in the atrium, giving rise later to the

sinoatrial node where the impulses originate. These impulses are transmitted to the rest of the conduction system, which is composed of the atrioventricular node, the His bundle, bundle branches, and as Purkinje fibers (Sun et al., 2007). WNT7A and WNT11 are upregulated in the developing conduction system, as early as embryonic day 8 (HH 34) in chicken embryo; however, there is no functional data to delineate the precise role of WNT signaling in the formation of the conduction system (Bond et al., 2003). Also, the proper patterning of the atrioventricular canal is important on timely delay the electrical impulses from the atria to the ventricles. Recent data suggests that  $\beta$ -catenin-dependent WNT signaling is an important regulator of this maturation process (Gillers et al., 2015). Concurrently, coronary artery formation is a critical part of the proper cardiac development and studies suggest that WNT9b plays an important role of this process via the  $\beta$ -catenin pathway (Zamora et al., 2007). A summary of the WNTs in cardiovascular development is shown in Table 1.

#### D. Vessels: WNT Signaling and the Control of Angiogenesis

The formation of blood vessels is under control of two physiological processes: vasculogenesis and angiogenesis. Vasculogenesis is the formation of blood vessels from the de novo production of ECs in embryos, after which angiogenesis takes place to provide new blood vessels from pre-existing ones to all organs and tissues in both physiological and pathologic conditions.

Vasculogenesis, the initial step of vasculature development, requires the differentiation of ECs from mesodermal precursors. This first step is under the control of many signaling cues, including WNT signaling. Previous studies have demonstrated a role of WNT2, WNT3A, and WNT5A in the differentiation of embryonic stem cells toward ECs (Wang et al., 2006, 2007; Yang et al., 2009). The latter seems to involve both the  $\beta$ -catenin and PKC $\alpha$  pathways (Yang et al., 2009). This will be covered in more detail in *section IX* of this review. The contribution of R-spondin-3 also seems to be important for vasculogenesis, as well as angiogenesis. A common role for R-spondin-3 has been identified in

Xenopus embryos and in mice and human ECs, thus highlighting the highly conserved nature of this signaling (Kazanskaya et al., 2008). R-Spondin-3 promotes the differentiation process of ESC toward angioblasts and activates the  $\beta$ -catenin pathway, resulting in an increased vascular endothelial growth factor (VEGF) expression (crosstalk with VEGF pathway, see *section X.C*) (Kazanskaya et al., 2008).

Angiogenesis is a complex process following sequential steps including the induction of tip cells, sprout elongation, vascular branching, and stabilization of the newly formed vascular network. After the initial differentiation of precursors cells to ECs, the initiation of sprouting is mediated by ECs that harbor different phenotypes: tip and stalk cells. The vascular sprout is under the guidance of tip cells at the vascular front, which have a migratory behavior. They are followed by stalk cells, which are proliferative ECs important for the extension of the sprout and involved in the creation of the vascular lumen.

1. *WNT Signaling Elements in Endothelial Cells.* WNT proteins can regulate the function of ECs during angiogenesis either via an autocrine or paracrine fashion, depending on whether if they are generated within endothelial or nonendothelial cells, respectively. As extensively reviewed elsewhere, many WNT ligands seem to influence EC functions (WNT2, -2B, -3, -4, -5A, -7A, -8A, -9A, -9B, -10B, and -11) (Zerlin et al., 2008; Franco et al., 2009). Moreover, ECs express a large panel of WNT receptors (FZD<sub>1, -2, -4, -5, -6, -7, -8, -9, and -10</sub>, and LRP5 and -6), but also several key elements involved in the modulation of WNT signaling (Ryk, sFRP1 and -3, DKK1 and -3) (Zerlin et al., 2008; Franco et al., 2009).

2. *Influence of WNT Signaling on Tip Cells.* Evidence for a role of WNT-signaling on tip cell induction and function is scarce. WLS, a key component of the WNT secretion machinery, is a cargo receptor that mobilizes WNT proteins from the Golgi to the cell surface (Bartscherer et al., 2006). The postnatal deletion of WLS in mouse ECs (Evi-ECKO) was associated with a decreased microvascular density in postnatal retina as well as in tumor tissues (Korn et al., 2014).

TABLE 1  
Wnts and cardiovascular development

Gene	Pathway	Role	Reference
WNT1	$\beta$ -catenin mediated	Cardiac neural crest cell development and outflow tract development	Brault et al. (2001)
WNT2B	non- $\beta$ -catenin mediated	Inhibition of cardiac progenitor cells	Wang et al. (2007)
WNT3A	$\beta$ -catenin mediated	Mesoderm formation	Liu et al. (1999)
WNT5A	non- $\beta$ -catenin mediated	Inhibition of cardiac progenitor cells Cardiomyocyte differentiation and outflow tract septation	Marvin et al. (2001) Schleifarth et al. (2007)
WNT7A	non- $\beta$ -catenin mediated	Conduction system development	Bond et al. (2003)
WNT9B	$\beta$ -catenin mediated	Epicardium development and coronary artery formation	Zamora et al. (2007)
WNT11	non- $\beta$ -catenin mediated	Cardiomyocyte differentiation Outflow tract septation Conduction system development	Pandur et al. (2002) Zhou et al. (2007) Bond et al. (2003)

Although the tip cell density at the vascular front was reduced in this transgenic model, no differences in the number of filopodia per tip cell were observed. This suggests that tip cell function is not impacted by WNT proteins (Korn et al., 2014). More recently, the MMP (matrix metalloproteinase) inhibitor Reck (reversion-inducing-cysteine-rich protein with Kazal motifs) and the adhesion G protein-coupled receptor Gpr124/ADGRA2 have been identified as key players of a WNT7A/WNT7B signaling complex for brain angiogenesis and blood-brain barrier (BBB) formation (see section VI.E) (Vanhollebeke et al., 2015; Noda et al., 2016). This atypical WNT/ $\beta$ -catenin signaling was shown to selectively control the function of tip cells in central nervous system (CNS) angiogenesis (Vanhollebeke et al., 2015). Furthermore, cell migration, an important feature for tip cells, has been shown to be impacted by a WNT/ $\beta$ -catenin-independent pathway, via phosphorylation of APC (Dejana, 2010; Harris and Nelson, 2010). Phosphorylated APC is present in cellular extensions during cell migration and is mandatory for cell migration and the organization of the cytoskeleton (Dejana, 2010; Harris and Nelson, 2010). Indirectly, WNT signaling could also regulate the selection of endothelial tip cells via interfering with Jagged-1. Jagged-1 is a Notch ligand that regulates the selection of tip cells by interfering with the Dll4/Notch signaling from the stalk to the tip cell (Fig. 6). Jagged-1 was significantly upregulated in ECs upon  $\beta$ -catenin-dependent WNT signaling (Reis et al., 2012). The study, however, supported that Jagged-1 is not directly regulated by  $\beta$ -catenin, because this upregulation was observed only after 48 hours of WNT3A stimulation while other genes were already regulated after 18 hours (Reis et al., 2012). Moreover, upon WNT/ $\beta$ -catenin stimulation, the expression of tip cell receptors (e.g., VEGFR2) was downregulated, whereas those expressed on stalk cells (e.g., VEGFR1) were upregulated, suggesting that WNT signaling rather promotes the induction of a stalk cell-like phenotype (Reis et al., 2012).

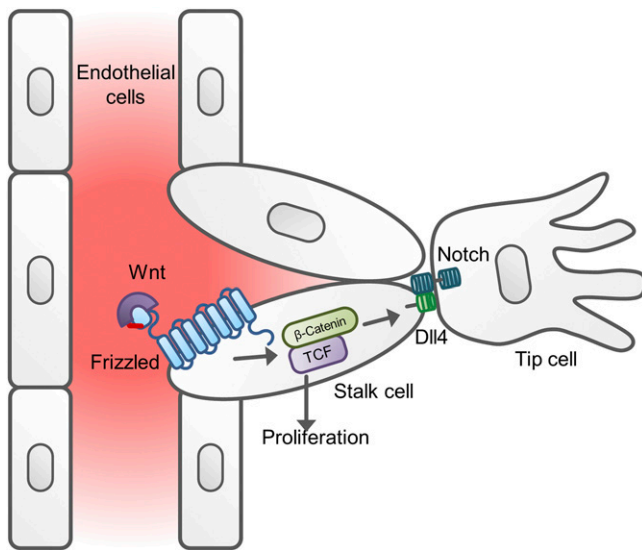
**3. Influence of WNT Signaling on Stalk Cells.** After the induction of tip cells, sprout elongation is mediated by stalk cells. The importance of WNT signaling for stalk cells has been highlighted by a study performed in the developing retinal vasculature of BAT-gal reporter mice. Indeed, a prominent  $\beta$ -catenin-dependent activity was found in stalk cells rather than in tip cells (Phng et al., 2009). Furthermore, several WNT ligands affect the proliferation of ECs either via the  $\beta$ -catenin-dependent or the  $\beta$ -catenin-independent pathways (Franco et al., 2009). WNT1, acting via the  $\beta$ -catenin-dependent pathway, seemed to inhibit the proliferation of human umbilical vein endothelial cells (Cheng et al., 2003). WNT3A, via both  $\beta$ -catenin-dependent and -independent WNT signaling, stimulates the proliferation and migration of human umbilical vein endothelial cells (Samarzija et al., 2009). WNT5A,

known to activate the  $\beta$ -catenin-independent signaling in ECs and to inhibit the  $\beta$ -catenin-dependent WNT signaling, has been shown to enhance angiogenesis in human ECs via an increased ECs proliferation and cell survival (Masckauchan et al., 2006; Cheng et al., 2008a). Increased WNT5A expression was also associated with increased tube formation and migration. On the other hand, the inhibition of WNT signaling by sFRP1 decreases the proliferation of ECs both in vitro and in vivo (Ezan et al., 2004). Overall, WNT  $\beta$ -catenin-dependent and independent pathways seem to have opposing effects on angiogenesis. In agreement with these findings, it has been hypothesized that the antagonistic impact of the WNT/ $\text{Ca}^{2+}$  signaling on the  $\beta$ -catenin-dependent pathway may serve as a fine-tuning system to balance the angiogenesis process. Conflicting results were observed, however, such as the promotion of EC proliferation and the stability of tube formation after activation of the  $\beta$ -catenin-dependent pathway by WNT1 on the one hand and the inhibition of EC proliferation and tube formation by WNT5A on the other (Goodwin et al., 2007). sFRP2 has been shown to increase EC migration, to stimulate tube formation in vitro and to induce angiogenesis in a mouse matrigel plug assay, via a calcineurin/nuclear factor of activated T-cells (NFAT) pathway (Courtwright et al., 2009).

**4. WNT Signaling and Stabilization and Specification of the Vascular Network.** Notch signaling is well known as a key regulator of vascular density. It is suggested that WNT could be involved in the fine tuning of Notch signaling (Phng et al., 2009). WNT signaling may therefore be in part responsible for the stability of the vascular network, whether to form or retract vascular connections. It has been shown that the loss of the Notch-regulated ankyrin repeat protein (*Nrarp*), *LEF-1*, or endothelial *Ctnnb1* caused vessel regression (Phng et al., 2009).

Shear stress itself seems to involve  $\beta$ -catenin-dependent WNT signaling for the expression of angiopoietin 2 (Li et al., 2014b). Contrary to angiopoietin 1, which is responsible for vessel maturation, migration, and survival, angiopoietin 2 is involved in cell death and the disruption of vascular networks (Fagian and Christofori, 2013).

Vessel maturation and stabilization are also under the control of mural cells. Pericytes are crucial perivascular cells supporting capillaries. Decreased pericyte coverage of the endothelial layer can lead to vessel loss. In the context of pulmonary hypertension (PAH), pericytes have a reduced expression of *FZD7* and *cdc42* -both genes being required for WNT/PCP activation, which is an important mechanism for EC motility and polarity (Yuan et al., 2015). Restoration of both genes in those pericytes was associated with improved endothelial-pericyte coverage and a larger vascular network (Yuan et al., 2015).



**Fig. 6.** The role of WNT signaling in sprouting angiogenesis. In this process, tip cells provide directional cues to the sprout cells that can proliferate and initiate the formation of a new vessel. WNT signaling can contribute to the proliferation and tube formation of the stalk cells. The effect of WNT signaling on the tip cells is likely to be indirect via the expression of delta-like ligand-4 (Dll4) in the stalk cells, which activates Notch signaling in the tip cells.

Furthermore,  $\beta$ -catenin-dependent Wnt signaling upregulates Sox17, a member of the SoxF family of transcription factors (Corada et al., 2014; Morini and Dejana, 2014). Sox17 activates Notch signaling via Dll4 and seems to be involved in the arterial differentiation of the vasculature. In absence of Sox17, ECs lack arterial markers and lose their ability to recruit mural cells. The upregulation of Sox17 induced by  $\beta$ -catenin-dependent WNT signaling therefore drives ECs toward an arterial phenotype (Corada et al., 2014; Morini and Dejana, 2014).

Wnt signaling has been established as a major driver for vessel formation and maturation. It does not only control the function of the ECs but also influence their microenvironment to fine tune the angiogenic process (see also *section X.C*). The major hurdle limiting our understanding is the lack of temporal resolution in many studies. We expect that the use of genetic models combined with advanced *in vivo* imaging techniques will help to decipher the Wnt-related molecular events during the highly dynamic angiogenic process.

### *E. WNT Signaling in the Formation of the Blood-Brain Barrier.*

The perfusion of the cerebral tissue is a critical process that needs to be strictly controlled to avoid the entrance of undesired blood components into the CNS. This is achieved due to the unique features of the CNS ECs, which are fundamental for the establishment and maintenance of the blood-brain barrier (BBB) (Abbott et al., 2010). The BBB comprises tight junction proteins that allow a very low permeability to non-CNS components,

but also specific transporters that enable the transport of nutrients (e.g., glucose, amino acids).

Supporting evidence for the contribution of WNT signaling in the development of cerebral vasculature and BBB has been found less than a decade ago (Liebner et al., 2008; Stenman et al., 2008; Daneman et al., 2009). The invasion of the CNS by the vasculature and the concomitant BBB development requires the presence of *Wnt7A* and *-B* in the developing neuroepithelium (Stenman et al., 2008). By using the Cre-recombinase technique, the deletion of *Wnt7A* and *-B* was feasible at the embryonic stage (to avoid early lethality as *Wnt7B* is required for placental development), and the embryos can develop up to E12.5. Subsequently these *Wnt7A/B* mutants die from a severe hemorrhage specifically localized to the CNS (Stenman et al., 2008). In addition, the expression of the glucose transporter (Glut1), an early BBB marker, was strongly downregulated in the *Wnt7A/B* mutants, suggesting the absence of a proper barrier when *Wnt7A* and *-B* are lacking (Stenman et al., 2008). The study suggested that *Wnt7A* and *-B* produced by the neuroepithelium can directly activate the ECs in the perineural vascular plexus surrounding the neural tube to promote angiogenesis and to acquire a BBB phenotype (Stenman et al., 2008). This was further confirmed in another study in which *in situ* hybridization experiments were performed. This study revealed that several WNT ligands are expressed by neural progenitor cells in the developing mouse brain, which could be responsible for the WNT signaling activation in ECs. *Wnt7A* and *-B* expression in particular was temporally associated with activation of WNT/ $\beta$ -catenin pathway in several areas of the developing CNS vasculature. The impact of *Wnt7A* on BBB was further explored by studying the transcriptional profiles of primary brain endothelial cells using microarrays. In accordance with the findings described above, *Wnt7A* upregulated several BBB influx transporters, including Glut1. However, the expression of tight junction molecules was not modulated (Daneman et al., 2009). Recent studies have revealed the existence of functional interactions between WNT7A/B and Reck and Gpr124/ADGRA2, an orphan G protein-coupled receptor, in genetically engineered mice and zebrafish and luciferase reporter assays (Noda et al., 2016). Although many questions remain on the exact molecular interactions taking place between those partners, this newly discovered WNT7A/B-Gpr124-Reck complex seems important for the WNT/ $\beta$ -catenin signaling, which is of relevance for cerebrovasculature development and BBB formation.

Further *in vivo* evidence was collected to confirm the major contribution of WNT/ $\beta$ -catenin signaling for CNS vasculature development and BBB formation. First, WNT inhibition in developing embryos, using the injection of adenoviruses encoding a soluble FZD<sub>8</sub>-FC fusion protein, led to many vascular defects that were limited to the CNS (Daneman et al., 2009). Second,

studies using reporter mice for WNT/ $\beta$ -catenin signaling, have identified active WNT/ $\beta$ -catenin signaling in ECs during brain angiogenesis, which progressively declined with the maturation of the BBB (Liebner et al., 2008; Daneman et al., 2009). In adult mice, most cerebral vessels did not exhibit WNT/ $\beta$ -catenin activity, suggesting that a fully mature BBB does not require a permanent activation of this pathway. Furthermore, conditional activation of endothelial  $\beta$ -catenin in vivo was associated with an increased claudin-3 expression (a tight junction protein) and a decreased plasmalemma vesicle-associated protein expression (PLVAP, a component of endothelial fenestrations) (Liebner et al., 2008). On the contrary, the conditional inactivation of  $\beta$ -catenin in endothelial cells in vivo was associated with a strong upregulation of PLVAP as well as an increased BBB permeability. This WNT3A/ $\beta$ -catenin/claudin-3 signaling was confirmed in vitro using primary endothelial cells isolated from mouse brains. Treatment of the cells with a WNT3A-conditioned medium led to a selective increase of claudin-3 expression in association with an activation of the  $\beta$ -catenin pathway. WNT5A conditioned medium had no impact on the expression of claudin-3 or other tight junction proteins (Liebner et al., 2008). In addition, the upregulation of claudin-3 was dependent on the transcriptionally active form of  $\beta$ -catenin rather than the structural association of  $\beta$ -catenin with cadherins at junctional levels, thus confirming that claudin-3 is a major key effector of WNT/ $\beta$ -catenin signaling at the BBB level (Liebner et al., 2008).

Although the contribution of  $\beta$ -catenin in this study was linked to an increased transcription of a tight junction protein, it is of importance to highlight the role of  $\beta$ -catenin (together with  $\alpha$ -catenin) in the composition of adherens junctions. The lack of association of  $\beta$ -catenin with cadherins would certainly be detrimental for BBB integrity. Unfortunately, the relative contribution of  $\beta$ -catenin to the adherens junctions compared with  $\beta$ -catenin-mediated WNT signaling in the context of BBB formation is poorly understood and should be subject to further study.

Apart from the WNT/ $\beta$ -catenin pathway, a contribution of WNT signaling for BBB function through the activation of the planar cell polarity complex (PCP) has been identified in immortalized human brain ECs (Artus et al., 2014). The PCP complex, which includes three interacting molecules, Par-3, Par-6, and an atypical PKC, is expressed in human brain ECs and colocalizes with tight junction proteins (zona occludens-1) (Artus et al., 2014). Stimulation of ECs with WNT5A was able to activate the Par/PKC $\alpha$ /PCP complex in this cellular model. Subsequently the cellular polarization increased and the cellular permeability decreased, thus highlighting the involvement of the Par/PKC $\alpha$ /PCP pathway to the WNT-mediated control of the BBB function.

WNT coreceptors are also crucial to control CNS vasculature. Deletion of both LRP5 and -6 in ECs severely impairs the CNS vasculature and is associated with cerebral bleeding (Zhou et al., 2014). In *Lrp5*<sup>-/-</sup> mice, there is a decreased capillary regression in the retinas, resulting especially in the retention of the hyaloid vasculature throughout life (Kato et al., 2002).

The absence of norrin (NDP, Norrie disease protein, or X-linked exudative vitreoretinopathy 2 protein) is detrimental for the regulation of angiogenesis as well as for BBB formation. Norrin can activate the WNT/ $\beta$ -catenin signaling pathway by binding to FZD<sub>4</sub> and LRP5/6. This complex has been shown to be of major importance for both blood retinal and blood-brain barriers (Wang et al., 2012). In the absence of norrin/FZD<sub>4</sub> signaling, vascular growth in retinas is retarded and the capillary network does not form (Wang et al., 2012). In the brains of *Fzd4*<sup>-/-</sup> mice, BBB integrity is altered in the cerebellum, olfactory bulb, spinal cord, and retina (blood-retinal barrier or BRB), but not in the cerebral cortex, striatum, or thalamus (Wang et al., 2012). Furthermore, when *Fzd4* deletion was induced during adulthood, the vasculature exhibited an increased expression of PLVAP and a reduced expression of claudin-5 and this was associated with loss of BBB and BRB integrity. This highlights that FZD<sub>4</sub> is crucial for the maintenance of mature BBB/BRB function. This conclusion has been supported by another study, in which the injection of an anti-FZD<sub>4</sub> monoclonal antibody, blocking its function, induced a BRB dysfunction (Paes et al., 2011).

In addition to its role in BBB/BRB, norrin/FZD<sub>4</sub> signaling plays a central role in retinal vascular growth. Norrie disease, due to loss-of-function mutations in the Norrie disease gene (NDP, the gene coding for norrin), is associated with a severely decreased vascularization of the retina (see section VII.C) (Warden et al., 2007). Mutations downstream of the norrin pathway, in *FZD4*, *LRP5*, or *TSPAN12* genes, are also associated with several retinopathies such as familial exudative vitreoretinopathy (see section VII.C) (Warden et al., 2007).

Although the important role played by norrin has been recognized for BBB formation, less is known regarding its impact in pathologic conditions. Its role was recently investigated in a rat model of subarachnoid hemorrhage (Chen et al., 2015). First, norrin expression increased over time after induction of subarachnoid hemorrhage, thus highlighting the importance of norrin signaling in this pathologic condition. Second, intracerebroventricular administration of norrin led to  $\beta$ -catenin nuclear translocation, increased expression levels of occludin, VE-cadherin, and zona occludens 1, as well as a reduced BBB permeability shown by a reduced brain water content, a reduced Evans blue extravasation, and improved neurologic functions (Chen et al., 2015). These effects were abolished when a pretreatment with a FZD<sub>4</sub> siRNA was

performed. Thus, norrin protects BBB integrity in the context of subarachnoid hemorrhage by activating the FZD<sub>4</sub>/β-catenin pathway (Chen et al., 2015).

Taken together, these data imply that multiple WNT signaling components are important for BBB (and BRB) formation but can also play crucial roles for BBB maintenance in both physiologic and pathologic conditions. Although the development of drugs targeting the WNT signaling could be of major importance to seal and injured BBB, WNT-targeting compounds inducing a transient opening of the BBB on the contrary could also be of interest to allow the passage of non-BBB permeable drugs into the CNS.

## VII. WNT Signaling in Vascular Disease

### A. Atherosclerosis

Atherosclerosis is a main cause of cardiovascular diseases. It is a chronic inflammatory condition that can lead to an acute clinical event due to plaque rupture and thrombosis. Although the incidence of atherosclerosis-related diseases and the associated mortality in Westernized countries have declined in the last decades, it remains a therapeutic priority (Lusis, 2000; Herrington et al., 2016). Clinical and preclinical studies have identified WNT signaling as being a key player in the development of this pathology. A schematic overview of the pathways described in this section is provided in Fig. 7.

#### 1. Clinical Evidence for WNT Signaling in Atherosclerosis.

A first clinical evidence linking WNT signaling to atherosclerosis was obtained after identifying a mutation in the *LRP6* gene in a family with autosomal dominant early coronary disease, hypertension, hyperlipidemia, and osteoporosis. The functional significance of the corresponding mutation, an amino acid substitution (Cys-Arg) that affects an epidermal growth factor-like domain, was further confirmed in vitro in cells transfected with the mutant *LRP6* and WNT reporter genes (Mani et al., 2007). A few years later, this 1062V *LRP6* variant was demonstrated to be an independent risk factor for carotid artery stenosis in hypertensive patients. (Sarzani et al., 2011).

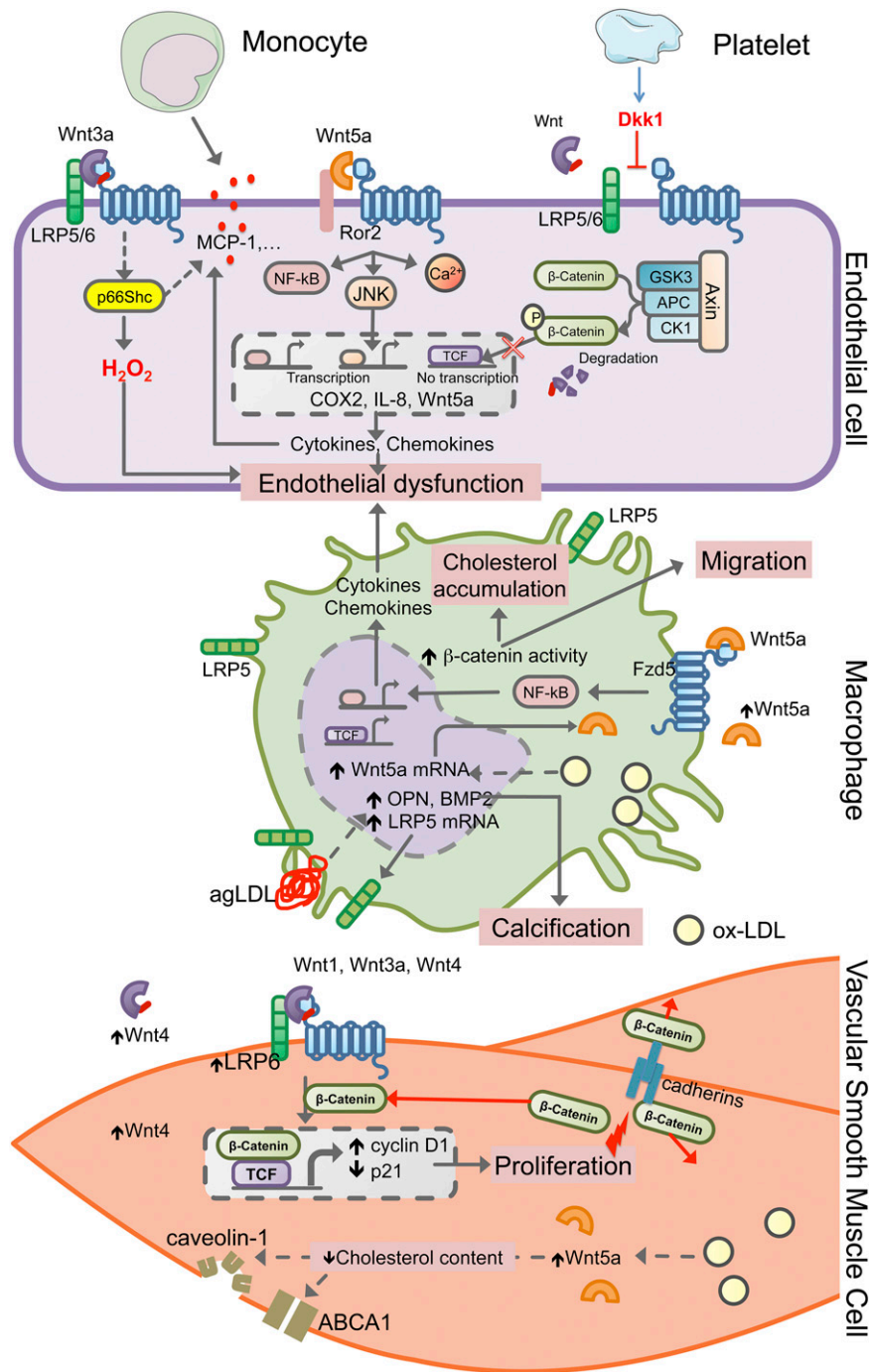
Next to *LRP6*, *WNT5A* has been shown to be also of importance in atherosclerotic lesions. First, *WNT5A* is highly expressed in the macrophage-rich regions of both human and murine atherosclerotic lesions (Christman et al., 2008). Second, *WNT5A* mRNA and proteins were more elevated in advanced than in less advanced arterial lesions (Bhatt and Malgor, 2014; Malgor et al., 2014). Circulating plasma *WNT5A* was also higher in atherosclerotic patients than in healthy controls (Malgor et al., 2014).

Furthermore, the *LRP5/6* inhibitor *DKK1* was also found to be increased in plasma and lesions of patients

with coronary artery disease and patients with carotid plaques (Ueland et al., 2009). Similarly to the findings described above on *WNT5A*, *DKK1* was more elevated in advanced and unstable disease. This result was confirmed in *ApoE*<sup>-/-</sup> mice, an experimental model for atherosclerosis (Ueland et al., 2009). Immunostaining of human material as well as in vitro experiments revealed platelets as being an important cellular source of *DKK1* (Ueland et al., 2009). Further mechanistic demonstrations of the role of *DKK1* in the context of atherosclerosis are discussed below.

2. *WNT Signaling and Endothelial Dysfunction.* Endothelial dysfunction is a well-established hallmark of atherosclerosis development. The contribution of *WNT5A* to endothelial dysfunction has been highlighted recently in diabetic patients. ECs from diabetic patients had higher *WNT5A* expression than from non-diabetic patients and this was associated with higher levels of activated c-jun N-terminal kinase (JNK). In patients, this was correlated with lower flow-mediated dilation, an indicator of endothelial function. In vitro, inhibition of *WNT5A* in ECs from patients with diabetes was able to restore insulin-induced eNOS phosphorylation and NO production and this was mediated by JNK. Therefore, endothelial dysfunction in human diabetes seems to involve the *WNT5A*/JNK signaling pathway (Breton-Romero et al., 2016).

Stimulation of human aortic ECs by *WNT5A* upregulated inflammatory genes rapidly (Kim et al., 2010a). Among the different genes, COX-2 and interleukin (IL-8) were strongly increased already after 1 hour of treatment (160- and 15-fold, respectively). Of particular interest, *WNT5A* did not upregulate COX-2 in other nonendothelial cell types, suggesting an endothelial-specific action of *WNT5A*. In addition, this *WNT5A*-induced inflammatory gene regulation was NF-κB dependent, as revealed by an enzyme-linked immunosorbent assay-based DNA binding assay for RelA and immunocytochemical analysis. A cytokine array further showed the enhanced expression of different cytokines, including GM-CSF, IL-1α, IL-3, IL-5, IL-6, IL-7, CCL2, and CCL8 at 24 hours after *WNT5A* stimulation compared with nonstimulated human aortic ECs. Within this experimental setting, *WNT3A* did not show a similar effect, suggesting a proinflammatory role of *WNT5A*/β-catenin-independent signaling (Kim et al., 2010a). Further experiments with calcium chelators and protein kinase C inhibitor identified that the *WNT5A*/Ca<sup>2+</sup>/PKC pathway was responsible for endothelial inflammatory regulation (Kim et al., 2010a). The impact of *WNT5A* was also functionally assessed in an endothelial permeability assay. The observed enhanced endothelial permeability induced by *WNT5A* could translate into an early inflammatory stage of the endothelium. Moreover, recent studies focused on endothelial barrier dysfunction are pointing toward an



**Fig. 7.** WNT signaling in atherosclerosis. The contribution of WNT signaling to the development and progression of atherosclerosis is described at the intima and media levels with a central role for the contribution of monocytes/macrophages (*section VII.A*). ABCA1, ATP-binding cassette transporter 1; agLDL, aggregated LDL; ox-LDL, oxidized LDL; MCP-1, monocyte chemotactic protein-1.

important role of WNT5A in the regulation of endothelial permeability (Skaria et al., 2016, 2017).

As described above, DKK1 is elevated in atherosclerotic lesions and is highly expressed in platelets. During platelet activation, DKK1 is quickly released and enhances  $\beta$ -catenin phosphorylation in ECs. Silencing of DKK in ECs leads to an increased endothelial  $\beta$ -catenin phosphorylation. Furthermore, neutralizing antibodies against DKK1 abolished platelet-induced

cytokine production in ECs (Ueland et al., 2009). These results suggest a deleterious role for DKK1 during atherosclerosis. Its increased release, at least in part by platelets, can inhibit the WNT/ $\beta$ -catenin pathway in ECs and could thus contribute to endothelial dysfunction at the initiation of the atherosclerotic lesion.

The adaptor protein p66Shc is known to play a major role in endothelial dysfunction and atherosclerosis (Napoli et al., 2003; Martin-Padura et al., 2008;

Franzeck et al., 2012; Kim et al., 2012). It was first described as an aging gene because *p66Shc* knockout mice have a 30% increased life span. Later studies showed that *p66Shc*<sup>-/-</sup> mice exhibited reduced oxidative stress, were protected from endothelial dysfunction during diabetes, and developed less atherosclerotic plaques (Migliaccio et al., 1999; Napoli et al., 2003; Camici et al., 2007). Vikram et al. (2014) discovered that WNT3A-stimulated dephosphorylation of  $\beta$ -catenin, and the subsequent  $\beta$ -catenin-dependent transcription was inhibited by knockdown of *p66Shc*. Furthermore, whereas WNT3A stimulation increased H<sub>2</sub>O<sub>2</sub> levels in ECs and impaired endothelium-dependent vasodilation, *p66Shc* knockdown was able to rescue them both. *P66Shc* knockdown was also associated with an inhibition of monocyte adhesion on WNT3A-stimulated ECs.

**3. WNT Signaling in Macrophages.** The importance of inflammation as a hallmark of atherosclerosis has been underappreciated for many years. Since two decades, however, atherosclerosis is recognized as an inflammatory disease, in which the role of macrophages is of major importance. A dysfunctional endothelium (see above) will ultimately lead to the adhesion, rolling, and finally migration of monocytes to the subendothelial layer, where they differentiate into tissue macrophages. By scavenging modified lipoprotein particles, those macrophages become foam cells, characteristic for atheromatous lesions, and release proinflammatory cytokines, reactive oxygen species, and matrix metalloproteinases. These molecular events can lead to an amplification of the local inflammatory response and the weakening of the atherosclerotic plaque (Libby, 2002; Cochain and Zerneck, 2017).

As described in the *section VII.A.1*, high levels of WNT5A have been found in atherosclerotic plaques, more specifically in macrophage-rich areas (Christman et al., 2008). This finding initiated research on the role of WNT5A in the process of macrophage activation. Because the uptake of oxidized-low density lipoprotein (oxLDL) by monocytes/macrophages is playing a detrimental role in atherosclerosis, its impact on WNT5A expression has been investigated. Although native LDL had no impact, oxLDL has been found to induce WNT5A mRNA expression in human monocyte-derived macrophages (Bhatt and Malgor, 2014). By interacting with FZD<sub>5</sub>, WNT5A induced the expression of proinflammatory cytokines. On the other hand, WNT3A had an anti-inflammatory effect via suppression of GSK3 $\beta$  (Schaale et al., 2011). Both WNT3A and WNT5A have been shown to activate the NF- $\kappa$ B pathway in murine and human macrophages. However, the WNT3A action was transient while WNT5A induced a more prolonged and sustained NF- $\kappa$ B activation (Schaale et al., 2011).

The role of LRP5 in macrophages in the context of atherosclerosis development was investigated by Borrell-Pages et al. (2011) over the last decade.

Immunohistochemical analyses revealed the expression of LRP5 in macrophages that was increased in advanced plaques compared with initial lesions. In vitro, upon exposure to aggregated LDL, but not native LDL, human monocytes and human monocyte-derived macrophages showed a significant increase of LRP5 mRNA and protein levels and this was additionally associated with an increased Wnt/ $\beta$ -catenin activity (Borrell-Pages et al., 2011). Cholesterol accumulation in human monocyte-derived macrophages and macrophage migration after exposure to aggregated LDL were decreased by silencing of LRP5 (Borrell-Pages et al., 2011). In addition, the expression of osteopontin and BMP2, both involved in the progression of atherosclerotic plaques, was upregulated in a LRP5-dependent manner upon aggregated LDL exposure (Dhore et al., 2001; Lund et al., 2009; Borrell-Pages et al., 2011) (see *section VII.3.B*). Later experiments also revealed the key role played by LRP5 to limit monocyte differentiation into macrophages (Borrell-Pages et al., 2014b). At first, immunohistochemical findings and in vitro results suggested a proatherosclerotic role for LRP5. However, the impact of LRP5 was further tested in vivo by the same authors, using *Lrp5* knockout mice fed a high-cholesterol diet. These mice had slightly elevated cholesterol levels, higher aortic lipid infiltration, and developed larger atherosclerotic lesions (Borrell-Pages et al. 2014, 2015a,b), similar to previous findings in ApoE<sup>-/-</sup> mice (Magoori et al., 2003). Furthermore, the lack of LRP5 increased the transcription of LRP6, increased macrophage infiltration, and induced a proinflammatory plasma profile, thereby suggesting an atheroprotective role for LRP5 (Borrell-Pages et al., 2015b). More recently, Borrell-Pages et al. (2016a) showed that human CD16<sup>+</sup> macrophages, which have an anti-inflammatory phenotype, had higher expression level of LRP5 than CD16<sup>-</sup> macrophages, the proinflammatory subtype (Murray and Wynn, 2011; Liu et al., 2014). It was also shown that LRP5<sup>+</sup> macrophages infiltrate the deep layers of atherosclerotic plaques, suggesting increased migratory and phagocytic activities. These anti-inflammatory macrophages expressing LRP5 were abundantly present in advanced human atherosclerotic plaques, suggesting the activation of a tissue-rescue system (Borrell-Pages et al., 2016a).

The identification of therapeutic targets promoting plaque regression is of major interest. Among the different potential options, targeting plaque macrophages appears to be a relevant approach due to its importance for inflammation, lipid uptake, and plaque destabilization. For this purpose, transcriptional differences between macrophages in regressing versus progressing plaques have been studied in animal models of plaque regression (Ramsey et al., 2014). The study demonstrated that the WNT/ $\beta$ -catenin pathway and SOX family members are activated in plaque macrophages during regression (Ramsey et al., 2014). In



summary, WNT signaling is not only a key pathway for atherosclerosis development, but also seems to be of importance for its regression. This highlights the relevance of new therapeutic strategies targeting components of the WNT signaling pathway in the treatment of atherosclerosis.

**4. WNT Signaling in Vascular Smooth Muscle Cells.** Smooth muscle cells, located in the vascular media, exhibit a contractile phenotype and a low proliferation rate. During atherosclerosis development, however, endothelial dysfunction and the associated inflammatory state of the vessel wall result in the activation and de-differentiation of the vascular smooth muscle cells (VSMCs) toward a synthetic phenotype. These synthetic VSMCs subsequently migrate into the intima, proliferate, and increase the production of extracellular matrix. The thickened intima that results from this pathologic process paves the way for the development of the atherosclerotic plaque (Owens et al., 2004; Mill and George, 2012).

The impact of  $\beta$ -catenin on VSMC proliferation is well known.  $\beta$ -Catenin/TCF signaling can upregulate the expression of several proproliferative genes such as cyclin D1 and decrease the level of the cell cycle inhibitor p21 (Wang et al., 2002; Quasnichka et al., 2006). Functional evidence linking this pathway to vascular wall injury has been demonstrated in a balloon injury model (Slater et al., 2004). More recently, a study using carotid ligation in a Wnt reporter mouse has demonstrated the involvement of  $\beta$ -catenin signaling in both media and intima 3 and 28 days after the injury (Tsaousi et al., 2011b). Moreover, it has also been suggested that the pool of  $\beta$ -catenin located at the plasma membrane, involved in cell adhesion complexes, could be mobilized after disruption of the cell-cell adhesion upon migration of VSMCs from the media to the intima (Ugnow et al., 2003; Slater et al., 2004).

Wnt/FZD signaling is involved in the control of VSMC proliferation. Its inhibition by FrzA, a member of the sFRP family that negatively modulates Wnt signaling, leads to antiproliferative actions both in vitro and in vivo (Ezan et al., 2004). The identification of the Wnt proteins involved in VSMC proliferation has been the focus of several studies. First of all, both WNT1 and WNT3A have been shown to induce the expression of cyclin D1 in VSMCs (Marchand et al., 2011). LRP6 could also promote VSMC proliferation. Indeed, the upregulation of LRP6 increased WNT1-induced TCF activation and the expression of a dominant negative mutant of LRP6 abolished WNT-induced survival, cyclin D1 activity, and cell cycle progression (Wang et al., 2004).

WNT4 was recently identified also to have an impact on VSMC proliferation. The mRNA expression of WNT4 was found to be elevated in proliferating VSMCs. Knockdown experiments as well as experiments using WNT4-conditioned medium confirmed the role of WNT4 in VSMC proliferation. The expression of WNT4 was

also found to be elevated in injured carotid arteries: in the media at 3 days and in the intima at 28 days after injury, in line with the intimal migration of VSMCs. The significance of WNT4 expression for atherosclerosis was further evidenced by the decreased intimal lesion size and intimal cell proliferation rate found in *Wnt4*<sup>+/-</sup> mice (Tsaousi et al., 2011a).

Since cholesterol accumulation is of major importance for the progression of atherosclerosis, the impact of WNT5A [known to be upregulated in atherosclerotic lesions and plasma (Christman et al., 2008; Bhatt and Malgor, 2014; Malgor et al., 2014)] on this process has been investigated on VSMCs treated with oxLDL (Qin et al., 2014). WNT5A protein was increased in a concentration and time-dependent manner by oxLDL treatment. After WNT5A knockdown, total cholesterol and free cholesterol content increased significantly upon exposure to oxLDL (Qin et al., 2014). Reciprocally, both levels were decreased after treatment of the cells with WNT5A. This action mediated by WNT5A was associated with a regulation of mRNA and protein expression of caveolin-1 and ATP binding cassette transporter A1, two important players of reverse cholesterol transport (Qin et al., 2014).

A recent study investigating miRNA expression profiles in patients with stable and unstable carotid plaque has revealed miR-210 as being the most significantly downregulated miRNA in local plasma material. It was shown that miR-210 inhibits APC and thus favors Wnt signaling in VSMCs of the fibrous cap, therefore promoting their proliferation and survival (Eken et al., 2017). Further studies are required to decipher the contribution of other miRNAs in the development of atherosclerosis.

The involvement of Wnt signaling at the different stages of atherosclerosis is highlighting its importance for this pathology (Hermans and Blankesteyn, 2015; Matthijs Blankesteyn and Hermans, 2015). Recently, it was shown that inhibition of Wnt signaling by sclerostin, a protein that binds to LRP4, LRP5, and LRP6, attenuated angiotensin II (AngII)-induced aortic aneurysm and atherosclerosis (Krishna et al., 2017). This was associated with a decreased macrophage infiltration and a decreased expression of  $\beta$ -catenin responsive genes (Krishna et al., 2017). This supports the idea of targeting the Wnt pathway to limit or stop the progression of atherosclerotic lesions.

## B. Vascular Calcification

Vascular calcification is a key process involved in the development of atherosclerosis as well as other cardiovascular pathologies such as congestive heart failure and chronic kidney disease (Demer and Tintut, 2008; Vervloet and Cozzolino, 2017). Vascular calcification has similarities with embryonic bone formation, in which WNT signaling is playing an important role (Wang et al., 2014b).

Early studies have identified a link between *Msx2*, a homeodomain transcription factor involved in bone anabolism, and WNT signaling (Shao et al., 2005, 2007; Cheng et al., 2008b). Indeed, *Msx2* has been found to upregulate WNT3A and WNT7A, while down-regulating the expression of the  $\beta$ -catenin-dependent inhibitor DKK1 in primary aortic myofibroblasts (Shao et al., 2005). Further in vivo analyses with mice overexpressing *Msx2* in the aorta, confirmed the suppression of aortic DKK1 and the upregulation of vascular WNT expression (Shao et al., 2005). It was thus hypothesized that *Msx2* activation was acting on the calcification process in a paracrine manner by signaling via the WNT pathway. In addition, mice overexpressing *Msx2* exhibited an increased bone volume compared with their wild-type littermates (Cheng et al., 2008b). In vitro investigations revealed WNT7-LRP6 signaling as being required for the induction of osteogenic differentiation of mesenchymal cells by *Msx2* (Cheng et al., 2008b). The signaling cascade was further investigated at the vascular level and bone morphogenetic protein 2 (BMP2) was identified as an upstream regulator of medial artery calcification in vivo (Shao et al., 2006, 2007). Injection of BMP2 in LDLR<sup>-/-</sup> mice was, as expected, able to increase aortic *Msx2* expression, aortic WNT signaling and aortic calcium content (Shao et al., 2006). The relevance of BMP2 for vascular calcification is supported by clinical data showing an elevation of serum BMP2 levels in chronic kidney disease patients (Rong et al., 2014). Mechanistic explanations were provided by in vitro experiments using rat vascular smooth muscle cells. Stimulation of these cells with BMP2 induced cell apoptosis and upregulation of  $\beta$ -catenin, *Msx2*, and *Runx2*. This effect was dependent on  $\beta$ -catenin signaling (Rong et al., 2014). It was suggested that TNF- $\alpha$ , a known activator of BMP2, could constitute a relevant stimulus of the BMP2-*Msx2*-WNT signaling in vascular pathologies (Shao et al., 2007).

Other pathologic triggers should also be considered for the study of WNT signaling in vascular calcification. The advanced glycation end products (AGEs) and their receptors RAGEs are of particular relevance for diabetic disorders as well as other inflammatory diseases. A recent study performed in human arterial smooth muscle cells has shown that AGEs/RAGEs can promote calcification via WNT/ $\beta$ -catenin signaling (Liu et al., 2016). It is hypothesized that RAGEs may activate genes, promoting the differentiation of arterial smooth muscle cells into osteogenic cells through the WNT/ $\beta$ -catenin pathway. The contribution of WNT/ $\beta$ -catenin signaling for the osteogenic transdifferentiation of vascular smooth muscle cells and calcification seems to be under the control of *Runx2*, a transcription factor of osteoblasts (Cai et al., 2016). Activation of the WNT/ $\beta$ -catenin pathway (e.g., with WNT3A) induces the expression of *Runx2*, which in turn, controls key

processes for osteoblast differentiation. On the contrary, blockade of WNT/ $\beta$ -catenin using DKK1 inhibits *Runx2* induction and calcium depositions in vascular smooth muscle cells (Cai et al., 2016). Moreover, when bone marrow-derived mesenchymal stem cells (MSCs) are in contact with calcified vascular smooth muscle cells, they display an osteoblast phenotype. This differentiation seems to be due to an activation of the WNT5A/ROR2 pathway (Xin et al., 2013; Guan et al., 2014).

Matrix metalloproteinases (MMPs) are remodeling the extracellular matrix and are therefore suggested to play a role during vascular calcification. Their contribution has been evidenced recently in uremic vascular calcifications (Hecht et al., 2016). Furthermore, the calcification of vascular smooth muscle cells by WNT activation seemed to involve an enhanced expression of MMP-2 and MMP-9 (Freise et al., 2016). Increased expression and activity of MMPs is suggested to further promote the process of vascular calcification.

Although most findings seem to support the deleterious effect of WNT signaling on vascular calcification, a recent study identified WNT16 as being able to attenuate TGF $\beta$ -induced chondrogenic transformation of vascular smooth muscle cells (Beazley et al., 2015). In this study, WNT16 was found to sustain Notch activity, which in turn maintained the contractile phenotype of vascular smooth muscle cells (Beazley et al., 2015). LRP6 was also found to limit the process of vascular calcification. Indeed, the conditional deletion of *LRP6* in the vascular smooth muscle cell lineage increased the aortic calcium content and decreased aortic distensibility in diabetic LDLR<sup>-/-</sup> mice (Cheng et al., 2015). The protective vascular function of LRP6 may be due to a downregulation of  $\beta$ -catenin-independent FZD signaling (Cheng et al., 2015).

### C. Norrie Disease

Norrin is a retinal growth factor with angiogenic and neuroprotective properties (see *section IX*) (Xu et al., 2004). Norrin binds to FZD<sub>4</sub> receptors and subsequently activates the WNT/ $\beta$ -catenin pathway. This activity is furthermore increased in the presence of Tspan12 within the norrin/FZD<sub>4</sub> receptor complex. A defect in norrin or FZD<sub>4</sub> has a detrimental impact on retinal vasculature during developmental angiogenesis (Ohlmann and Tamm, 2012). The so-called Norrie disease, associated with mutations in the NDP gene, causes blindness as well as progressive deafness and mental retardation (Berger et al., 1992). This also applies for familial exudative vitreoretinopathy, retinopathy of prematurity, and other retinal hypovascularization diseases (Ye et al., 2010; Ohlmann and Tamm, 2012). Next to norrin and FZD<sub>4</sub>, LRP5 and TSPAN12 have been found to be also associated with familial exudative vitreoretinopathy, thus highlighting the importance of the norrin/FZD<sub>4</sub> signaling for proper retinal

vasculature development (Wang et al., 2012; Gilmour, 2015). The cysteine-rich domain (CRD) of FZD<sub>4</sub> has been shown to play a critical role in norrin-FZD<sub>4</sub> binding (Smallwood et al., 2007). Specific mutations of the FZD<sub>4</sub> in the cysteine-rich domain led to a defective norrin/ $\beta$ -catenin signaling in *Xenopus* embryos (Zhang et al., 2011).

The retinal vasculature in mice lacking norrin, FZD<sub>4</sub> and Tspan12 is massively impaired. The superficial retinal vascular plexus is delayed and incomplete compared with WT and they all lack the intermediate and deep vascular plexi (Zuercher et al., 2012). Further analyses of the retinal vasculature development revealed that, in a norrin knockout model, the vascular density and number of branching points were decreased, whereas the number of filopodia was significantly increased (Zuercher et al., 2012). However, the morphology of the filopodial protrusions was different compared with WT with more narrow angles and a reduced amount of long tip cell protrusions (Zuercher et al., 2012). In addition, an excessive recruitment of mural cells, required to stabilize the nascent vasculature, was observed in the retinal vessels from the *norrin* knockout mice, suggesting a potential interaction with PDGF- $\beta$ /PDGFR- $\beta$  signaling. The overall decreased vascular density and impaired vascular growth is suggested to be related to a decreased proliferation of the retinal ECs in the *norrin* knockout mice. This was evidenced directly in the retina after systemic injection of bromodeoxyuridine, as well as during an in vitro assay in which norrin was identified as a mitogenic stimulus for engineered HEK293 cells (Zuercher et al., 2012).

By using mouse genetic and cell culture models, it was demonstrated that Muller glia cells are responsible for norrin production, which then stimulates FZD<sub>4</sub> receptors located on retinal ECs (Ye et al., 2009). Subsequently, the norrin/FZD<sub>4</sub>/LRP5/6 signaling is responsible for a transcriptional program in ECs to promote vascular growth and the endothelial-mural cell interactions. This program is at least partly under the control of the transcription factor Sox17. It was shown that the transfection of Sox17 was able to rescue the ability of *Fzd4* knockout retinal ECs to form capillary-like structures in a Matrigel assay (Ye et al., 2009). In addition, although FZD<sub>4</sub> is also expressed in photoreceptors and some other retinal neurons, it was shown that the impaired visual function in *Fzd4* knockout mice is linked to a defect in retinal vasculature rather than an impaired signaling in retinal neurons (Ye et al., 2009).

Finally, norrin/FZD<sub>4</sub>/LRP5/6 signaling is also involved in other retinopathies, opening thus new avenues for therapeutic interventions. In addition, this signaling complex seems to be also of importance for other nonretinal disorders, such as female infertility (Hsieh et al., 2005; Luhmann et al., 2005).

Its contribution in this field needs to be further investigated.

#### D. Pulmonary Arterial Hypertension

Pulmonary arterial hypertension (PAH) is a life-threatening disease associated with increased pulmonary pressures, subsequently followed by development of right-sided heart failure. The pathology is characterized by a microvascular rarefaction and an increased pulmonary vascular resistance resulting from wall thickening due to smooth muscle cell proliferation (Rabinovitch, 2012).

The relevance of WNT signaling within this context was revealed by the expression profile of laser-microdissected pulmonary arterial resistance vessels obtained from idiopathic PAH and donor lung tissue (Laumanns et al., 2009). Pathway analysis demonstrated a significant upregulation of the WNT/PCP pathway. Moreover,  $\beta$ -catenin expression was also upregulated in ECs of pulmonary arteries from PAH patients compared with control subjects (Laumanns et al., 2009).

Thickening of the pulmonary arterial wall is observed in PAH due to an increased proliferation of vascular smooth muscle cells in the media. This has been found to be associated with higher levels of active  $\beta$ -catenin within pulmonary artery smooth muscle cells (PASMCs) in PAH patients (Takahashi et al., 2016). In addition, PASMCs from PAH patients also harbor higher expression levels of the platelet derived growth factor (PDGF)-BB and its receptors. PDGF-induced growth of PASMCs that turned out to be dependent of  $\beta$ -catenin activation and GSK3 $\beta$  inhibition (Takahashi et al., 2016). WNT5A was able to repress PDGF-BB-induced proliferation in PASMCs from healthy patients but not from PAH patients (Takahashi et al., 2016), highlighting a potential defective signaling in PASMCs during PAH.

Idiopathic PAH can also result from mutations in bone morphogenetic protein (BMP) receptor II (BMPRII). Such mutations are associated with apoptosis of pulmonary artery ECs as well as microvascular rarefaction during idiopathic PAH (de Jesus Perez et al., 2009). This is of particular interest as the proliferation, survival, and migration of pulmonary artery ECs induced by BMP2 is dependent of  $\beta$ -catenin to mediate GSK3 $\beta$  inactivation, but also dependent of Smad1 phosphorylation to recruit DVL and activate the RhoA/Rac1 pathway (de Jesus Perez et al., 2009). The relationship between the BMPRII and WNT signaling pathways has been further studied using primary cells from patients (induced pluripotent stem cell-derived mesenchymal and ECs) to identify molecular signatures of PAH (West et al., 2014). The results confirmed the increased WNT signaling in the cells of PAH patients and established a link with decreased BMPRII signaling (West et al., 2014). In particular, expression analyses

from PAH patient lung tissue and BMPRII mutant mice revealed an increased expression of the WNT inhibitor sFRP2 (West et al., 2014).

Finally, the progression and severity of PAH pathology is associated with a dysregulation of miRNA expression (Courboulin et al., 2011; Rhodes et al., 2013). Recently, a systematic investigation has been carried out via miRNA profiling of lung tissue from end-stage idiopathic PAH (Wu et al., 2016a). A pathway enrichment analysis identified WNT/ $\beta$ -catenin signaling as being the most affected. Five miRNAs (let-7a-5p, miR-26b-5p, miR-27b-3p, miR-199a-3p, and miR-656) relevant for PAH pathogenesis were significantly increased and correlated with hemodynamics and histopathological parameters of PAH (Wu et al., 2016a). Moreover, many WNT family genes (*FZD<sub>4</sub>*, *FZD<sub>5</sub>*, *CTNNB1*) and downstream targets (CCDN1, VEGFA, axin2) were upregulated in end-stage idiopathic PAH lung tissue (Wu et al., 2016a).

In conclusion, WNT signaling pathways appear to exert a key role of the preservation of pulmonary vascular homeostasis. Future studies are required to investigate the therapeutic potential of WNT signaling for PAH (de Jesus Perez et al., 2014).

### *E. Conclusions and Suggestions for Further Research*

Wnt signaling is not only an important key pathway for atherosclerosis development but seems to be also of importance for its regression. The activation of Wnt signaling in atherosclerosis has been firmly established, not only in experimental models but also in patients by measuring pathway components in the circulation. This activation is likely to contribute to endothelial dysfunction, the inflammatory response, and VSMC proliferation and appears to involve both  $\beta$ -catenin-mediated and non- $\beta$ -catenin-mediated WNT signaling. Most of the studies published to date on this subject made use of genetic interventions, so a suggestion for further research would be to include pharmacological interventions. Because of the involvement of multiple signaling pathways, intervening in the WNT production and/or at the level of the receptor complex appears to be a good starting point for these experiments. In the case of vascular calcification, most of the experimental data presented so far point to activation of the  $\beta$ -catenin-mediated signaling. Therefore, it would be interesting to study interventions in this branch of the Wnt signaling pathway. There is a rapidly growing number of drugs specifically developed for targeting  $\beta$ -catenin-mediated WNT signaling (please refer to *section XI*), and these drugs appear to be interesting candidates to be tested in vitro and in animal models of vascular calcification. PAH is a condition for which new therapeutic approaches are urgently needed. The field has been hampered for a long time by the lack of animal models that adequately represent the pathologic aspects of this condition. Fortunately, new animal models

for PAH have been proposed (Colvin and Yeager, 2014) that can be used to test the effects of therapeutic interventions in WNT signaling on the pulmonary vascular resistance.

## **VIII. WNT Signaling in Cardiac Disease**

### *A. WNT Signaling in Myocardial Infarction*

Myocardial infarction (MI) is one of the most frequent acute cardiovascular events. It usually occurs as a consequence of the progression of an atherosclerotic plaque toward an unstable phenotype, where the fibrous cap ruptures and releases the content of the plaque into the circulation. However, myocardial infarction can also be the result of plaque erosion with distal embolization or coronary vasospasm (Regitz-Zagrosek et al., 2016). In any case, due to the interrupted blood flow the affected region of the heart is deprived of oxygen and nutrients, resulting in cell loss in that area.

Despite the fact that stem cells have been identified in the heart, the repair of the infarct area does not result in the significant regeneration of the lost cardiac muscle (Laflamme and Murry, 2011). Instead, it shows many similarities to the wound healing process in the skin: The initial response consists of an inflammatory reaction involving polymorphonuclear neutrophils and macrophages. The function of these cells is to clear the infarct area from the necrotic debris, which is an essential first step in the repair of the infarct. The inflammatory phase is followed by the formation of granulation tissue, which typically is rich in (myo)-fibroblasts and newly formed blood vessels, embedded in a loosely organized extracellular matrix. The myofibroblasts in the granulation tissue can be derived from different sources such as resident fibroblasts, circulating fibrocytes, or epicardial cells undergoing epithelial-to-mesenchymal transition (EMT) (Daskalopoulos et al., 2012). Because of their unique combination of contractile and matrix synthesizing properties, the myofibroblasts play an important role in the formation of a compact and strong replacement of the lost cardiac cells (van den Borne et al., 2010). This granulation tissue gradually develops into scar tissue by losing cells and increasing the amount and the cross-linking of extracellular matrix (Hermans et al., 2016).

*1. Effects of Modulation of WNT Expression and Secretion.* As indicated in *section VI*, WNT signaling is particularly active during cardiac development. In contrast, little WNT signaling takes place in the adult heart under normal physiologic conditions. This changes during pathologic remodeling of the heart, where many fetal genes, including components of WNT signaling, become reactivated (Kuwahara and Nakao, 2011). In a mouse model of MI, where the underlying pathology of human infarction is mimicked by a surgical ligation of a coronary artery, the

modulation of the expression of multiple genes of the WNT pathway has been reported. This includes the upregulation of WNT2, -4, 10-b, and -11 expression and the downregulation of WNT7B (Barandon et al., 2003; Aisagbonhi et al., 2011; Paik et al., 2015; Morishita et al., 2016). Moreover, in the same studies the increased expression of FZD<sub>1</sub>, -2, -5, and -10 and the decreased expression of FZD<sub>8</sub> was demonstrated (Barandon et al., 2003; Aisagbonhi et al., 2011). Recently, Mizutani et al. (2016) used in situ hybridization to monitor the expression of all 19 WNTs in a cryoinjury model in neonatal mice. They found that WNT2B and WNT5A and, to a lesser extent, WNT9a were upregulated in the injured epicardial layer. WNT3A, -4, -5B, -6, -8A, -9B, and -10B were upregulated in the myocardium of the injured hearts, whereas WNT8B, -10A, -11, and -16 were similarly expressed in injured and uninjured hearts. These results demonstrate the modulation of WNT signaling in injured hearts but also highlight the potential effects of age (neonatal vs. adult) and injury model (cryoinjury vs. coronary ligation) on the expression pattern of WNT genes. For a detailed overview of the role of different WNT proteins in cardiac function, we refer the reader to an excellent review on this subject (Dawson et al., 2013).

In several studies, the effect of modulation of WNT expression or secretion on infarct healing was addressed. Applying coronary artery ligation and cryoinjury in a mouse model of cardiomyocyte-specific overexpression of WNT10B, an increase in neovascularization of the infarct zone, a smaller scar size with fewer myofibroblasts and an improved ventricular function were observed (Paik et al., 2015). Similar beneficial results were reported in a mouse model of MI, in which WNT11 was overexpressed via adeno-associated virus-mediated overexpression, where a marked reduction in the inflammatory response in the infarcted heart was reported (Morishita et al., 2016). An attenuated inflammatory response and increased neovascularization could also be induced by the macrophage-specific deletion of the WNT transporter WLS (*csfm-icre;Wls<sup>fl/fl</sup>* mouse). The WLS-deficient macrophages displayed an M2 phenotype, known to stimulate repair and angiogenesis (Palevski et al., 2017). Interference with the lipidation of WNT proteins by intravenous administration of the PORCN inhibitor GNF-6231 to a mouse model of MI also reduced infarct size, prevented adverse remodeling, and reduced the decline in cardiac function. Similar beneficial effects were observed in a recent study, in which the PORCN inhibitor WNT-974 (formerly known as LGK974) was administered by oral gavage to infarcted mice, starting at 1 week after MI induction. The investigators observed an unanticipated proregenerative response in heart muscle and a collagen-VI-dependent antifibrotic effect of the treatment and concluded that PORCN inhibition could be a promising therapeutic strategy to block pathologic

remodeling in MI patients (Moon et al., 2017). On the other hand, direct injection of recombinant WNT3A protein in the border zone of the infarct area was shown to have detrimental effects on infarct healing, because this treatment was found to inhibit the proliferation of cardiac side population cells (Oikonomopoulos et al., 2011). From these studies it can be concluded that more general interventions in WNT processing and secretion result in better infarct healing, but that more studies are needed to determine the positive and negative contribution of individual WNTs to the wound healing process.

*2. Modulation at the Level of Secreted Frizzled-Related Proteins.* As indicated in section IV.B of this review, sFRPs are thought to act primarily as negative modulators of Wnt signaling. Barandon et al. (2003) were the first to demonstrate the upregulated expression of sFRP1 after MI. In transgenic mice overexpressing FrzA, the bovine homolog of sFRP1, these authors demonstrated the beneficial effect of FrzA on infarct size and cardiac function after MI. These beneficial effects were attributable to a reduction in cardiomyocyte apoptosis and leukocyte infiltration and an improved capillary density in the scar. Similar results were obtained when bone marrow cells of sFRP1 overexpressing mice were transplanted in wild-type recipients, which subsequently were subjected to MI (Barandon et al., 2011). A genetic variant of sFRP1 was reported to be associated with an increased risk of MI in a Chinese Han population (Tao et al., 2016). An anti-inflammatory and antiapoptotic role for sFRP5 has also been described in a mouse model of ischemia/reperfusion damage (Nakamura et al., 2016), supporting a beneficial effect of sFRPs on infarct healing.

The role of sFRP2 in infarct healing has been addressed in several publications. sFRP2 was shown to be a paracrine factor secreted from stem cells and to activate an antiapoptotic gene expression profile in cardiomyocytes (Mirotsov et al., 2007). Direct injection of recombinant sFRP2 in the infarcted myocardium of rats was reported to have antifibrotic effects and improved cardiac function (He et al., 2010). sFRP2 was also found to contribute to the engraftment of mesenchymal stem cells (MSC), and injection of sFRP2-overexpressing MSC in the border zone of infarcted mice improved the wound healing and cardiac function (Alfaro et al., 2008). On the other hand, mice lacking the sFRP2 gene also were shown to have decreased fibrosis and improved cardiac function after being subjected to MI compared with wild types (Kobayashi et al., 2009). Moreover, therapy with sFRP2 antibody reduced apoptosis and fibrosis in a hamster model of heart failure (Mastri et al., 2014). These contradictory results of the different studies may be explained by the multiple modes of action of sFRPs (Bovolenta et al., 2008), please refer to section IV.B for details.

3. *Interventions at the Receptor Complex.* WNT receptor complexes are formed by members of the FZD protein family in close association with a coreceptor such as LRP5/6 or ROR2. Using a rat model of myocardial infarction induced by permanent coronary artery ligation, our research group was the first to describe the upregulated expression of a member of the WNT/FZD signaling system, FZD<sub>2</sub>, peaking at 7 days post-MI. The highest FZD<sub>2</sub> expression was initially localized in the border zone of the infarct and expanded toward the center of the infarct, together with the infiltration of the area with myofibroblasts (Blankesteyn et al., 1997). The high level of expression of FZD<sub>2</sub> and <sub>4</sub> in cardiac fibroblasts was confirmed in an unbiased G protein-coupled receptor RT-PCR screen (Snead and Insel, 2012). A similar expression kinetic was observed for the signal transduction molecule DVL1 in a rat MI-model, supporting the concept of activated WNT signaling in the wound healing process (Chen et al., 2004). Later on it was shown that the expression of more members of the FZD family is regulated post-MI: FZD<sub>1</sub>, <sub>2</sub>, <sub>5</sub>, and <sub>10</sub> were found to be upregulated, whereas FZD<sub>8</sub> was downregulated at 7 days post-MI (Barandon et al., 2003). However, to our knowledge, no studies have been published in which the effects of genetic interventions in FZD genes on infarct healing were addressed.

At present, the options for pharmacological intervention in WNT signaling at the level of the FZD receptor are very limited; for details, please refer to *section XI*. In our group, we investigated the antagonistic effects of peptide fragments of WNT3A and WNT5A on WNT signaling. We identified UM206, a 13-amino acid peptide derived from WNT5A, and tested the effects of subcutaneous infusion of this peptide on infarct healing in a mouse model of permanent coronary occlusion. Administration was performed via osmotic minipump, starting directly after MI induction, and the effect was evaluated at 5 weeks post-MI. UM206 administration reduced LV dilatation, increased cardiac performance, and prevented the development of heart failure. Histologic analysis showed a smaller and thicker infarct and increased myofibroblast numbers in the infarcts (Laeremans et al., 2011). Attenuation of infarct expansion was also observed in UM206-treated swine subjected to cardiac ischemia/reperfusion injury and followed for 5 weeks. In this study, however, the myofibroblast numbers in the infarct area were reduced rather than increased, as was observed in the mice. Moreover, in the UM206-treated group cardiac function was not better than in vehicle-treated controls (Uitterdijk et al., 2016). There are still many open questions regarding the mechanism of action of the peptide inhibitor, because the region from which the peptide is derived does not have a direct interaction with the FZD CRD in the XWNT8/mFZD8 structure published by Janda et al. (2012). However, the

attenuation of infarct expansion observed in both studies suggests that the WNT pathway can be a promising target to improve infarct healing a limit post-MI remodeling.

In two recent studies, the effect of the LRP5/6 coreceptors on infarct healing was investigated. As described in detail in *section III.C*, these coreceptors are specifically involved in WNT/ $\beta$ -catenin signaling. In a swine model of ischemia-reperfusion, LRP5 expression was found to be increased only in animals fed a high-cholesterol diet, but not in the group with normal cholesterol diet. Translocation of  $\beta$ -catenin to the nucleus, as well as increased expression of the WNT target genes Osteopontin and BMP2, confirmed the activation of WNT/ $\beta$ -catenin signaling in this group. In the same study, deletion of LRP5 was found to have a deleterious effect on infarct healing in mice. Moreover, human hearts explanted due to ischemic cardiomyopathy expressed higher levels of LRP5 and  $\beta$ -catenin compared with dilated cardiomyopathy (Borrell-Pages et al., 2016b). These results could be confirmed using mice carrying a cardiomyocyte-specific deletion of LRP5 and 6 (Wo et al., 2016). No studies on the effects of interventions in the coreceptor ROR2 in infarct healing have been published to date.

In several recent studies the role of members of the LRP5/6 inhibitor family DKK in myocardial infarction has been addressed. Plasma levels of DKK1 were found to be higher in patients with ST-segment elevation MI compared with non-ST-segment elevation MI infarcts and can serve as an independent predictor of major adverse cardiovascular events (Wang et al., 2013a). Injection of purified DKK1 protein into the border zone of the infarct area in mice exacerbated the ischemic injury and increased the expression of markers of fibrosis at 4 weeks post-MI. However, cardiac function was not affected by this treatment (Wo et al., 2016). DKK1 and DKK2 were found to have opposite effects on angiogenesis, whereas injection of DKK2 protein in the border zone enhanced the neovascularization of the infarct area with concomitant improved cardiac function (Min et al., 2011). In cultured cardiac fibroblasts, DKK2 reduced proliferation and differentiation into myofibroblasts in a WNT/ $\beta$ -catenin dependent manner. This effect could be antagonized by the overexpression of miR-154, which was found to interact directly with DKK2 (Sun et al., 2016). Global deletion of the *Dkk3* gene was found to promote apoptosis, inflammation, and remodeling, whereas cardiomyocyte-specific overexpression of DKK3 [ $\alpha$ -myosin heavy chain ( $\alpha$ -MHC) promoter] had the opposite effect (Bao et al., 2015). In an aptamer-based search for novel candidate biomarkers for cardiovascular disease, DKK4 was found to be upregulated in hypertrophic cardiomyopathy patients at 10 and 60 minutes after a planned septal ablation, a procedure causing cardiac ischemia. The increased circulating DKK4 levels could be confirmed in

patients suffering from spontaneous myocardial infarction (Ngo et al., 2016). Although these combined studies do not show a uniform role for the different members of the DKK family in infarct healing, they clearly emphasize the importance of WNT/ $\beta$ -catenin signaling in multiple processes associated with cardiac remodeling and infarct healing.

**4. The  $\beta$ -Catenin Destruction Complex.** Under resting conditions,  $\beta$ -catenin is continuously degraded by the  $\beta$ -catenin destruction complex, keeping its intracellular levels low. As described in detail in *section V.B*, this involves two subsequent phosphorylation steps by CK1 and GSK3, respectively, preparing the protein for ubiquitination and degradation. The role of the two isoforms of GSK3 (GSK3 $\alpha$  and  $-\beta$ ) in the remodeling after MI has been investigated using genetic models. Because a full GSK3 $\beta$  knockout is embryonic lethal (Lal et al., 2015), an inducible cardiomyocyte-specific GSK3 $\beta$  deletion was used for these studies (mer-Cre-mer under the control of a  $\alpha$ -MHC promoter). Despite similar infarct sizes, gene deletion at 5 days post-MI resulted in reduced dilatation of the left ventricle and a better preservation of LV function in the KO mice compared with the control group at 8 weeks post-MI. The beneficial effect on cardiac function may be supported by the induction of hypertrophy in the remote myocardium (Woulfe et al., 2010). In contrast to GSK3 $\beta$ , global deletion of the GSK3 $\alpha$  gene does not affect the development or the life span of mice (Lal et al., 2015). However, when MI was induced in these mice, increased mortality due to infarct rupture, augmented LV dilatation and attenuated cardiac function were observed. Moreover, the infarct size was increased due to increased apoptosis in the border zone of the infarct (Lal et al., 2012). Remarkably, cardiomyocyte-specific deletion of GSK3 $\alpha$  had the opposite effect on infarct healing, with a better preservation of cardiac function and more viable cardiomyocytes in the border zone. The latter could be attributed to both lower cardiomyocyte apoptosis and higher proliferation rates of the cardiomyocytes (Ahmad et al., 2014). Cardiomyocyte-specific deletion of both GSK3 $\alpha$  and GSK3 $\beta$  (mer-Cre-mer under the control of a  $\alpha$ -MHC promoter), however, resulted in spontaneous dilatation of the left ventricle and decreased cardiac function (Zhou et al., 2016). Because the two GSK3 homologs are very similar and no subtype-specific drugs are available (Lal et al., 2015), this observation may question the usefulness of GSK3-inhibitors in the treatment of acute MI.

To our knowledge, there are no published studies available in which the role of CK1 in infarct healing is addressed by genetic interventions. However, an alternative and translationally interesting approach is the use of the Food and Drug Administration-approved compound pyrvinium. This compound is registered as an anti-helminthic drug, but it has been shown to inhibit WNT signaling by activating CK1 $\alpha$  with an

IC<sub>50</sub> of around 10 nM (Thorne et al., 2010). A single intramyocardial administration of pyrvinium improved adverse cardiac remodeling and limited the LV dilatation by increasing the proliferation of differentiated cardiomyocytes in the remote myocardium at 30 days post-MI. However, an increased mortality was observed due to toxicity of the intracardiac administration of pyrvinium and no significant improvement of cardiac function was found (Saraswati et al., 2010). In another study, pyrvinium was shown to reduce cardiac fibroblast survival particularly under conditions of low glucose. Oral administration of a pyrvinium pamoate suspension between day 1 and 14 after infarction reduced fibrosis and scar formation in infarcted hearts at 4 and 14 days post-MI, combined with a better ejection fraction than the vehicle-treated mice (Murakoshi et al., 2013). The latter study suggests that oral rather than intracardiac administration would be the preferential route for this drug.

**5.  $\beta$ -Catenin-Mediated Gene Transcription.** Nuclear accumulation of  $\beta$ -catenin and modulation of the transcription of genes involved in proliferation and survival by this intracellular signaling protein are the final steps in the WNT/ $\beta$ -catenin signaling pathway. The availability of WNT/ $\beta$ -catenin reporter mice has provided the opportunity to visualize the activation of  $\beta$ -catenin-mediated WNT signaling in the infarct area. By using a LacZ-reporter mouse driven by the axin2 promoter, the amount of LacZ<sup>+</sup> cells in the border zone was found to increase markedly between day 7 and 21 post-MI. Costainings showed that LacZ<sup>+</sup> cells coexpressed Sca (hematopoietic stem cells), CD31 (ECs), CD45 (leukocytes), von Willebrand factor (ECs), and  $\alpha$ -smooth muscle actin (myo)fibroblasts), indicating the activation of WNT signaling in multiple cell types (Oerlemans et al., 2010). In a model in which the LacZ expression was driven by a TCF/LEF-responsive promoter (the TOPGAL mouse line), a similar LacZ expression was observed in the border zone and at the epicardial side of the infarct. Many of the  $\alpha$ -smooth muscle actin-positive cells were found to originate from epicardial ECs that underwent endothelial-to-mesenchymal transformation, as shown by cell lineage tracing (Aisagbonhi et al., 2011). In this study, the signal had disappeared at 3 weeks post-MI, which suggests a more rapid completion infarct healing, e.g., due to a smaller infarct size. Recently, the use of an MRI probe consisting of iron-binding human ferritin heavy chain and GFP, under the control of a TCF/LEF sensitive promoter, was described in a rat model of MI. The construct was delivered via an AAV9 vector to the infarcted heart by local injection. Iron accumulation could be detected at the border zone of the infarct of the transduced hearts at 4 weeks post-MI. Histologic analysis confirmed the colocalization of the iron and GFP signals. Inhibition of WNT/ $\beta$ -catenin signaling using the compound SEN195 completely abolished the iron

signal. The combined studies provide evidence for an activation of WNT/ $\beta$ -catenin signaling at the borders of the infarct in the first weeks after MI, involving multiple cell types (Matteucci et al., 2016).

Several studies have been executed in which the effect of modulation of  $\beta$ -catenin levels on infarct healing was studied. Adenoviral gene transfer of a constitutively active form of  $\beta$ -catenin in cardiomyocytes and cardiac fibroblasts induced proliferation in the latter, whereas hypertrophy and binucleation were observed in the former cell type. In the fibroblasts, an augmented differentiation toward myofibroblasts was observed. Injection of the adenovirus in the border zone of an infarcted rat heart resulted in reduced infarct size due to less apoptosis and cell cycle activation in both cardiac fibroblasts and myocytes (Hahn et al., 2006). The importance of WNT1-mediated activation of WNT/ $\beta$ -catenin signaling in the epicardium, promoting epithelial-mesenchymal transition, was also observed using a conditional epicardial  $\beta$ -catenin knockout model (Duan et al., 2012). However, cardiomyocyte-specific deletion of  $\beta$ -catenin ( $\alpha$ MHC-Cre PR1 model) has also been reported to have a beneficial effect on survival and left-ventricular function. This was accompanied by a different scar phenotype where cardiac progenitor cells in the subepicardial layer were found to express cardiomyocyte lineage markers (Zelarayan et al., 2008). Furthermore, pharmacological inhibition of the  $\beta$ -catenin-mediated transcription by administration of the small molecule WNT signaling modulator ICG-001 resulted in improved ejection fraction in female rats at 10 days post-MI (Sasaki et al., 2013). Based on the combined results of these studies, there is no consensus whether  $\beta$ -catenin signaling should be stimulated or inhibited after MI.

### B. WNT Signaling in Valvular Disease

Heart valves have a typical building plan that is highly conserved in evolution and makes them optimally suited to control the direction of blood flow within the heart. Heart valves consist of three layers: the spongiosa is situated in the middle, flanked by the fibrosa layer on the non-flow side, and the atrialis or ventricularis on the flow side of the valve. The valves are covered by a layer of valvular ECs. The atrialis/ventricularis is rich in radially oriented elastin fibers, whereas the fibrosa contains high amounts of densely packed collagen fibers that maintain the structural integrity of the closed valve. The spongiosa contains proteoglycans and glycosaminoglycans, allowing some movement of the atrialis/ventricularis and the fibrosa (Orton et al., 2012). The most abundant cell type in the different layers of the valve is the valvular interstitial cell, involved in maintenance of the valve by synthesis and degradation of matrix molecules. Under normal circumstances, valvular interstitial cells resemble

quiescent fibroblasts, but upon injury they differentiate into myofibroblasts.

Valvular defects are a common cause of inadequate pump function of the heart. Generally, a distinction is made between valvular stenosis, common in the aortic valve and leading to obstructed outflow, and valvular degeneration, frequently found in the atrioventricular valve and leading to regurgitation of blood from the ventricle into the atrium in the systolic phase. The prevalence of aortic valve stenosis is particularly high in the elderly, with 15%–25% of subjects over 65 year of age being affected (Mathieu et al., 2014). Clinically significant mitral regurgitation due to a myxomatous (degenerated) mitral valve has been reported in 1.7% of the general population, but this rises to 10% in the population over 75 year of age (Rajamannan, 2014). The most prominent histologic abnormalities appear to be the formation of bone tissue in the calcified aortic valves (Liu and Xu, 2016), whereas cartilage formation can be detected in myxomatous mitral valves (Rajamannan, 2014).

The driving force behind valvular stenosis is calcification of the valve leaflets, the resulting stiffening of the valve leading to an obstruction in the outflow. In many ways this calcification resembles the formation of bone (Rajamannan, 2010; Liu and Xu, 2016). The control of bone formation by WNT/ $\beta$ -catenin signaling has been firmly established. Genetic intervention studies have shown that  $\beta$ -catenin is essential for the differentiation of mature osteoblasts and multiple WNT genes are expressed in either osteoblast precursors or adjacent cells during embryonic development (Hartmann, 2006). Moreover, mutations in LRP5 are associated with either high bone density (Boyden et al., 2002) or a genetic form of bone loss called osteoporosis pseudoglyoma syndrome (Gong et al., 2001), underscoring the central role of WNT/ $\beta$ -catenin signaling in the regulation of bone density. These observations have induced extensive studies of involvement of WNT signaling in valvular defects. The expression of osteoblastic genes is controlled by the balance between Notch and WNT/ $\beta$ -catenin signaling (Mathieu et al., 2014), and indeed elevated levels of LRP5 were reported in calcified aortic valves and, to a lesser extent, degenerative mitral valves (Caira et al., 2006). Moreover, a positive association between circulating DKK1 levels and calcified aortic stenosis was recently reported in patient with angiographically normal coronary arteries, but this association was less clear in the presence of concomitant atherosclerosis of the coronaries (Motovska et al., 2015).

Involvement of WNT/ $\beta$ -catenin signaling is not restricted to valvular calcification but was recently implicated in myxomatous valve disease as well. Myxomatous valve diseases is characterized by a thickening of the valvular leaflets and alterations in the extracellular matrix. It is the result of altered growth factor signaling, among which Wnt, BMP, and Notch



are important participants. Excessive deposition of proteoglycans and a concomitant degradation of collagen and elastin are characteristics of myxomatous valve disease, where the phenotype of the spongiosa resembles that of cartilage (Orton et al., 2012). Loss of  $\beta$ -catenin signaling in valvular interstitial cells activated the transcription factor Sox9 and subsequent expression of chondrogenic matrix genes (Fang et al., 2014). Inactivation of *axin2*, a negative regulator of WNT/ $\beta$ -catenin signaling, showed immature heart valves and a progressive myxomatous valve degeneration in mice at 4 months of age, underscoring the importance of this signaling pathway in valve homeostasis (Hulin et al., 2017).

### C. WNT Signaling in Cardiac Arrhythmias

Cardiomyocytes are attached to each other by specialized structures called intercalated disks. These intercalated disks not only provide strong mechanical connections between cardiomyocytes but also allow the fast propagation of electrical signals through the myocardial tissue, which is an essential feature for their simultaneous and coordinated contraction. Three main structures can be identified in the intercalated disk: desmosomes, anchoring the adjacent cells; adherens junctions, connecting actin filaments to the cell adhesion complex; and gap junctions, involved in the electric and metabolic coupling of neighboring cells. Although these structures are often studied separately, it is becoming increasingly clear that all intercalated disk components closely cooperate and should be seen as an integrated functional unit of the cardiomyocyte (Vermij et al., 2017). Therefore the term *area composita* was introduced to emphasize the integrated nature of desmosomes and adherens junctions (Patel and Green, 2014).

Apart from their role in WNT signaling, members of the catenin family are an important component of both desmosomes and adherens junctions. In the latter, N-cadherins are single membrane-spanning proteins which form intercellular connections via interaction of the extracellular domains extending from two neighboring cells. On the cytoplasmic side, N-cadherin is attached to the actin cytoskeleton via a complex consisting of  $\alpha$ - and  $\beta$ -catenin and plakoglobin, also known as  $\gamma$ -catenin. Desmosomes are sturdy, symmetrical anchoring structures that provide structural support to the cell-cell junction. They are not involved in the connection with the actin cytoskeleton but they connect to intermediate filaments. Desmosomes consist of the single membrane spanning cadherins desmoglein and desmocollin. Desmin is bound to these cadherins via a complex formed by plakoglobin, plakophilin, and desmoplakin. Gap junctions consist of complexes of gap junction proteins from the connexin (Cx) family, forming a tubular structure. The typical ventricular gap junction consists of a complex of six Cx43 proteins in the

plasma membrane, connected to a similar complex in the neighboring cell. These structures allow the rapid propagation of action potentials and transport of small molecules between cells (Patel and Green, 2014). Other members of the connexin family, including Cx40 and Cx45, have been identified in fast-conducting structures such as the His/Purkinje system (Lo, 2000).

There are several lines of evidence that the expression of Cx43 is under the control of WNT signaling. First, activation of WNT/ $\beta$ -catenin signaling in neonatal rat cardiomyocytes by coculturing with WNT1-secreting cells or with the GSK3-inhibitor lithium chloride (LiCl) resulted in an increase in Cx43 expression (Ai et al., 2000). Rapid electrical stimulation of neonatal rat cardiomyocytes resulted in nuclear translocation of  $\beta$ -catenin within 10 minutes and an increase in Cx43 expression within 60 minutes, suggesting a WNT/ $\beta$ -catenin-mediated regulation. This was accompanied by a significant increase in conduction velocity in these cells. Moreover, in a cardiomyopathic mouse model showing arrhythmias and remodeling of gap junctions, decreased levels of  $\beta$ -catenin and Cx43 were observed (Nakashima et al., 2014). Recently, mutations in the lamin A/C gene (*LMNA*) causing cardiomyopathy with conduction abnormalities were shown to decrease WNT/ $\beta$ -catenin signaling, which was accompanied by an increase in sFRP expression and reduced expression of Cx43 (Le Dour et al., 2017).

The involvement of aberrant WNT signaling in arrhythmogenic cardiomyopathy (AC) has been extensively studied and documented. AC is an inheritable cardiac disease characterized by life-threatening ventricular arrhythmias and heart failure and is a major cause of death in young athletes. Originally the condition was thought to be concentrated in the right ventricle, giving rise to the name arrhythmogenic right ventricular cardiomyopathy, which is now replaced by AC. A typical pathologic finding in these hearts is the replacement of cardiomyocytes by adipose and fibrous tissue (Pilichou et al., 2016). In most families, AC is inherited in an autosomal dominant fashion with incomplete penetrance. Mutations in five genes encoding the desmosomal proteins plakoglobin, desmoplakin, plakophilin-2, desmoglein-2, and desmocollin-2 have been reported in about half of the affected patients, therefore AC is generally considered a desmosomal disease (Sen-Chowdhry et al., 2005; Basso et al., 2011). The link between AC and WNT/ $\beta$ -catenin signaling can be found in desmosomal crosstalk with the nucleus. Decreasing desmoplakin expression via siRNA in cultured atrial myocytes resulted in nuclear translocation of plakoglobin, which competes with  $\beta$ -catenin for interaction with the TCF/LEF transcription factors. This induced a twofold reduction in WNT/ $\beta$ -catenin signaling and induced the expression of adipogenic and fibrogenic genes as observed in AC patients. An AC phenotype was also found in mice by the heterozygous

deletion of the desmoplakin (*Dsp*) gene (Garcia-Gras et al., 2006) and in mice with a cardiomyocyte-specific silencing of  $\beta$ -catenin and plakoglobin (MerCreMer under control of the  $\alpha$ MHC promoter) (Swope et al., 2012). Genetic fate mapping experiments have shown that the adipocytes in AC hearts originate from second heart field progenitor cells, in which the attenuated WNT/ $\beta$ -catenin signaling resulting from nuclear plakoglobin induced an adipogenic fate (Lombardi et al., 2009). The importance of WNT/ $\beta$ -catenin signaling in AC was recently illustrated in two mouse models with mutated desmosomal proteins (Chelko et al., 2016). The mice showed GSK3 accumulation in the intercalated disks, similar to those observed in AC patients. Treatment with SB216763, a GSK3 inhibitor, normalized the protein distribution in the intercalated disks and improved ventricular fibrosis and inflammation, pointing to GSK3 as a therapeutic target in AC.

#### D. WNT Signaling in Cardiac Hypertrophy

Cardiac hypertrophy is an adaptive response of the heart to a wide range of external factors and neurohormonal/mechanical stimuli, such as pressure overload,  $\beta$ -adrenergic stimulation, MI, etc. These factors activate different signaling pathways and key molecules, including, but not restricted to, NFAT, PI3K/Akt, GATA4, CaMKII, PKC, ERK, JNK, calcineurin (Hunter and Chien, 1999), and WNT (Blankesteyn et al., 2008). Hypertrophy can have profound effects on pathologic cardiac remodeling, which progresses to impairment of cardiac function and eventually leads, when left untreated, to lethal congestive heart failure. In various studies, cardiac hypertrophy has been linked with changes in WNT signaling and here we will discuss the involvement of various WNT components in the mediation of pro- and antihypertrophic stimuli.

1. *Involvement of WNT, FZD, and LRP5/6 and DKK.* The data on WNT ligand involvement in the development and progression of myocardial hypertrophy is quite scarce. He et al. (2015) used an isoprenaline-induced hypertrophy mouse model and reported that both  $\beta$ -catenin-dependent and -independent WNT signaling cascades are activated in hypertrophic hearts, as was suggested by increased WNT3A and WNT5A expression, respectively. Both  $\beta$ -catenin and non- $\beta$ -catenin signaling cascades were confirmed to play an essential role in cardiomyocyte hypertrophy as shown by other groups. Exogenous stimulation of primary cardiomyocytes with WNT5A produces a pronounced increase in cardiomyocyte growth and protein synthesis, whereas the WNT component Dapper-1 was found to play a crucial role in this effect (Hagenmueller et al., 2014). Interestingly, the same group has also shown that WNT3A mediates its prohypertrophic effects on cardiomyocytes via Dapper-1, showing that the  $\beta$ -catenin-dependent pathway (via DVL2) is heavily involved (Hagenmueller et al., 2013). On the other hand, Jin

et al. (2015b) proposed a beneficial effect of WNT5A on hypertrophy of the right ventricle (RV). Using a hypoxia-induced pulmonary hypertension mouse model, they established that exogenous treatment with recombinant mouse WNT5A suppresses cardiomyocyte growth via inhibition of the  $\beta$ -catenin/cyclin D1 axis.

Our group used the method of subtractive hybridization and showed more than 20 years ago that FZD<sub>2</sub> is immediately upregulated following aortic banding in rats, whereas levels remain elevated for at least 10 days after surgery (Blankesteyn et al., 1996). The involvement of FZD<sub>2</sub> in cardiac hypertrophy was also observed by Cerutti et al. (2006), who performed microarray studies and showed a positive correlation between FZD<sub>2</sub> expression and LV mass index in different hypertensive/hypertrophic animal models [spontaneously hypertensive rat, Lyon hypertensive rat, and heterozygous TGR9(mRen2)27 rat].

Alapati et al. (2013) made a link between LRP5/6 and RV hypertrophy, which is exacerbated in a hyperoxia-induced neonatal lung injury mouse model. Hyperoxia-induced neonatal lung injury has pronounced effects on the lungs, leading to pulmonary hypertension and RV hypertrophy, and this is linked to activation of WNT/ $\beta$ -catenin signaling. By making use of a WNT-LRP5/6 complex blocker, called Mesd, they show that the activation of WNT signaling is suppressed and both pulmonary hypertension and RV hypertrophy are attenuated. Thus blockade of LRP5/6 can produce anti-hypertrophic effects on the RV.

Phillips et al. (2011) investigated the effects of a double KO of *Dkk1* and *Dkk2* in mice during early development. The double *Dkk1/2* KO mouse genotype is perinatally lethal; however, it is interesting that embryonic hearts are thickened at embryonic day (E) 10.5. At E12.5 it is found that the double null mice demonstrate increased numbers of cardiomyocytes (thus myocardial hyperplasia), an effect that is long lasting. On the other hand, the epicardium is also affected by the altered WNT signaling activity; however, any epicardial changes observed early are resolved later on (by E15.5).

More attention has been devoted to the DKK3 isoform. Cerutti et al. (2006) were probably the first to demonstrate a negative correlation between LV mass augmentation and DKK3 in hypertensive rats. The study of Zhang et al. (2014) suggested that DKK3 is heavily involved in the regulation of pathologic hypertrophy: Patients suffering from dilated cardiomyopathy (DCM), as well as mice that are subjected to 4 weeks of aortic banding show robustly downregulated DKK3 expression levels, whereas  $\beta$ -myosin heavy chain (MHC) and atrial natriuretic factor, both markers of hypertrophy, are decreased in both cases. Additionally, DKK3 expression is suppressed in cultured neonatal cardiomyocytes following AngII or phenylephrine stimulation. When DKK3 is knocked down in these cells,

cardiomyocyte hypertrophy worsens but when DKK3 expression is induced this effect is reversed. Lastly, mice overexpressing DKK3 were subjected to aortic banding. The authors report that hypertrophy is drastically attenuated 8 weeks after surgery in these mice compared with their WT counterparts, clearly demonstrating the cardioprotective effects of DKK3 (Zhang et al., 2014). In addition, DKK3 is also a crucial player in the development of familial dilated cardiomyopathy (Lu et al., 2016). Levels of DKK3 expression are paradoxically found to be elevated in two mouse models of DCM (cTnT<sup>R141W</sup> and adriamycin-induced DCM), opposite to the findings of Zhang et al. (2014). Lu et al. (2016) show that deletion of DKK3 exacerbates cardiac remodeling; however, when DKK3 is transgenically overexpressed, it upregulates the  $\beta$ -catenin-dependent signaling (via observed effects on DVL1,  $\beta$ -catenin, c-myc, and AXIN2) and downregulates the  $\beta$ -catenin-independent signaling (via suppression of JNK, CAMKII, and HDAC4). DKK3 appears to be a cardioprotective factor in this case; however, it is an enigma how it can induce rather than downregulate the WNT cascade to mediate this effect.

**2. The Role of Secreted Frizzled-Related Proteins in Cardiac Hypertrophy.** Most of the studies implicating sFRP in mediating cardiac hypertrophy are quite recent. Sklepkiwicz et al. (2015) demonstrated that an active sFRP1 expression is crucial for LV remodeling and hypertrophy, and is especially relevant in the aging organism. *Sfrp1* KO mice exhibit robust hypertrophy already at 6 months of age compared with their WT counterparts, as shown by increased HW/BW ratio and LV wall thickness. This difference was even more pronounced at 12 months of age, and this coincided with upregulated WNT1/-3/-7b mRNA expression, induced  $\beta$ -catenin protein levels, and LEF1 expression in the KOs. Furthermore, hypertrophy in the *Sfrp1* KO was accompanied by profound changes in LV diastolic and systolic function, as well as presence of  $\beta$ -catenin in the intercalated disks, possibly contributing to myocardial stiffening (Sklepkiwicz et al., 2015).

Downregulation of plasma membrane calcium ATPase isoform 4 (PMCA4), either by genetic intervention or by pharmacological antagonism, revealed that sFRP2 expression is induced in fibroblasts. In addition, the mice lacking PMCA4 (and thus overexpressing sFRP2, a protein exerting antihypertrophic activity) demonstrated a high survival 3 months after aortic banding. sFRP2 secreted by fibroblasts was shown to be the crucial regulator (Mohamed et al., 2016).

Askevold et al. (2014) suggested that sFRP3 could inhibit cardiac hypertrophy by targeting WNT signaling. HF patients demonstrated elevated sFRP3 levels compared with control patients, a finding that was correlated with increased mortality. Indeed, sFRP3 and -4 are shown to be upregulated in failing human hearts, leading to an attenuation of WNT signaling

(Schumann et al., 2000). Nevertheless, keeping in mind conflicting reports on the effect of sFRP3 on WNT signaling (Zi et al., 2005; Galli et al., 2006), further studies are warranted to ascertain the potential of sFRP3 as an antihypertrophic therapy tool.

Exposure of cardiomyocytes to AngII is reported to induce sFRP5 expression, along with BNP and TNF $\alpha$ , which are indicators of hypertrophic growth. Interestingly, when cardiomyocytes were preincubated with sFRP5 before the AngII challenge, sFRP5 could salvage hypertrophy, as shown by suppressed BNP and TNF $\alpha$  protein levels (Jin et al., 2015a).

**3. The  $\beta$ -Catenin Destruction Complex.** The destruction complex is the limiting factor of  $\beta$ -catenin-mediated WNT signaling, regulating the free  $\beta$ -catenin levels and preventing its stabilization and nuclear translocation. Various studies have demonstrated an involvement of the destruction complex components in the development of cardiac hypertrophy (Kerkela et al., 2007). Adult hypertrophic hearts show suppressed levels of APC mRNA expression compared with their healthy counterparts. This finding is in accordance with induced  $\beta$ -catenin protein levels in the hypertrophic hearts, signifying an activation of the WNT cascade (Rezvani and Liew, 2000).

Our group has provided compelling evidence for the essential role of DVL1 in the regulation of cardiac hypertrophy. *Dvl1* KO mice were subjected to aortic banding and were followed for 7, 14, and 35 days. Aortic banding induces hypertrophy, as shown by increased HW/tibia length ratios, in WT mice 7, 14, and 35 days post-op, but this is evident after only 35 days in the *Dvl1* KO mice, implying that deletion of the *Dvl1* gene delays induction of hypertrophy. In the same way, posterior wall thickness was increased in WT mice but only mildly and later in the KOs. In addition, 7 days after operation, the expression of both ANF and BNP (markers of hypertrophy development) was more than doubled in the WT compared with their KO counterparts. These results suggest a detrimental effect of DVL1 following aortic banding. GSK3 $\beta$  activity was found to be upregulated in the *Dvl1* KO mice, which was reflected in lower  $\beta$ -catenin mRNA and protein expression. In contrast, *Dvl1* WT mice showed an increase in  $\beta$ -catenin expression (as well as an upregulation of p-Akt, which is known to induce hypertrophy), implying an activation of the WNT/ $\beta$ -catenin cascade under normal conditions (van de Schans et al., 2007). In agreement with the aforementioned findings, Malekar et al. (2010) showed that overexpression of DVL1 can have deleterious effects on the development of hypertrophy and cardiac remodeling in mice, 4 weeks after aortic banding. Cardiomyocyte size in *Dvl1* overexpressing mice was increased by a staggering 86% and the HW/BW ratio was significantly increased compared with WT; this leads to LV dilatation, an almost halving in EF and an impairment in contractility. Both

$\beta$ -catenin-dependent (via  $\beta$ -catenin, cyclin D1, c-myc, and axin2) and -independent (via JNK, CAMKII, and PKC) signaling routes were found to be activated in the DVL1 overexpressing mice, contributing, probably synergistically, to a severe cardiomyopathy phenotype.

When the *Dvl1* overexpressing mice were crossed with *CAMKII $\delta$*  KO mice, the hypertrophy and cardiomyopathy phenotype was lost; it is interesting to note that the authors could not demonstrate any difference in PKC, JNK, or  $\beta$ -catenin activation following the deletion of *CAMKII $\delta$* / $\gamma$  in the *Dvl1*-overexpressing mice (Zhang et al., 2015c). The transgenic deletion of *CaMKII $\delta$*  $\gamma$  halts hypertrophy of *Dvl1*-overexpressing mice, as verified by substantial increases in heart size, myocyte area, HW/tibia length, fibrosis, and hypertrophy marker gene expression (ANF and  $\beta$ -MHC). The findings demonstrated clearly that all branches of WNT signaling are taking part in the mediation of cardiac hypertrophy.

The group of Hardt investigated the involvement of DVL2 in cardiac hypertrophy (Hagenmueller et al., 2013). The authors indicate Dapper-1 as a key regulator in DVL2-mediated hypertrophy and LV remodeling. Mice overexpressing Dapper1 showed elevated HW/BW and exacerbated cross-sectional area of the myocytes, as well as induction of hypertrophy markers (ANF, BNP, and  $\beta$ -myosin). These mice exhibited high DVL2 and  $\beta$ -catenin expression. In the meantime, in vitro work showed that Dapper-1 is an essential coregulator of the  $\beta$ -catenin-dependent WNT-mediated hypertrophy in cardiomyocytes. Dapper-1 downregulation leads to an attenuation of WNT signaling cascade ( $\beta$ -catenin and c-myc protein levels), whereas its overexpression increases both  $\beta$ -catenin and TCF/LEF activity.

GSK3 is a ubiquitous Ser/Thr kinase that plays an essential role in a multitude of cellular functions (Kerkela et al., 2007). GSK3 phosphorylates a wide range of substrates and suppresses their activities, mediating decisively both  $\beta$ -catenin-dependent and -independent cascades. GSK3 $\beta$  has been shown to play an essential role in cardiac hypertrophy (Hardt and Sadoshima, 2002). It should be noted that the other isoform of GSK3, GSK3 $\alpha$ , is also known to play crucial roles in cardiac hypertrophy and appears to have a rather antihypertrophic effect mediated mainly via ERK (Zhai et al., 2007) and E2F (Matsuda et al., 2008). Although several reports demonstrate that GSK3 $\alpha$  might actually be even more important in aortic banding-induced hypertrophy than GSK3 $\beta$  (Cheng et al., 2011a), it is the latter that mediates its effects also via the WNT signaling pathway. However, an extensive discussion of GSK3 $\alpha$  is beyond the scope of the current review.

Interestingly, it is not exclusively the WNT/ $\beta$ -catenin signaling component of GSK3 $\beta$  that is involved in this prohypertrophic mediation (Hirotani et al., 2007); GATA4 (Liang and Molkentin, 2002), NFAT (Antos

et al., 2002), CREB, PI3K/Akt, PKA, and ERK (El Jamali et al., 2004), eIF2 (Hirotani et al., 2007), JAK/STAT (Clerk et al., 2007), and others play equally essential roles. However, as discussed earlier, the activity of GSK3 $\beta$  is regulated by its phosphorylation status: phosphorylation at the Ser<sup>9</sup> residue leads to inhibition of GSK3 $\beta$  (Sutherland et al., 1993), resulting in activation of WNT signaling, whereas phosphorylation at the Thr<sup>216</sup> residue is inducing the activity of the kinase (Liang and Chuang, 2007).

Haq et al. (2003) reported that either hypertrophic stimulation (by phenylephrine and endothelin-1) of neonatal rat ventricular myocytes, or aortic banding in rats, activates PKB to phosphorylate GSK3 $\beta$  at Ser<sup>9</sup>. This phosphorylation induces inactivation of GSK3 $\beta$ , stabilization of  $\beta$ -catenin, and as a consequence an increase in myocyte size and protein synthesis. A similar effect was demonstrated by Morisco et al. (2000) following an isoprenaline challenge of myocytes, leading to the hypothesis that the  $\beta$ -adrenergic system mediates its prohypertrophic effects via the Akt/GSK3 $\beta$  signaling.

A rather contradictory finding by Haq et al. (2001) was that GSK3 $\beta$  signaling is suppressed in failing hearts (end-stage HF) but not in hypertrophic ones, implying that GSK3 $\beta$  upregulation in overload in humans might be short-lived (<4 weeks?). Tateishi et al. (2010) studied the effect LiCl-mediated GSK3 $\beta$  inhibition in a model of pressure overload-induced cardiac hypertrophy in the rat. LiCl treatment resulted in higher LV weight, increased posterior wall thickness, and increased mRNA expression of the prohypertrophic marker atrial natriuretic factor compared with untreated rats exposed to aortic banding. Thus inhibition of GSK3 $\beta$  is found to exacerbate hypertrophy. Data from *Gsk3 $\beta$*  null mice seem to support these observations. Deletion of *Gsk3 $\beta$*  in mice led to severe hypertrophic cardiomyopathy and cardiac-related death of mice pups due to a wide range of cardiac defects (Kerkela et al., 2008). These mice exhibited substantially induced proliferation of cardiomyocytes, leading to increased LV/RV free wall and IVS thickness. The molecular basis of these findings was proposed to be an increased expression and nuclear localization of GATA4, cyclin D1, and c-Myc, all three regulators of cell proliferation. Hirotani et al. (2007) generated Tg mice with an  $\alpha$ MHC-driven cardiac-specific expression of dominant negative GSK3 $\beta$  (Tg-GSK3 $\beta$ -DN). These mice exhibit physiologic (adapted) hypertrophy after 2.5, 4, 6, or 8 months, while also showing increased contractility compared with WT counterparts. When they are subjected to aortic banding, the GSK3 $\beta$  inhibition does not appear to produce any further effects implying that GSK3 $\beta$ -DN is mainly mediating basal state hypertrophy and not aortic banding-related hypertrophy (but still, decreased GSK3 $\beta$  activity appears to protect the heart from decompensated hypertrophy). On the other hand when

GSK3 $\beta$  was transgenically overexpressed (conditional induction regulated by doxycycline), mice developed LV dysfunction and augmented heart weight /tibia length ratios, as well as increased apoptosis, stressing that decreased GSK3 $\beta$  activity in the diseased heart is cardioprotective. Interestingly, a GSK3 inhibitor called 6-bromoindirubin-3'-oxime (BIO) induced neonatal and adult cardiomyocyte proliferation, despite the fact that the myocyte is regarded in general as a terminally differentiated cell (Tseng et al., 2006). It seems that the activation of the WNT cascade might be stimulating dedifferentiation and proliferation of mammalian myocytes, indicating another mechanism for GSK3's effects on hypertrophy, without though excluding the possibility of BIO having off-target activity, because it is not selective for GSK3 isoforms (Cheng et al., 2011a).

Ser<sup>9</sup> replacement by Ala (which renders GSK3 $\beta$  insensitive to phosphorylation and thus inhibition) suppresses cardiac hypertrophy induced by overexpression of calcineurin, 9 days of  $\beta$ -adrenergic stimulation with isoproterenol, or 3 weeks of aortic banding (Antos et al., 2002), as shown by increased heart weights and upregulated expression of markers of hypertrophy (BNP, ANF,  $\beta$ -MHC). Using a doxycyclin-inducible overexpression of the same GSK3 $\beta$ -mutant, Sanbe et al. (2003) observed a similar suppression of the hypertrophic response to pressure overload. Expression of inactivation-resistant GSK3 $\alpha/\beta$  isoform was shown to be cardioprotective following beta-adrenergic challenge with isoprenaline (Webb et al., 2010). Normal heart weight as well as LV thickness were preserved in these mice, underscoring the importance of a normally functioning GSK3 $\beta$  system. On the other hand, abnormal upregulation of GSK3 $\beta$  can be deleterious for the myocardium. GSK3 $\beta$  features as a crucial negative regulator of hypertrophic growth of the LV. As reported by Michael et al. (2004), transgenic mice with an approximately ninefold overexpression of wild-type GSK3 $\beta$  showed dramatically reduced growth with structural and functional abnormalities in both ventricles at 2 and 5 months of age (including reduced wall thickness and internal long-axis dimension of the LV). An interesting observation was that the differences in heart size of WT and GSK3 $\beta$ -Tg were much smaller, which may suggest that the differences observed later in life are due to disruption of hypertrophic growth (mediated obviously by GSK3 $\beta$ ) rather than from proliferation of cardiomyocytes, which primarily takes place during embryonic development.

The results described above suggest that the blockade and the overexpression of GSK3 $\beta$  are equally noxious to the heart under conditions inducing hypertrophy. Thus, a precise balance of GSK3 $\beta$  is of paramount importance. What is characteristic about GSK3 $\beta$  is that there is quite some interest for the identification of potent and selective GSK3 $\beta$  activators and inhibitors, to suppress or activate, respectively, WNT signaling. High-

throughput screening methods are under way and there is plenty of potential for novel compounds that will be able to adequately stimulate or block (depending on the context) this kinase (Cohen and Goedert, 2004; An et al., 2010; Takahashi-Yanaga, 2013). On the other hand, it is paradoxical that this interest on the bench level does not materialize in clinical trials investigating GSK3 $\beta$ -modifying agents that could be used as potentially novel tools against cardiac hypertrophy (Cheng et al., 2011a). A search in clinicaltrials.org showed that the only agents affecting GSK3 $\beta$  under investigation are the "old school" valproate, LiCl, and the novel NP031112 (GSK3 inhibitor), however, none is under evaluation in a cardiovascular study.

4.  *$\beta$ -Catenin-Mediated Gene Transcription.* As expected by its key role in the regulation of WNT signaling, there is a myriad of studies that have focused on  $\beta$ -catenin in an effort to shed light on the mechanisms regulating cardiac hypertrophy. It is well established that the multiple functions exhibited by  $\beta$ -catenin are determined by its phosphorylation and stabilization status: the spatial localization of  $\beta$ -catenin is heavily dependent on its Tyr<sup>142</sup> and Tyr<sup>654</sup> phosphorylation (Harris and Peifer, 2005). The study of null mutations of  $\beta$ -catenin is precluded by the fact that such mutations are embryonically lethal (Haegel et al., 1995). Actually, both gain and loss of function of  $\beta$ -catenin lead to severe developmental defects (Grigoryan et al., 2008). Thus, the design of conditional mutants is the only alternative to investigate the role of  $\beta$ -catenin in (patho)physiology in the mouse. Two approaches are available: 1) one producing tissue-specific null mutation and so, a conditional loss-of-function of WNT signaling and 2) another one producing a constitutively stabilized  $\beta$ -catenin by deleting exon 3 of  $\beta$ -catenin gene, leading to a gain-of-function of WNT signaling (Grigoryan et al., 2008).

In a hallmark paper, Haq et al. (2003) showed not only that the WNT/ $\beta$ -catenin signaling is important for the growth of cardiomyocytes in the context of hypertrophy, but also that  $\beta$ -catenin activation is necessary, although not necessarily independent because PKB also seems to be profoundly involved, for the induction of hypertrophy in vitro.  $\beta$ -Catenin levels are upregulated in cardiomyocytes that are challenged with various hypertrophic stimuli (phenylephrine, endothelin-1, or aortic banding), whereas  $\beta$ -catenin stabilization can induce protein synthesis and cardiomyocyte enlargement, but not ANF expression, sarcomere organization, or cardiomyocyte proliferation (Haq et al., 2003). Still, it appears rather unlikely that these effects are WNT-mediated. Overexpression of  $\beta$ -catenin via adenoviral gene transfer also robustly affects cellular size, protein synthesis and nuclear polyploidization, all indications of hypertrophy of cardiomyocytes, without having any effects on proliferation of cardiomyocytes, while apoptosis of cardiomyocytes was also reported as a beneficial effect of

$\beta$ -catenin upregulation (Hahn et al., 2006). Similar effects were observed on cardiac fibroblasts, albeit less pronounced. Conversely, gene transfer of  $\beta$ -catenin mutants in which transcriptional activity is disrupted (NCad $\Delta$ C), reversed the aforementioned effects on both myocytes and cardiac fibroblasts. Zhang et al. (2009a) used siRNA to knockdown  $\beta$ -catenin in cardiomyocytes. They observed a suppression of phenylephrine-induced protein synthesis, a reduction of the transcription levels of the hypertrophic marker ANF, and a significantly depressed cardiomyocyte cell area. The study also demonstrated that the ANF promoter contains a LEF1 binding site, to which  $\beta$ -catenin is recruited during phenylephrine stimulation.

The  $\beta$ -catenin/TCF/LEF axis was shown to be an essential component of hypertrophic growth in myocytes by Chen et al. (2006). Mice carrying a myocyte-specific deletion of  $\beta$ -catenin (Lef-120) exhibit an attenuated hypertrophic response (as exhibited by dramatically reduced myocyte growth) and substantially improved cardiac function after aortic banding compared with mice with preserved  $\beta$ -catenin expression. Cardiac-specific deletion of  $\beta$ -catenin (via Cre recombinase under the control of a  $\alpha$ MHC promoter) shows a demised TAC-induced hypertrophy 4 weeks after banding (Qu et al., 2007). Prohypertrophic gene expression was dramatically altered in these mice, with enhancement of fetal gene expression (ANF, BNP,  $\beta$ -MHX, SERCA, etc.) being evident. Baurand et al. (2007), on the other hand, challenged the concept of low  $\beta$ -catenin expression being beneficial by showing an exacerbation of spontaneous cardiac hypertrophy in cardiomyocyte-specific  $\beta$ -catenin-depleted mice ( $\alpha$ MHC-Cre-PR1 model); mice exhibiting stabilized  $\beta$ -catenin were reported to show attenuation of AngII-induced cardiac hypertrophy, as shown by cardiomyocyte size and ANF expression. Interestingly, the reduced hypertrophy in the latter mice came to the expense of declined heart function (as indicated by reduced ejection fraction). These contradicting results could possibly be explained by the fact that different models (aortic banding vs. AngII stimulation) activate different signaling pathways, resulting in different responses on the cardiomyocytes.

A final point of interest is the exact mechanism by which  $\beta$ -catenin modulates the hypertrophic state of cardiomyocytes. As discussed before,  $\beta$ -catenin resides at the adherens junctions in the catenin/cadherin complex and plays important roles in cell adhesion and cell-cell communication (Harris and Peifer, 2005). mXin $\alpha$ , a protein of the intercalated disk (an important point where sarcomeric organization takes place during cardiac hypertrophy), was found to contain a  $\beta$ -catenin-binding domain, implying that  $\beta$ -catenin can play a key role in bundling of actin filaments (Choi et al., 2007). Interestingly,  $\beta$ -catenin is found to accumulate in the intercalated disks of myocytes in hypertrophic

cardiomyopathy hamsters, possibly leading to induced wall stiffness of the myocardium and deleterious structural and functional effects associated with hypertrophic cardiomyopathy (Masuelli et al., 2003). Zheng et al. (2013) reported the exact opposite observation, showing that  $\beta$ -catenin presence is suppressed in the intercalated disks and increased in the myocyte nucleus of spontaneously hypertensive heart failure (SHHF) rats (Zheng et al., 2013). This finding might at first look paradoxical; however, it could be explained by the variability of the two models (as the SHHF is more of a heart failure model, whereas the hypertrophic cardiomyopathy is rather hypertrophic model without progression to heart failure).

In conclusion, stabilized  $\beta$ -catenin is sufficient (and as shown, necessary) for the induction of hypertrophic growth in cardiomyocytes. Nevertheless, the precise roles of  $\beta$ -catenin are still not fully understood with variable reports from in vitro and constitutive mutation studies. More in-depth investigations are required to clarify the exact mechanisms by which  $\beta$ -catenin (and WNT signaling in general) exhibits its sophisticated effects on the hypertrophic response. This will provide new options for interfering with the signaling to halt cardiac hypertrophy and prevent its adverse effects.

### *E. WNT Signaling and Heart Failure*

Heart failure (HF) is a progressively disabling chronic disease, which comes as an aftermath of a variety of pathologies, most importantly MI and hypertrophy (covered above). Initially, following injury, endogenous compensatory mechanisms prevent cardiac failure; however, eventually, cardiac remodeling produces a detrimental effect on cardiac architecture and function and leads to lethal HF. HF is a major public health issue, and the total number of HF patients around the globe is estimated to be more than 26 million (Ambrosy et al., 2014). According to the latest statistics from the American Heart Association (Benjamin et al., 2017), about 20% of people suffering an MI after 65 years of age will develop HF, and approximately one in two patients diagnosed with HF will die within 5 years. The prognosis for the United States alone is even worse because the HF prevalence is expected to rise 46% by 2030. It is obvious that the financial impact of HF is enormous: in 2012 the total cost associated with HF in the United States was more than \$30 billion, and the estimations are expected to increase to \$69 billion by 2030. Currently, available anti-HF pharmacotherapy is not curative but mainly directed at the symptoms and it only attenuates the deterioration to end-stage HF, without completely preventing it. WNT signaling has been shown to be involved in mediating adverse cardiac remodeling and the progression to HF, thus interventions in the pathway may provide new targets for HF therapy.

1. *Extracellular Signaling—Secreted Frizzled-Related Proteins.* Inactivation of the *Sfrp1* gene has been reported to cause hypertrophy, fibrosis, and depressed cardiac function in old (6 and 12 month old) mice. In these mice,  $\beta$ -catenin, WISP-1, and LEF1 levels are induced. In agreement with these findings, the authors show that sFRP1 is downregulated and WISP-1 is induced in human dilated cardiomyopathy and ischemic cardiomyopathy (Sklepkiwicz et al., 2015). As mentioned before, transgenically overexpressed FrzA (sFRP1) exhibits a cardioprotective effect following MI. Infarct scar is strengthened, myofibroblast presence is augmented, whereas more and thicker blood vessels/capillaries lead to better perfusion of the ischemic tissue. These observations, in combination to the improved cardiac function as attested by echography, suggest a cardioprotective profile of sFRP1 overexpression, which could be exploited to prevent HF progression (Barandon et al., 2003). In the same context, sFRP1 levels were shown to be downregulated in patients suffering from HF but this effect was reversed following left ventricular assist device (LVAD) support (Felkin et al., 2011). The role of sFRP2 in heart failure development is less clear: On the one hand, injection of sFRP2 was shown to have beneficial effects on cardiac function in a hamster model of congestive heart failure with reduced myocyte apoptosis and fibrosis (Mastri et al., 2014), whereas injection of sFRP2 into the rat infarct leads to suppression of fibrosis, prevention of anterior wall thinning, and attenuation of cardiac adverse remodeling (He et al., 2010). A recent in vitro study by Lin et al. (2016) proposes a cardiac fibroblast-related explanation of the multifaceted effects of sFRP2, so it cannot be excluded that the aforementioned sFRP2-mediated effects are due to its impact on cardiac fibroblasts and not noncardiac fibroblast cell types.

Schumann et al. (2000) were the first to report differences in sFRP isoform expression in human failing hearts, with 2- to 3-fold upregulated mRNA levels for the endogenous WNT signaling antagonists sFRP3 and sFRP4 (which happen to be proapoptotic) but not for sFRP1 and sFRP2. In addition,  $\beta$ -catenin levels were found to be decreased in the failing hearts compared with nonfailing ones, all contributing to decreased WNT/ $\beta$ -catenin signaling of hearts progressing to HF. Askevold et al. (2014) reported a positive correlation between increased mRNA and circulating serum levels of sFRP3 in failing hearts compared with controls. From the GISSI-HF study they included 153 HF patients and showed that increased circulating sFRP3 levels were associated with adverse outcomes in HF. Intriguingly, a more recent study by Askevold et al. (2015) with a larger patient population showed that there might actually be a nonlinear association between circulating sFRP3 levels and outcomes. The authors demonstrate that mid-tertile serum concentrations of sFRP3 are

correlated with reduced mortality in a population of old HF patients. This finding could mean that too low or too high sFRP3 levels might be leading to deleterious effects (associated with higher mortality) but intermediate sFRP3 levels correspond to low inflammatory response with reasonable levels of remodeling mechanisms, leading to improved cardiac function and thus leading to healthier patients. Furthermore, the discrepancy between the two studies could be explained by the fact that the population of the latter was generally older and suffering from more severe HF (as well as lower renal function). It should be noted, however, that Motiwala et al. (2014) report a lack of significance of sFRP3 with the progression and complications of HF. Lastly, it should be mentioned that sFRP appears to mediate the lamin A/C gene (*LMNA*)-related cardiomyopathy, a condition that leads to LV dysfunction and predisposition to HF. Six-month-old *Lmna*<sup>H222P/H222P</sup> mice feature elevated sFRP1 and sFRP2 transcripts (as well as upregulated DKK3 mRNA expression) and robustly decreased  $\beta$ -catenin protein expression, displaying the deleterious effects of WNT signaling abnormal suppression (Le Dour et al., 2017).

2. *Cytoplasmic Signaling Components—DVL and GSK3 $\beta$ .* The group of Hardt demonstrated a positive association between overexpression of DVL1, HF development, and increased mouse mortality, which is mediated via both  $\beta$ -catenin-dependent and -independent signaling pathways (Malekar et al., 2010; Zhang et al., 2015c). Both studies are covered in-depth in section VII.D.

Double KO of *GSK3 $\alpha$*  and *GSK3 $\beta$*  exhibits disastrous effects on the mouse myocardium. As reported by a recent study of Zhou et al. (2016), adult cardiomyocytes with double GSK3 deletion feature DNA damage and apoptotic cell death linked to mitotic catastrophe. This appears to be contributing to the in vivo exacerbated pathologic hypertrophy and fibrosis, leading to acute HF and death around 40 days of life. Several other studies have focused on manipulations via GSK3 $\beta$  signaling; however, a large part of them does not appear to be mechanistically related to WNT signaling but to act mainly via the PI3K/Akt pathway (Haq et al., 2001; Yao et al., 2008; Braz et al., 2009). Kirk et al. (2014) demonstrated that cardiac resynchronization therapy reactivates GSK3 $\beta$  in tachypacing-induced HF (200 beats/min) in dogs and enhances myofilament and contractile function. The report of Braz et al. (2009) was in the opposite direction. The authors showed that a GSK3 $\beta$  inhibition (leading to an activation of WNT/ $\beta$ -catenin signaling) could exhibit anti-HF beneficial effects as shown in recovering dog hearts following cardiac resynchronization therapy. Once again, there is no indication that WNT signaling is heavily involved in any of these effects. Lastly, transgenic overexpression of GSK3 $\beta$  followed by TAC operation was shown to drive deleterious effects on cardiac

function and progress to HF in the Tg mice (Hirohata et al., 2007). Recovery of this was correlated with increased phosphorylation of GSK3 $\beta$  (leading to its inhibition) and increased levels of  $\beta$ -catenin.

3. *Signaling Inside the Nucleus— $\beta$ -Catenin.* Very recently, Hou et al. (2016) reported interesting evidence relating to the activation of WNT signaling in human (ischemic heart disease and idiopathic dilated cardiomyopathy) and murine (desmin-related cardiomyopathy model) failing hearts. In all three cases,  $\beta$ -catenin nuclear accumulation was evident, paralleled by activation of TCF7L2 and induction of c-myc mRNA transcripts. When the authors subjected cultured neonatal rat cardiomyocytes to TCF7L2 overexpression, the myocyte growth was augmented and c-myc was upregulated, whereas knockdown (via shRNA) of TCF7L2 lead to the opposite effects, indicating the positive correlation of WNT/ $\beta$ -catenin signaling activation with hypertrophy and HF development. Nuclear levels of  $\beta$ -catenin were substantially upregulated in the myocytes of spontaneously hypertensive heart failure (SHHF) rats, whereas  $\beta$ -catenin presence in the intercalated disks was reduced. This latter finding is very important, because it could mean that low intercalated disk  $\beta$ -catenin expression is correlated with sarcomerogenesis control and the transition from compensated hypertrophy to decompensated hypertrophy and HF development (Zheng et al., 2013). A recent report of Nakagawa et al. (2016) is in the same line. Conditional gain-of-function mutation of  $\beta$ -catenin in endothelial cells of Bmx/CA mice (in which *BMX-CreER* controls  $\beta$ -catenin expression in endothelial cells) led to acute HF (with extreme dilatation and fibrosis) that is unrelated to cardiomyocytes and consequently to early mortality (100% of the mice die before 60 weeks of age) (Nakagawa et al., 2016). Lastly, epicardial activation of  $\beta$ -catenin signaling could be correlated to HF. Nuclear levels of  $\beta$ -catenin, LEF1, and TCF-1/3/4 are shown to be induced in the epicardium coming from end-stage pediatric allografts. Notably, CFs originating from epicardial fibrosis exhibit induced  $\beta$ -catenin/TCF4 axis activation, and thus epicardial WNT/ $\beta$ -catenin could possibly be targeted to alleviate pediatric HF (Ye et al., 2013).

#### F. Conclusions and Directions for Future Research

The effect of interventions in Wnt signaling in models of cardiac disease has been the subject of extensive investigation in the last decade. From these studies it can be concluded that the expression of components of the WNT signaling pathway is modulated in a variety of cardiac pathologies, suggesting an activation of the pathway. Unfortunately, the results are often ambiguous when it comes to the question whether WNT signaling should be activated or inhibited. This is particularly the case in studies in which genetic interventions were applied. Interventions in a specific

member of the large family of genes which act in WNT signaling may be compensated by the expression of other family members, hampering the interpretation of the results.

In contrast, from the studies in which pharmacological inhibitors of the pathway have been applied in models of myocardial infarction a rather clear picture is emerging: intervention at the level of either PORCPN (Bastakoty et al., 2016; Moon et al., 2017), FZD (Laeremans et al., 2011; Uitterdijk et al., 2016), the  $\beta$ -catenin destruction complex (Murakoshi et al., 2013), or  $\beta$ -catenin-mediated gene transcription (Sasaki et al., 2013) all have shown beneficial effects on infarct size and, in most cases, on cardiac function. Unfortunately, the numbers of studies in which pharmacological interventions have been applied in other models of cardiac disease are too small to draw firm conclusions, so it would be desirable to test the available compounds in models of cardiac hypertrophy and arrhythmias. Moreover, little is known about the optimal level where the pathway should be targeted: Upstream targeting (at the level of WNT production or interaction with the receptor) can target both  $\beta$ -catenin-dependent and -independent signaling, whereas downstream targeting (at the level of signal transduction) could introduce selectivity for a specific signaling pathway. To clarify this issue, future research should concentrate on interventions at different levels of the pathway in a single experiment to compare the effects on infarct composition and cardiac function. These studies should pay specific attention to the induction of cardiac renewal as a potential mechanism for the beneficial effects of WNT inhibition.

## IX. WNT Signaling in Stem Cells

One of the unique features of stem cells is their dual ability to self-renew as well as differentiate into cells of different lineages. Stem cells can have varying degrees of potency ranging from a pluripotent cell that is able to give rise to all three germ layers to a more limited tissue progenitor capable of generating a specific target cell (e.g., myeloid progenitor in blood). The potency of progenitors during embryonic development as well as in the adult animal is regulated by a wide range of cell-dependent and cell-autonomous factors. Circulating and local signals are known to affect the self-renewal and lineage specification of progenitors in a niche. WNT signaling is active in stem cell niches and can regulate the potency, fate, and renewal of progenitors. WNTs can regulate pluripotency (Willert et al., 2003; Sato et al., 2004; Dravid et al., 2005) and direct fate specification of pluripotent cells. For instance, WNTs can regulate pluripotency of embryonic stem cells but can also direct specification of germ cell lineage from the embryonic stem cells. Thus WNTs can promote the maintenance of stem cell potency or renewal or can drive differentiation



of progenitors to adopt specific fates in a context-dependent manner. This seemingly dual function of the WNT signaling of maintaining supporting stem cell pluripotency as well as enhance differentiation can be explained at least partially by different coactivator usage (Miyabayashi et al., 2007).

#### A. WNT Signaling Controls Stem Cell Differentiation

In the adult mammal, stem cells or progenitors are thought to reside in specialized microenvironments called niches. Signaling within a niche determines the fate of a stem cell to self-renew or differentiate into target parenchymal cells of that tissue. An understanding of signaling pathways thus provides critical insight into mechanisms of stem cell maintenance or exhaustion. WNTs are an important family of proteins that regulate the biology of stem cells and determine cell fate decisions. WNTs can regulate differentiation of progenitors to various directions, including neuronal, hepatic, myogenic, chondrogenic, osteogenic, adipogenic, and cardiogenic cell fate (Visweswaran et al., 2015). It has been shown that WNT signaling promotes neurogenic differentiation. More specifically, in cortical mouse neural precursor cells, WNT7A promoted neuronal differentiation (Hirabayashi et al., 2004). Furthermore, WNT5A appears to drive neuronal differentiation via FZD<sub>3</sub> and <sub>-5</sub>, and signaling through the WNT5A-JNK pathway (Jang et al., 2015). WNT activation can also enhance myogenic differentiation. Various WNT molecules have been shown to promote myogenic markers, with WNT11 being the strongest (Belema Bedada et al., 2005). Hepatic differentiation appears to be influenced by WNT signaling as well. In humans, depending on the stem cells source, the WNT signaling pathways can have different effects. They are activated in hepatic differentiation of embryonic stem cells, but downregulated in hepatic differentiation of adipose tissue-derived stem cells (Heo et al., 2013). Additionally, WNT signaling regulates stem cell differentiation in osteogenic and chondrogenic lineages as well. The data on the WNT pathways on bone development have been contradictory, with reports suggesting enhancing as well as inhibitory effects (Visweswaran et al., 2015).

With regard to WNTs in stem cell differentiation into cardiac lineage, the data suggest that they play a critical role as mentioned in detail in *section IX.C*. It is important to highlight that the WNT/ $\beta$ -catenin pathway regulates the early differentiation of murine embryonic stem cells (Anton et al., 2007). Furthermore, regulation of WNT3A transcription serves as a turning point for cardiomyogenic differentiation (Deb et al., 2008).

#### B. WNT Signaling and Cardiovascular Regeneration in Lower Vertebrates

The remarkable cardiac regeneration ability in the lower vertebrates, including frogs, newts, and axolotls,

was initially studied more than 50 years ago (Rumyantsev, 1966; Rumyantsev and Marakjan, 1968). More recent studies provided evidence of full regeneration of the adult hearts in newts over a period of 60 days (Witman et al., 2011). Although earlier studies showed only limited regenerative capacity, those studies were likely prematurely terminated for analysis (Porrello and Olson, 2014). In addition, the location of the injury could suggest a differential regenerative capacity of the progenitor cells or cardiomyocytes. However, zebrafish show an exceptional regenerative capacity following different modalities of cardiac injury (Kikuchi and Poss, 2012). The different modalities include surgical removal of 20% of the apex of the ventricle, cryoablation-induced injury of 25% of the apex of the ventricle, or ablation of up to 70% of cardiomyocytes by transgenic expression of diphtheria toxin (Poss et al., 2002; Chablais et al., 2011; Wang et al., 2011a). Regardless of the type of injury, the cellular mechanisms involved in heart regeneration appear to be closely similar, regardless of the injury models. The epicardium appears to be essential for these cellular changes to occur. Interestingly, genes that are normally expressed during development, such as Wilms' tumor 1b, appear to be upregulated during the regeneration process. Although there is ample evidence of the role of WNT signaling pathways participating in mammalian heart regeneration and remodeling, little is known regarding their role heart regeneration in lower vertebrates. WNT/ $\beta$ -catenin signaling appears to be upregulated following ventricular regeneration after resection (Stoick-Cooper et al., 2007). In addition, a WNT target gene is also activated in the zebrafish after injury (Kizil et al., 2009). However, further studies are needed to elucidate the role of WNT signaling in lower vertebrate heart regeneration.

#### C. WNT Signaling in Cardiomyocyte Differentiation of Stem/Progenitor Cells

As mentioned in *section VI*, the studies on WNT signaling during cardiac development in vertebrates have yielded mixed results. In chicken and frog embryo experiments, the activation of  $\beta$ -catenin-dependent WNT signaling inhibited cardiac differentiation and WNT inhibitors had the opposite effect (Marvin et al., 2001; Schneider and Mercola, 2001). In addition, application of LiCl, an activator of  $\beta$ -catenin-dependent WNT signaling, increased the myogenic differentiation of mesenchymal stem cells derived from cardiac patients (Brunt et al., 2012). Non- $\beta$ -catenin-mediated signaling plays also a role in promoting cardiomyocyte differentiation. Mesenchymal stem cells transduced with WNT11 appeared to upregulate GATA-4, likely through signaling via PKC or JNK pathway (He et al., 2011). From a developmental standpoint, these insights have been applied in other projects with a direct potential clinical impact. It has been proposed that

human embryonic stem cells could serve as a source of cardiomyocytes. Subsequently, cultured embryonic stem cells form clusters called embryonic bodies that appear to differentiate in random fashion, even to a few beating cardiomyocytes. However, addition of WNT3A to these cultures significantly increased the onset of beating activity and expression of cardiac genes (Kwon et al., 2007). Furthermore, addition of a WNT signaling inhibitor resulted in complete abolition of the beating embryonic bodies (Kwon et al., 2007). These findings suggest that  $\beta$ -catenin-dependent WNT signaling not only drives the cardiomyocyte formation in embryonic stem cells but also is a necessary pathway for such events.

#### D. WNT Signaling in Cardiac Fibrosis and Repair

The importance of WNT signaling in development cannot be fully appreciated without elaborating on their role in cardiac repair and fibrosis (Deb, 2014). As discussed in detail in *section VIII.A*, expression of several WNTs, such as WNT1, -2, -4, -7A, -10B, and -11 increases significantly shortly after an ischemic cardiac injury (Kobayashi et al., 2009; Aisagbonhi et al., 2011; Duan et al., 2012). The effects of WNT/ $\beta$ -catenin signaling appears to be profibrotic and antagonism of this pathway with sFRPs appears to have beneficial effects on fibrosis, cardiac function, and left ventricular remodeling (Kobayashi et al., 2009). However, the specific mechanisms of beneficial effects of WNT antagonism following injury remains unclear, although it is tempting to speculate that this induces cardiac regeneration. Which cell populations express different WNTs in the injured hearts and how WNT/FZD signaling changes in different cell populations after cardiac injury will need to be further studied, especially to develop therapeutic strategies in the ischemic heart. Cardiac hypertrophy, in contrast to myocardial infarction, is characterized by an increase in cardiomyocyte cell size and increase fibrosis as well. Gene expression of WNTs does not appear to be altered after cardiac hypertrophy (ter Horst et al., 2012), but when patients with a hypertrophic heart develop heart failure, WNT signaling appears to be depressed (Kobayashi et al., 2009). In addition,  $\beta$ -catenin loss-of-function mutations in the myocytes have been associated with enhanced cardiac function, and gain-of-function mutations have been associated with reduced cardiac function resembling dilated cardiomyopathy (Baurand et al., 2007; Zelarayan et al., 2008). These findings suggest that WNTs signaling in cardiac myocytes promotes cardiac hypertrophy and suggests that inhibition of the  $\beta$ -catenin-dependent WNT pathway signaling might be a pharmacological target for cardiac hypertrophy.

#### E. Conclusions and Future Perspectives

Although there is ample evidence for the involvement of WNT signaling in stem cell homeostasis, e.g., in the

intestine, its role in cardiovascular stem cell regulation is only beginning to be explored. Because regeneration of heart tissue would be a major step forward in the treatment of damage, e.g., caused by cardiac ischemia, further exploration of interventions in Wnt signaling aiming at the regeneration of heart tissue will be an exciting area of research for the near future. However, it will be challenging to specifically address the regeneration process in the heart, without affecting stem cells in other organs. A detailed molecular analysis of the signaling pathways involved in cardiac regeneration is needed to identify targeting points that are sufficiently specific to avoid unacceptable side effects in other stem cell populations.

### X. Crosstalk between WNT Signaling and Other Signal Transduction Pathways

#### A. Transforming Growth Factor $\beta$

Rather than operating in isolation, WNT signaling appears to be strongly intertwined with other signaling pathways, including the TGF $\beta$ /BMP and YAP/TAZ signaling. Multiple examples of crosstalk between these pathways can be found, and this crosstalk appears to be taking place at different levels in the signaling cascade (Guo and Wang, 2009). Activation of this signaling network is particularly relevant for the control of fibrosis of different organs, where differentiation of effector cells from different origins into myofibroblasts plays a key role (Piersma et al., 2015). Here we will discuss this crosstalk at multiple levels: the extracellular level, at the intracellular signal transduction, and in the nucleus.

1. *Extracellular Crosstalk between WNT and TGF $\beta$  Signaling.* There are multiple studies in which the mutual regulation of the expression of WNTs and TGF $\beta$  were demonstrated (van den Bosch et al., 2016). In a model of experimental autoimmune myocarditis, the myofibroblast differentiation and subsequent cardiac fibrosis induced by TGF $\beta$  was shown to be dependent on the rapid secretion of WNT proteins in a TGF $\beta$ -activated kinase-1 (TAK-1)-dependent fashion (Blyszczuk et al., 2017). An increase in WNT production was also observed in TGF $\beta$ -stimulated vascular smooth muscle cells overexpressing the intracellular signal transduction molecule SMAD3 (DiRenzo et al., 2016). A similar induction of WNT5A and WNT11 expression by TGF $\beta$  was recently reported in fibrotic liver and lung smooth muscle cells, respectively (Kumawat et al., 2016; Beljaars et al., 2017). However, the crosstalk between WNT and TGF $\beta$  can also follow the opposite direction as shown by the WNT3A-induced expression of TGF $\beta$  in mouse fibroblasts (Carthy et al., 2011).

2. *Crosstalk at the Level of the Signal Transduction.* TGF $\beta$  signals through TGF $\beta$  type I and II receptors, members of the tyrosine kinase receptor family. Upon binding of a TGF $\beta$  homodimer, a heterodimeric receptor

complex is formed that can phosphorylate the signaling modulator Smad2/3. These Smads can in turn associate with Smad4, travel to the nucleus, and form a transcription module with multiple cofactors including p300 and CBP. Smad3 was found to facilitate the rapid nuclear translocation of  $\beta$ -catenin upon TGF $\beta$  stimulation of mesenchymal stem cells (Jian et al., 2006). On the other hand, the transcriptional activity of Smad3 was significantly enhanced in cardiac fibroblasts that are conditionally depleted of GSK3 $\beta$  using the tamoxifen-inducible *Col1a2-cre* model (Lal et al., 2014). A direct interaction of Smad3 with a complex of axin and GSK3 was shown to result in the phosphorylation of Smad3 at Thr<sup>66</sup>, triggering its degradation via the ubiquitin/proteasome pathway (Guo et al., 2008). These observations suggest a mutual interaction between the two pathways.

The complexity of this interaction is further enhanced by the involvement of a third pathway, YAP/TAZ, that has been shown to be strongly intertwined with both TGF $\beta$  and WNT signaling in the differentiation of myofibroblasts (Piersma et al., 2015). YAP and TAZ are two effectors of Hippo signaling, another highly conserved signaling pathway involved in the regulation of heart size and stem cell fate (Xiao et al., 2016). YAP has been shown to interact with Smad7, an inhibitor of the intracellular TGF $\beta$  signaling, thereby repressing the signaling to the nucleus (Piersma et al., 2015). Moreover, in the WNT off state YAP and TAZ are associated with the  $\beta$ -catenin destruction complex where they facilitate the ubiquitination of  $\beta$ -catenin by  $\beta$ -TrCP. Activation of WNT signaling results in dissociation of YAP and TAZ from the destruction complex and release of  $\beta$ -catenin (Azzolin et al., 2014). TAZ was also reported to be able to bind to DVL, thereby attenuating the activation of WNT signaling and to associate directly with  $\beta$ -catenin, thereby inhibiting its nuclear localization (Imajo et al., 2012).

**3. Nuclear Crosstalk between WNT, TGF $\beta$  and YAP/TAZ Signaling.** A specific feature of these three signaling pathways is that they rely on the nuclear translocation of their intracellular signaling proteins to activate gene transcription. As indicated in *section V.B.4*,  $\beta$ -catenin does not possess DNA binding capabilities. The same holds true for YAP/TAZ: both form transcriptional modules with more general transcriptional coactivators such as p300 and/or CBP. Interestingly, YAP/TAZ transcription factors can be added to the TGF and WNT complexes, giving rise to additional or even synergistic activation of the gene expression. This crosstalk appears to be of specific importance in EMT and myofibroblasts activation (Piersma et al., 2015).

### B. Renin Angiotensin System

Besides its central role for cardiovascular homeostasis, the renin angiotensin system (RAS) is also involved in other organ systems and is interconnected to many

signaling systems both in physiologic and pathologic conditions (de Gasparo et al., 2000; Bader and Ganten, 2008).

AngII, the main effector of the renin angiotensin system, is formed by the enzymatic degradation of angiotensinogen by renin and angiotensin converting enzyme, respectively. AngII has been shown to modulate the mRNA expression of FZD<sub>2</sub> in rat vascular smooth muscle cells in a time-dependent manner (Castoldi et al., 2005). The initial rapid upregulation of FZD<sub>2</sub> after AngII stimulation was accompanied by a rapid upregulation of TGF $\beta$ -1, suggesting a potential crosstalk between AngII and FZD<sub>2</sub>, most probably indirectly via the contribution of TGF $\beta$ -1 signaling (see *section X-A*) (Castoldi et al., 2005). Following this early finding, more direct evidence linking the RAS to WNT signaling was identified more recently.

The (pro)renin receptor (PRR), one of the latest identified RAS family members, is able to bind renin and its precursor prorenin. Shortly after its discovery, PRR was recognized as the full-length form of a smaller protein described earlier, associated with the V-ATPase (vacuolar H<sup>+</sup>-ATPase), a multiprotein complex involved in the acidification of vesicles (Nguyen, 2011). More recently PRR was proposed to be a component of the WNT receptor complex independently of its function for renin. PRR seems to act as an adaptor between WNT receptors and the V-ATPase complex (Buechling et al., 2010; Cruciat et al., 2010). Coimmunoprecipitation experiments showed that PRR could bind FZD<sub>2</sub>, FZD<sub>8</sub>, and LRP6. In addition, loss of function and pharmacological inhibition of V-ATPase in vitro and in vivo revealed its necessity for WNT/ $\beta$ -catenin signaling (Cruciat et al., 2010). Results from this study have thus shed light on an unsuspected role for PRR/V-ATPase and acidification during WNT/ $\beta$ -catenin signaling. The authors hypothesized that the WNT/PRR complex is internalized upon stimulation of the FZD receptor, followed by an acidification of the endocytosed complex that allows the phosphorylation of LRP6 and the subsequent  $\beta$ -catenin activation (Cruciat et al., 2010). In addition to its involvement in the WNT/ $\beta$ -catenin pathway, the PRR has been identified to be also part of the WNT/PCP pathway in *Drosophila* (Buechling et al., 2010; Hermle et al., 2010).

Recent in vitro and in vivo results have identified that multiple RAS genes encoding angiotensinogen, renin, angiotensin-converting enzyme, and the receptors for AngII are downstream targets of WNT/ $\beta$ -catenin signaling. The overexpression of  $\beta$ -catenin or different WNT ligands upregulates the expression of RAS genes, whereas blockade of WNT/ $\beta$ -catenin signaling with a small molecule inhibitor effectively downregulates RAS genes. In vivo, the inhibition of the WNT/ $\beta$ -catenin/RAS axis was able to reduce renal inflammation and fibrosis, thus opening new therapeutic avenues (Zhou et al., 2015).

### C. Vascular Endothelial Growth Factor

Vascular endothelial growth factors (VEGF-A, VEGF-B, VEGF-C, and VEGF-D) are key players influencing angiogenesis through VEGF receptors (VEGFR1, VEGFR2, and VEGFR3). This VEGF system has many sites of interaction with other signaling systems such as TGF $\beta$ , Notch, and FGF (Holderfield and Hughes, 2008; Dejana, 2010; Lieu et al., 2011). Studies performed in the last decade have also revealed synergistic or antagonistic interactions with WNT signaling.

The interaction between VEGF and WNT signaling was first identified in colonic neoplasia (Zhang et al., 2001). Similarly, the importance of the WNT/ $\beta$ -catenin signaling pathway in VEGF signaling has also been evidenced in human retinal pigment epithelial cells subjected to hypoxia. In this model, angiogenesis induced by tissue factor was decreased in presence of the WNT/ $\beta$ -catenin signaling pathway inhibitor IWR-1-endo (Wang et al., 2016c).

Later, researchers performed a high-throughput RNA interference screen to identify novel modulators of the WNT/ $\beta$ -catenin pathway and thus potential new therapeutic targets (Naik et al., 2009). A library of siRNAs was tested in a luciferase assay for activation of WNT/ $\beta$ -catenin transcription with WNT-stimulated human cells. Follow up studies with the best hits identified VEGFR1 (but not VEGFR2 or VEGFR3) as a positive regulator of WNT signaling (Naik et al., 2009). The study showed that this VEGFR1-WNT synergy was independent of GSK3 $\beta$ . A VEGFR1-tyrosine phosphorylation of  $\beta$ -catenin itself was proposed by the authors as the mechanism for this synergistic effect (Naik et al., 2009).

Further evidence for a crosstalk between WNT and VEGF signaling was obtained in the field of developmental angiogenesis. Zebrafish embryos deficient for *Rspo1* or its receptor *Kremen 1* exhibited primary vessels resulting from vasculogenesis, but did not demonstrate further vascular development (Gore et al., 2011). This was found in association with a decreased expression of VEGFC. Additional experiments revealed that the expression of VEGFC was dependent of *Rspo1* and WNT and that the proangiogenic effects of *Rspo1*/WNT signaling are mediated by VEGFC/VEGFR3 signaling (Gore et al., 2011). At an earlier developmental stage, it has been shown that VEGF signals through the WNT/ $\beta$ -catenin pathway to favor the vasculogenic differentiation of mesenchymal stem cells (Zhang et al., 2016c).

Although WNT signaling is well known for its contribution to vascular development, its interplay with VEGF signaling remains poorly investigated. Additional studies are required to confirm the previous observations and to further decipher the complex WNT-VEGF interactions.

### D. Conclusions and Future Perspectives

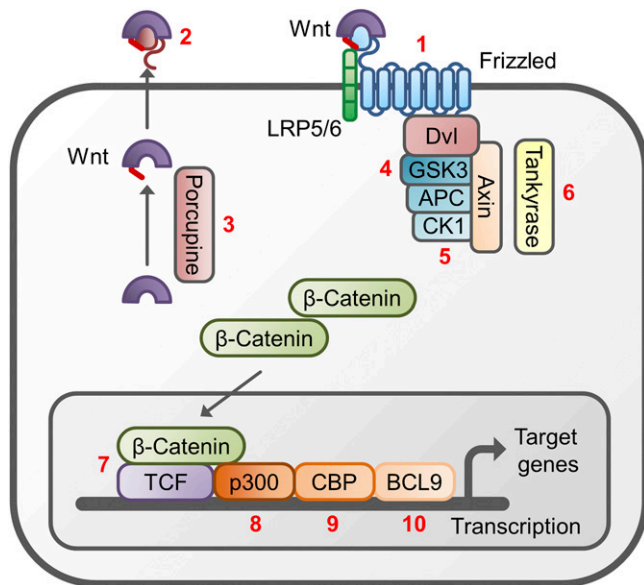
It is becoming increasingly clear that signaling pathways do not operate in isolation but are in fact strongly intertwined. The WNT signaling pathway appears to be no exception, as illustrated by its interactions with TGF $\beta$  signaling, the RAS and VEGF described in this section. It is quite conceivable that some of the effects that we observe by a therapeutic intervention in, e.g., the RAS are in fact the result of inhibition of an interacting signaling pathway. Although these interactions add significantly to the complexity of the study of WNT signaling, they are important because they can be a source of adverse effects of interventions. This calls for a more holistic approach in the preclinical phase of drug discovery in general to reduce the number of drugs that fail in clinical trials.

## XI. Therapeutic Interventions

Because of the involvement of WNT signaling in conditions varying from cancer to neurodegenerative diseases, many compounds have been developed that target the WNT signal transduction pathway at different levels. Although many of these compounds are aiming at the WNT/ $\beta$ -catenin branch of the signaling, other compounds act more upstream at the level of WNT synthesis/secretion or at the receptor complex. For an overview of the sites of interaction, please see Fig. 8. The interventions are schematically represented in Tables 2–5.

### A. WNT Synthesis–Secretion

The first level of the WNT signaling cascade is undoubtedly the production and secretion of the WNT ligand. Following its production, WNT is not released as is, but undergoes glycosylation and palmitoylation, two processes which modify WNT and are indispensable for the secretion of the active WNT ligand (Komekado et al., 2007; Kurayoshi et al., 2007). PORCN, residing in the endoplasmic reticulum of WNT-producing cells, is a critical factor in this transformation and secretion of WNTs (Komekado et al., 2007). A wide range of PORCN inhibitors has been developed with demonstrated effects on WNT secretion, thus mediating the prevention of WNT signaling activation. Additionally, as shown by in vivo studies with transgenic mice, genetic loss of *Porc*n leads to dramatic inhibition of endogenous WNT production, but mice can still respond to exogenous WNTs, indicating that the receptor compartment is not affected (Barrott et al., 2011). Studies with human astrocytes showed that involvement of PORCN in the WNT production might be cell type and WNT subtype dependent (Richards et al., 2014). Chen et al. (2009a) were the first to show that four compounds, named IWP-1 to -4 (inhibitors of WNT production), can attenuate the



**Fig. 8.** Different sites of pharmacological intervention in WNT signaling. Details are provided in *section XI*. (1) Targeting of FZD proteins (e.g., vantictumab or UM206); (2) WNT scavengers (e.g., ipafricept); (3) Porcupine inhibitors (e.g., LGK974 or IWP-2); (4) Glycogen synthase kinase-3 $\beta$  (GSK3 $\beta$ ) inhibitors (e.g., LiCl, valproic acid, 6-BIO); (5) Casein kinase-1 (CK1) inhibitors (e.g., pyrvinium); (6) Tankyrase inhibitors (e.g., XAV939, IWR-1); (7) Inhibitors of the interaction between  $\beta$ -catenin and the TCF transcription factors (e.g., ICRT3, -5, and -14, nonsteroid anti-inflammatory drugs); (8, 9, 10) Inhibitors of the interaction of the transcription complex with the cofactors p300, CBP, and BCL9 (e.g., windorphen, ICG-001, and SAH-BCL9).

secretion of the WNT ligands. These four IWP molecules induce PORCN-mediated inhibition of WNT 1, 2, and 3a production. A few years later they reported another 13 IWP Porcn inhibitors and attention was drawn toward IWP-L6, which was shown to be 100 times more potent than the originally designed IWP-2 (Wang et al., 2013b). In addition, LGK974, a potent and specific PORCN-inhibitor, was first discovered by the research group of Harris; the compound is also referred to as WNT974. The orally bioavailable molecule inhibits PORCN, as well as operating more downstream too by blocking WNT-mediated LRP5/6 phosphorylation, and is very effective against various types of cancer both in vitro and in vivo (Liu et al., 2013). Interestingly, LGK974 can work in synergy with the PI3K inhibitor GDC-0941 and produce antitumor effects in MDA-MB-231 breast cancer cells (Tzeng et al., 2015). Furthermore, LGK974 has also entered a phase I clinical trial (<https://clinicaltrials.gov/ct2/show/NCT01351103>), including patients with colorectal cancer, pancreatic adenocarcinoma, and other cancer types with well-established association with the WNT signaling cascade. The beneficial effects of this compound, as well as the PORCN inhibitor GNF-6231, on infarct healing was previously discussed (*section VIII.A.1*). Quite recently, ETC-159 was demonstrated to strongly inhibit PORCN, block WNT secretion, attenuate the expression of the  $\beta$ -catenin target genes TCF7, c-myc and axin2, and to

possess robust antitumor capabilities versus teratocarcinomas and colon cancers, as well as preventing remissions (Madan et al., 2016a). Proffitt et al. (2013) propose the compound WNT-C59 as a robust and safe inhibitor of PORCN. All PORCN isoforms and all WNT members that can activate the  $\beta$ -catenin-dependent pathway were reported to be substantially inhibited by WNT-C59 and this is reflected by its excellent antitumor effects in mice, where it prevents MMTV-WNT1 growth (Proffitt et al., 2013). WNT-C59 has the additional advantage of being orally bioavailable. Treatment with WNT-C59 leads to the robust attenuation of renal fibrosis in a unilateral ureteral obstruction mouse model. This is effectuated by a complex crosstalking mechanism and since the disruption of porcupine leads to blunted WNT secretion, this has the inhibition of inflammatory cytokine activation and thus a suppression of ECM-depositing fibroblasts expression as a consequence (causing an attenuation in fibrosis) (Madan et al., 2016b). Novel PORCN inhibitors, such as compound 53 have shown promising preliminary results, but their physicochemical and pharmacokinetic properties restrict their applicability. Thus, more research is required for their improvement, before they are further studied in vitro and in vivo (Xu et al., 2016). Lastly, one very important point that should be taken into consideration when using factors acting at the level of PORCN is their relative nonselectivity. Targeting PORCN usually leads to a global WNT secretion suppression, both the ones forming the  $\beta$ -catenin-dependent pathway (WNT1, -2, -3, -8A/B, and -10A/B), as well as the  $\beta$ -catenin-independent WNTs-4, -5A/5B, -6, -7A/B, and -11), thus there is always the danger for off-target effects.

WNT16 expression can be directly affected by a compound called 4,4-di-*O*-methylellagic acid (4,4-DiOMEA). The expression of WNT16 mRNA levels is correlated with concentrations of 4,4-DiOMEA, and this molecule exhibits robust antiproliferative effects on colon cancer cell lines, even ones that are resistant to well-established chemotherapy drugs such as 5-FU (Ramirez de Molina et al., 2015).

### B. Extracellular Pharmacological Targeting of WNT Signaling: WNT-FZD Interaction

The interaction of the WNT ligands with their corresponding FZD receptors is the hallmark event initiating the signal transduction. A long list of compounds have been tested and have been found to influence the WNT-FZD interaction, whereas a probably even longer list has been identified via high-throughput methods (Koval and Katanaev, 2012) and awaits further investigation. As discussed in *section III.E*, the interaction of WNT and FZD components is complex. The scientific community has been assuming a rather simplified view of the kinetics, the selectivity of some WNTs for some FZD, the nature and varied types of

TABLE 2  
Interventions in WNT synthesis and secretion

Intervention	Use	Proposed Target	Effect	Affinity	Reference
<i>Porcn</i> genetic deletion	In vivo (mice)	<i>Porcn</i> gene	Inhibition of WNT3A secretion	n/a	Barrott et al. (2011)
IWP-1	In vitro	PORCN protein	Inhibition of WNT secretion (WNT1, -2, and -3A?)	IC50: 58 nM	Chen et al. (2009a)
IWP-2	In vitro	PORCN protein	Inhibition of WNT-1, -2, -3a secretion	IC50: 27 nM	Chen et al. (2009a)
IWP-3	In vitro	PORCN protein	Inhibition of WNT secretion (WNT1, -2, and -3A?)	IC50: 40 nM	Chen et al. (2009a)
IWP-4	In vitro	PORCN protein	Inhibition of WNT secretion (WNT1, -2, and -3A?)	IC50: 25 nM	Chen et al. (2009a)
IWP-L6	In vitro	PORCN protein	Inhibition of WNT secretion	IC50: 0.5 nM	Wang et al. (2013b)
LGK974	In vitro	PORCN protein	Inhibition of WNT1, -2, -3, -3A, -6, -7A, and -9A secretion	IC50: 0.3–0.4 nM (depending on the cell assay)	Liu et al. (2013)
ETC-159	In vitro	PORCN protein	Inhibition of WNT1, -2, -3A, -6, -7B, -8A, -9A, -9B, and -10B secretion	IC50: 2.9 nM ( $\beta$ -catenin reporter assay) IC50: 18.1 nM (mouse PORCN inhibition)	Madan et al. (2016a)
WNT-C59	In vitro and in vivo (mice)	PORCN protein	Inhibition of WNT1, -2, -3A, -6, -7B, -8A, -9A, -9B, and -10B secretion	IC50: 74 pM (WNT-3a-mediated TOPFLASH assay)	Proffitt et al. (2013)
Compound 53	In vitro	PORCN protein	Inhibition of WNT-3a secretion	IC50: 0.5 nM	Xu et al. (2016)
4,4-DiOMEA	In vitro	PORCN protein?	Inhibition of WNT-16 secretion	IC50: 7.6 $\pm$ 1.5 nM	Ramirez de Molina et al. (2015)

complexes formed, the cofactors playing secondary roles in the complex formation, the role of G proteins, etc. Nevertheless, it appears that our knowledge is far from complete and more research is needed in our understanding of receptor complex formation. Thus, one should make sure to take into account all factors playing a major role in the formation of the WNT-FZD complex, before devising a plan to prevent or promote the complex formation (Schulte, 2015).

Blockade of FZD receptors with antibodies is an attractive therapeutic strategy, because antibodies usually bind their target with high affinity and selectivity. Various groups have used antibodies directed against several FZD isoforms. In a seminal study, Gurney et al. (2012) showed that the antibody OMP-18R5 (vantictumab) could be a novel tool in blocking multiple FZD receptor targets. Initially, OMP-18R5 was thought to bind to FZD<sub>7</sub> only; however, it was later shown to act antagonistically against FZD<sub>1</sub>, -2, -5, and -8 as well. OMP-18R5 is effective against a wide range of human cancers (lung, pancreas, breast, teratocarcinoma), and it was shown to act synergistically with first-line chemotherapy (Gurney et al., 2012). A chimeric humanized monoclonal antibody directed against FZD<sub>10</sub> was developed, code-named OTSA101. The plain (nonradiolabeled) antibody is a weak antagonist of FZD<sub>10</sub>; however, when radiolabeled with Yttrium 90 (OTSA101-DTPA-90Y), it exhibits a robust antitumor effect. Even a single intravenous injection of the labeled monoclonal antibody suppressed the growth of sarcoma tumor cells in a mouse xenograft model, without leading to substantial toxicity (Fukukawa et al., 2008). Currently, OTSA101 is in Phase I clinical trial stage (<https://clinicaltrials.gov/ct2/show/NCT01469975>; “SYNFRIZZ”) with patients suffering

from synovial sarcoma. Furthermore, Nagayama et al. (2005) described a polyclonal antibody (TT641 pAb) against FZD<sub>10</sub> that acts on its extracellular domain. An attenuated growth of synovial sarcoma tumor cells in vitro, as well as of xenografts after injection in mice was observed using this antibody. In addition, Paes et al. (2011) generated an FZD<sub>4</sub> monoclonal antibody (1.99.25). The antibody binds the FZD<sub>4</sub> CRD and thus neutralizes the WNT3A-mediated  $\beta$ -catenin signaling in a retinopathy mouse model. A polyclonal antibody directed against FZD<sub>5</sub> has been developed, with evidence showing that it attenuates the proinflammatory cytokines IL-6, IL-15, and RANKL in rheumatoid arthritis (Sen et al., 2001), while a blocking antibody against FZD<sub>7</sub> has been used both in vitro and in vivo in Wilm’s tumor, with beneficial effects (Pode-Shakked et al., 2011).

OMP-54F28 (ipafricept) is a novel fusion protein that targets FZD<sub>8</sub>-interacting WNT ligands. It has been constructed by fusing the CRD of FZD<sub>8</sub> with the Fc domain of immunoglobulin. The latter competes with the native FZD<sub>8</sub> receptor for its ligands and thereby antagonizes WNT signaling (thus acting more as a scavenger of WNT ligands, rather than an antagonist of FZD). The first preclinical studies showed beneficial effects of OMP-54F28 against a variety of solid tumors (Le et al., 2015). Currently there is one clinical trial that has been completed, one is in progress, and two are recruiting patients, in the context of the use of OMP-54F28 in various human cancers (<https://www.clinicaltrials.gov/ct2/results?term=omp54f28&Search=Search>). The first data show beneficial therapeutic effects against ovarian cancer when the antibody is used along with paclitaxel and carboplatin (Fischer et al., 2015), whereas the antibody did not exhibit pronounced toxicity in

TABLE 3  
Extracellular targeting of the Wnt signaling pathway

Intervention	Use	Proposed Target	Effect	Affinity	Reference
OMP-18R5 (mAb)	In vitro and in vivo (mice)	FZD <sub>1</sub> , FZD <sub>2</sub> , FZD <sub>5</sub> , FZD <sub>7</sub> , FZD <sub>8</sub>	Inhibition of WNT-FZD interaction	n/a	Gurney et al. (2012)
OTSA101 (mAb) + Yttrium 90	In vitro, in vivo (mice) and CT	FZD <sub>10</sub>	Inhibition of WNT-FZD interaction	n/a	Fukukawa et al. (2008)
TT641 (pAb)	In vitro and in vivo (mice)	FZD <sub>10</sub>	Inhibition of WNT-FZD interaction	n/a	Nagayama et al. (2005)
1.99.15 (mAb)	In vitro and in vivo (mice)	FZD <sub>4</sub>	Inhibition of WNT-FZD interaction	n/a	Paes et al. (2011)
pAb against FZD5	In vitro	FZD <sub>5</sub>	Inhibition of WNT-FZD interaction	n/a	Sen et al. (2001)
Ab against FZD7	In vitro and in vivo (chick embryo)	FZD <sub>7</sub>	Inhibition of WNT-FZD interaction	n/a	Pode-Shakked et al. (2011)
OMP-54F28 fusion protein	In vitro, in vivo (mice) and CT	FZD <sub>8</sub> -interacting WNT ligands	Inhibition of WNT-FZD interaction	n/a	Le et al. (2015), Fischer et al. (2015)
UM206	In vitro and in vivo (mice)	FZD <sub>1</sub> and FZD <sub>2</sub>	Inhibition of WNT-FZD interaction	IC <sub>50</sub> : 2.10 nM for rFZD1; IC <sub>50</sub> : 0.0169 nM for rFZD2	Jimeno et al. (2014) Laeremans et al. (2011) Uitterdijk et al. (2016)
Foxy5 + FZD <sub>5</sub> receptor-blocking Ab	In vitro and in vivo (mice)	FZD <sub>5</sub>	Mimicking of WNT5A (effect independent of $\beta$ -catenin)	n/a	Safholm et al. (2006) Safholm et al. (2008)
Box5	In vitro	FZD <sub>5</sub> (?)	Blockade of WNT5A signaling	n/a	Jenei et al. (2009)
3235–0367	In vitro	FZD <sub>8</sub> CRD	Inhibition of WNT-FZD interaction	IC <sub>50</sub> : 7.1 $\mu$ M	Lee et al. (2015)
1094–0205	In vitro	FZD <sub>8</sub> CRD	Inhibition of WNT-FZD interaction	IC <sub>50</sub> : 5 $\mu$ M	Lee et al. (2015)
2124–0331	In vitro	FZD <sub>8</sub> CRD	Inhibition of WNT-FZD interaction	IC <sub>50</sub> : 10.4 $\mu$ M	Lee et al. (2015)
NSC36784	In vitro	FZD <sub>8</sub> CRD	Inhibition of WNT-FZD interaction	IC <sub>50</sub> : 6.5 $\mu$ M	Lee et al. (2015)
NSC654259	In vitro	FZD <sub>8</sub> CRD	Inhibition of WNT-FZD interaction	IC <sub>50</sub> : 5.7 $\mu$ M	Lee et al. (2015)
Niclosamide	In vitro	FZD <sub>1</sub> (internalization)	Inhibition of WNT-FZD interaction	IC <sub>50</sub> : 0.5 $\mu$ M	Chen et al. (2009b)
	In vitro	LRP6	LRP6 degradation	IC <sub>50</sub> : 0.33–0.75 $\mu$ M (depending on the cell line)	Lu et al. (2011b)
Curcumin	In vitro	FZD <sub>1</sub>	Inhibition of WNT-FZD interaction (?)	n/a	Yan et al. (2005)
	In vitro	WNT3A; LRP6	Inhibition of WNT-FZD interaction (?)	n/a	Zheng et al. (2017)
	In vitro	WNT10B; FZD <sub>2</sub> ; LRP5	Enhancement of WNT-FZD interaction	n/a	Ahn et al. (2010)
anti-Sclerostin Ab	In vitro	Sclerostin binding to LRP5/6	Prevention of Sclerostin-mediated disruption of LRP5/6-FZD complex formation	n/a	van Dinther et al. (2013)
Salinomycin	In vitro	LRP6 (?)	Inhibition of WNT1/FZD <sub>5</sub> /LRP6 and WNT3/FZD5/LRP6 complexes	IC <sub>50</sub> : 163 nM (WNT-1)	Lu et al. (2011)
	In vitro	LRP6	Inhibition of WNT/FZD/LRP6 complex (?)	n/a	Lu and Li (2014)
Leptin	In vivo (mice)	LRP6; WNT4 and WNT7 (?); GSK-3 $\beta$	Enhancement of WNT4/FZD/LRP6 and WNT7A/FZD/LRP6 complexes	n/a	Benzler et al. (2013)
Silibinin	In vitro	LRP6	Inhibition of WNT3A/FZD/LRP6 complex	IC <sub>50</sub> : 34–122 $\mu$ M (depending on the cell line)	Lu et al. (2012)
anti-LRP6 Abs	In vitro	LRP6	Inhibition of WNT1 and WNT3A-mediated cascade (WNT/FZD/LRP6 complex?)	n/a	Ettenberg et al. (2010)
	In vitro	LRP6	Inhibition or enhancement of WNT1/2/2B/4/6/7A/7B/8A/9B/10A/10B-mediated cascade (WNT/FZD/LRP6 complex?)	n/a	Gong et al. (2010)

(continued)

TABLE 3—Continued

Intervention	Use	Proposed Target	Effect	Affinity	Reference
28aa peptide	In vitro and in vivo (mice)	LRP5/6	Mimicking of LRP5/6-domain → Enhancement of WNT signaling (via N-cadherin)	n/a	Hay et al. (2012)
DKK1 and DKK2	In vivo (Xenopus embryos)	LRP6	Inhibition of WNT signaling	n/a	Mao et al. (2001)
anti-DKK1 Ab	In vitro and in vivo (mice)	DKK1	Neutralization of DKK1 → Enhancement of WNT signaling	n/a	Sato et al. (2010b)
BHQ880 mAb	In vitro, in vivo (mice) and CT	DKK1	Neutralization of DKK1 → Enhancement of WNT signaling	n/a	Fulciniti et al. (2009)
DKK1 vaccine	In vivo (mice)	DKK1	Enhancement of DKK1 levels → Inhibition of WNT signaling	n/a	Iyer et al. (2014) Qian et al. (2012)
Iminooxothiazolidines (Compounds 1–34)	In vitro	sFRP1	Inhibition of sFRP1 → Enhancement of WNT signaling	EC <sub>50</sub> : 7.2 μM (Compound 1)	Shi et al. (2009)
WAY-316606	In vitro	sFRP1	Inhibition of sFRP1 → Enhancement of WNT signaling	IC <sub>50</sub> : 0.5 μM EC : 0.65 μM	Bodine et al. (2009)
N-substituted piperidinyl diphenylsulfonamide	In vitro	sFRP1	Inhibition of sFRP1 → Enhancement of WNT signaling	<sup>50</sup> IC <sub>50</sub> : 0.04–0.87 μM ED50: 0.07–1.9 μM	Moore et al. (2010) Moore et al. (2010)

patients with advanced solid tumors, although bone effects were observed (Jimeno et al., 2014).

Our laboratory has been active for more than a decade in developing interventions aiming at the WNT-FZD interaction. We focused on identifying molecules that block FZD proteins, thereby preventing the WNT and FZD complex formation, eventually leading to the attenuation of signal transduction. We previously showed that UM206, a 13-amino acid-long peptide fragment of WNT3A/WNT5A, can have profound effects on FZD-receptor blockade, leading to a suppression of WNT signaling pathway (Laeremans et al., 2011). UM206 possesses two Cys residues and it was shown that substituting both of them with another amino acid can have detrimental effects for the activity of the molecule, which is indicative for a possible covalent binding between the two Cys of UM206 and the Cys-rich domain of FZD. Furthermore, UM206 may be rather selective, as shown by the fact that it blocked FZD<sub>1</sub> and FZD<sub>2</sub> receptors in the presence of WNT3A, but not FZD<sub>3</sub> or FZD<sub>4</sub>. Due to these pronounced effects of UM206 on the WNT signaling pathway, we decided to investigate its effects following MI in mice. UM206 treatment demonstrated beneficial effects on cardiac remodeling and prevented HF development 35 days post-MI, an outcome attributed to increased myofibroblast numbers in the infarct area and better scar healing. Reduced dilatation of the left ventricle and improved infarct healing were also observed in a pig MI model where UM206 was infused for 5 weeks post-MI, albeit that the myofibroblasts numbers were actually reduced in this

study (Uitterdijk et al., 2016). It has to be noted, however, that direct binding of UM206 to the CRD of FZD has not been observed, and, given that the peptide is derived from a region of WNT5A that appears to be not in direct contact with FZD (Janda et al., 2012), it is more likely that peptide inhibits the formation of the complex of ligand and (co)-receptors in a different but yet unidentified way.

Foxy5 is a formylated hexapeptide that was shown to act via FZD<sub>5</sub> receptors, leading to impaired migration of MDA-MB-468 breast cancer tumor cells. The authors pretreated cells with a FZD<sub>5</sub>-specific blocking antibody and observed an attenuation of the effects of Foxy5, a phenomenon implying that the compound is most probably mediating its effects via the FZD<sub>5</sub> receptor but not via FZD<sub>2</sub> (Safholm et al., 2006, 2008). The same group investigated later another compound called Box5, which shares the same amino acid sequence with Foxy5 but possesses a butyloxycarbonyl group (instead of a formyl group) at the N terminal of its hexapeptide. Box5 directly antagonizes the WNT5A-mediated cellular signaling by attenuating WNT5A-mediated Ca<sup>2+</sup> and PKC signaling but, although it was not proven, there is the assumption that it also acts via a blockade of FZD<sub>5</sub> receptor similar to Foxy5 (Jenei et al., 2009).

Recently, a nonpeptidic compound was described that can interact with FZD<sub>4</sub>. The compound, named FzM1, was shown to bind to the intracellular loop three of FZD<sub>4</sub> and blocking the Norrin-stimulated activation of TCF/LEF with micromolar affinity. Interestingly, the compound is also able to act as a chaperone for the



TABLE 4  
Interventions in the intracellular compartment

Intervention	Use	Proposed target	Effect	Affinity	Reference
<i>1. Interventions Targeting Dishevelled</i>					
3289–8625	In vitro and in vivo (Xenopus embryos and mice)	DVL PDZ	Blockade of DVL → Inhibition of WNT signaling	IC <sub>50</sub> : 10.5 μM	Grandy et al. (2009)
NSC668036	In silico and in vivo (Xenopus embryos)	DVL PDZ	Blockade of DVL → Inhibition of WNT signaling	n/a	Shan et al. (2005)
J01-017a	In silico	DVL PDZ	Blockade of DVL → Inhibition of WNT signaling	n/a	Shan et al. (2012)
FJ9	In vitro and in vivo (mice)	DVL PDZ	FZD <sub>7</sub> -DVL PDZ interaction disruption → Inhibition of WNT signaling	n/a	Fujii et al. (2007)
PolyR-DBM	Ex vivo and in vitro	DVL-CXXC5	FZD7-DVL PDZ interaction disruption → Inhibition of WNT signaling	n/a	Kim et al. (2015)
KY-02327	In silico, in vitro, ex vivo, in vivo (mice)	DVL-CXXC5	FZD7-DVL PDZ interaction disruption → Inhibition of WNT signaling	IC <sub>50</sub> : 3.1 μM	Kim et al. (2016)
KY-02061	In silico, in vitro, ex vivo, in vivo (mice)	DVL-CXXC5	FZD7-DVL PDZ interaction disruption → Inhibition of WNT signaling	IC <sub>50</sub> : 24 μM	Kim et al. (2016)
pen-N3	In silico and in vitro	DVL PDZ	Blockade of DVL → Inhibition of WNT signaling	IC <sub>50</sub> : 11 ± 4 μM	Zhang et al. (2009b)
<i>2. Interventions Targeting Axin</i>					
XAV939	In vitro and in vivo (mice)	axin	Induction of protein levels and stabilization of axin → Enhancement of axin-GSK3β complex formation → Inhibition of Wnt Signaling	IC <sub>50</sub> : 0.114–2.194 μM (PARP1/2); IC <sub>50</sub> : 0.004–0.011 μM (TNKS1/2)	Huang et al. (2009), Wang et al. (2014a)
IWR-1 and IWR-2	In vitro and in vivo (zebrafish and mice)	axin	Induction of protein levels and stabilization of axin → Enhancement of axin-GSK3β complex formation → Inhibition of Wnt Signaling	IC <sub>50</sub> IWR-1: 0.18 μM; IC <sub>50</sub> IWR-2: 0.23 μM	Chen et al. (2009), Wang et al. (2014a)
XAV939 + 5-FU or cisplatin	In vitro	axin	Induction of protein levels and stabilization of axin → Enhancement of axin-GSK3β complex formation → Inhibition of Wnt Signaling	n/a	Wu et al. (2016b)
XAV939 + ICAT + niclosamide	In vitro	axin; β-catenin/ p300 complex; FZD1	Stabilization of axin (XAV939), β-catenin/p300 complex inhibition (ICAT), FZD1 internalization (niclosamide) → Inhibition of Wnt Signaling	n/a	Ono et al. (2014)
E7449	In vitro and in vivo (mice)	axin/PARPs	TNKS proteins inhibition → Stabilization of axin → Inhibition of Wnt signaling	IC <sub>50</sub> PARP1/2: 1–2 nM, IC <sub>50</sub> TNKS1/2: 50–120 nM	McGonigle et al. (2015)
WXL-8	In vitro and in vivo (mice)	axin/TNKSs	TNKS proteins inhibition → Stabilization of axin → Inhibition of Wnt signaling	IC <sub>50</sub> : 9.1 nM (TNKS1)	Ma et al. (2015)
JW67, JW74 and JW55	In vitro and in vivo (Xenopus embryos and mice)	axin	TNKS proteins inhibition → Induction of protein levels and stabilization of axin → Enhancement of axin-GSK3β complex formation → Inhibition of Wnt Signaling	IC <sub>50</sub> JW67: 1.17 mM; IC <sub>50</sub> JW64: 790 nM; IC <sub>50</sub> JW55: 470 nM	Waalder et al. (2011), (2012)
Compounds 13 and 14	In silico and in vitro	axin/TNKSs	TNKS proteins inhibition → Stabilization of axin → Inhibition of Wnt signaling	IC <sub>50</sub> = 2 nM (for TNKS2)	Nathubhai et al. (2017)
WIKI4	In vitro	axin/TNKSs	TNKS proteins inhibition → Halting of axin ubiquitination → Stabilization of axin → Inhibition of WNT signaling	IC <sub>50</sub> : 15 nM (TNKS2)	James et al. (2012)
G007-LK	In vitro and in vivo (mice)	axin/TNKSs	TNKS proteins inhibition → Stabilization of axin → Inhibition of WNT signaling	IC <sub>50</sub> : 0.08 μM	Lau et al. (2013)
AZ1366 + EGFR-inhibitor	In vitro and in vivo (mice)	axin/TNKSs	TNKS proteins inhibition → Stabilization of axin → Inhibition of WNT signaling	n/a	Scarborough et al. (2017)

(continued)

TABLE 4—Continued

Intervention	Use	Proposed target	Effect	Affinity	Reference
AZ1366 + irinotecanin IWRs (1-5)	In vitro and in vivo (mice) In vitro and in vivo (zebrafish)	axin/TNKSs	TNKS proteins inhibition → axin induction → TNKS proteins inhibition → Induction of protein levels and stabilization of axin → Enhancement of axin-GSK3β complex formation → Inhibition of Wnt Signaling	n/a IC <sub>50</sub> : 0.18–2.0 μM	Quackenbush et al. (2016) Chen et al. (2009)
NVP-TNKS656	In silico and in vitro	axin/TNKSs	TNKS proteins inhibition → Induction of protein levels and stabilization of axin → Enhancement of axin-GSK3β complex formation → Inhibition of Wnt Signaling	IC <sub>50</sub> : 0.006 μM (TNKS2)	Shultz et al. (2013)
Compounds 9 and 25	In silico and in vitro	axin/TNKSs	TNKS proteins inhibition → Induction of protein levels and stabilization of axin → Enhancement of axin-GSK3β complex formation → Inhibition of Wnt Signaling	IC <sub>50</sub> : <0.003 μM (compound 9); IC <sub>50</sub> : 0.005 μM (Wnt cell) (compound 25)	Johannes et al. (2015)
SEN461	In vitro and in vivo (Xenopus embryos and mice)	axin (not via TNKS?)	Induction of protein levels and stabilization of axin → Enhancement of axin-GSK3β complex formation → Inhibition of Wnt Signaling	IC <sub>50</sub> : 18 μM (TNKS1); IC <sub>50</sub> : 2.9 μM (TNKS2); IC <sub>50</sub> : 0.2–1.9 μM (Ca cell assays)	De Robertis et al. (2013) De Robertis et al. (2014)
Tigecycline (monotherapy or + paclitaxel)	In vitro and in vivo (mice)	axin1	Induction of protein levels and stabilization of axin1 → Enhancement of axin-GSK3β complex formation → Inhibition of Wnt Signaling	n/a	Li et al. (2015)
SKL2001	In vitro	axin	Disruption of the axin/β-catenin interaction → Activation of Wnt signaling	n/a	Gwak et al. (2012)
<i>3. Interventions Targeting CK1</i>					
Pyruvium	In vitro and in vivo (Xenopus embryo and mice)	CK1	Induction of CK1α → Enhancement of axin-GSK3β complex formation → Inhibition of Wnt Signaling	n/a	Saraswati et al. (2010), Thorne et al. (2010)
CKI-7	In vitro (Xenopus embryos and <i>C. elegans</i> )	CK1/DVL	Inhibition of CK and DVL → Inhibition of Wnt Signaling	n/a	Peters et al. (1999)
IC261	In vitro	CK1	Targeting of CK1ε → Inhibition of Wnt Signaling	IC <sub>50</sub> : 0.5–86 μM (depending on cell line treated)	Kim et al. (2010b)
SB203580	In vitro	CK1	Targeting of CK1δ/ε → Inhibition of Wnt Signaling	?	Laco et al. (2015)
SR-3029	In vitro and in vivo (mice)	CK1	Targeting of CK1δ → Inhibition of Wnt Signaling	n/a	Rosenberg et al. (2015)
<i>4. Interventions Activating GSK3β</i>					
Curcumin	In vitro	GSK3β	Akt inhibits p-GSK3β → activation of GSK3β → Enhancement of destruction complex → Inhibition of Wnt Signaling	IC <sub>50</sub> : 25 mM (after 24 hours treatment) and 18.4 mM (after 48 hours treatment)	Choi et al. (2010)
9-Hydroxycanthin-6-one	In vitro and in vivo (zebrafish embryos)	GSK3β	Induction of GSK3β → Inhibition of Wnt Signaling (without involvement of CK)	IC <sub>50</sub> : 36.7 - > 40 μM (depending on cell line)	Ohishi et al. (2015)
All-trans retinoic acid (ATRA)	In vitro	GSK3β	Induction of GSK3β → Inhibition of Wnt Signaling	n/a	Zhu et al. (2015)
S-ibuprofen	In vitro	GSK3β	Induction of p-GSK3β → Inhibition of Wnt Signaling (NF-κB also involved)	n/a	Greenspan et al. (2011)
<i>5. Interventions Inhibiting GSK3β</i>					
Lithium	In vitro	GSK3β (and β-catenin)	Inhibition of GSK3β (+ up-regulation of β-catenin) → Activation of Wnt Signaling	n/a	Wexler et al. (2008)
Valproic acid	In vitro	GSK3β	Inhibition of GSK3β → Activation of Wnt Signaling	n/a	Chen et al. (1999)
6-bromindirubin-3'-oxime (BIO)	In vitro and in vivo (mice)	GSK3β	Competitive inhibition of GSK3β → Activation of Wnt Signaling	n/a	Kohler et al. (2014)

(continued)

TABLE 4—Continued

Intervention	Use	Proposed target	Effect	Affinity	Reference
	In vitro (incl. stem cells)	GSK3 $\beta$	Inhibition of GSK3 $\beta$ $\rightarrow$ Activation of Wnt Signaling	n/a	Sato et al. (2004), Wen et al. (2010), Tseng et al. (2006)
IBU-PO	In vitro	GSK3 $\beta$	Induction of p-GSK3 $\beta$ $\rightarrow$ Inhibition of GSK3 $\beta$ $\rightarrow$ Activation of Wnt Signaling	n/a	Farias et al. (2005)
Curcumin	In vitro	GSK3 $\beta$ (and axin)	Inhibition of GSK3 $\beta$ (and axin) $\rightarrow$ Activation of Wnt Signaling	n/a	Ahn et al. (2010)
SB-216763 and SB-415286	In vitro	GSK3 $\alpha/\beta$	Inhibition of GSK3 $\beta$ $\rightarrow$ Activation of Wnt Signaling	ID <sub>50</sub> : 34 nM (SB- 216763); IC <sub>50</sub> : 78 nM (SB-415286)	Coghlan et al. (2000)
Kenpaullone	In vitro (stem and progenitor cells)	GSK3	Inhibition of GSK3 $\rightarrow$ Activation of Wnt Signaling	n/a	Lange et al. (2011)
CHIR 99021	In vitro (incl. embryonic stem cells)	GSK3 $\alpha/\beta$	Inhibition of GSK3 $\beta$ $\rightarrow$ Activation of Wnt Signaling	IC <sub>50</sub> : 5 nM (GSK3 $\beta$ )	Bennett et al. (2002), Ye et al. (2012), Hou et al. (2013) Naujok et al. (2014)
CG0009	In vitro	GSK3 $\beta$	Induction of Ser9 p-GSK3 $\beta$ and inhibition of Tyr215 p-GSK3 $\beta$ $\rightarrow$ Inhibition of GSK3 $\beta$ $\rightarrow$ Activation of Wnt Signaling	IC <sub>50</sub> : 0.49–>100 $\mu$ M (depending on cell type in the assay)	Kim et al. (2013)
L803-mts	In vitro and in vivo (mice)	GSK3	Inhibition of GSK3 $\rightarrow$ Activation of Wnt Signaling	IC <sub>50</sub> : 40 $\mu$ M	Plotkin et al. (2003), Kaidanovich- Beilin et al. (2004)

misfolded FZD<sub>4</sub> protein observed in familiar exudative vitreoretinopathy, restoring the impaired transport of this mutated receptor to the plasma membrane (Generoso et al., 2015).

Lee et al. (2015) identified five different novel and very potent molecules (1094-0205, 2124-0331, 3235-0367 and the two most potent ones, NSC36784 and NSC654259) that bind to the CRD of FZD<sub>8</sub> and can regulate WNT signaling. All five compounds could inhibit the WNT3A-mediated signaling in low micromolar concentrations. Interestingly, all five compounds showed LRP6-inhibitory activity, although the authors could not exclude that the FZD CRD inhibitors might be able to target other FZD receptor's CRDs (other than FZD<sub>8</sub>).

Niclosamide is an anthelmintic drug, used against tapeworm infestations; nevertheless, its antitumor properties have been demonstrated in a variety of cancers. It has been shown to act via WNT signaling (Li et al., 2014c), albeit not exclusively, and the targeting appears to be dependent on the context: it caused internalization of FZD<sub>1</sub> in an osteosarcoma cell line (U2OS) (Chen et al., 2009b) and attenuation of DVL2 in a colorectal cell line (Osada et al., 2011). Conversely, studies on prostate and breast cancer cell lines suggested that LRP6 suppression is a direct effect of treatment with niclosamide (Lu et al., 2011b). These findings show it is important to make the choice of niclosamide treatment heavily dependent on the type of cancer to be treated (Osada et al., 2011). Interestingly, curcumin (which will be discussed in more depth later) was found to suppress the expression of FZD<sub>1</sub> eightfold in MDA-1986 squamous cell carcinoma cell line, a finding that was paradoxical, because curcumin was

always regarded to work more downstream (Yan et al., 2005). In this context, Zheng et al. (2017) very recently showed that curcumin might have additional inhibitory effects on WNT3A and LRP6. Previously, it was shown that WNT10B, FZD<sub>2</sub>, and LRP5 might be activated, leading to amplification of the cascade (Ahn et al., 2010). These findings strongly suggest that curcumin could also exhibit its effects more upstream in the pathway, affecting the binding of the WNT ligand to its receptor (FZD) and to its coreceptor (LRP). In addition, there is evidence stressing potential deleterious effects of curcumin (even promoting cancer in some cases), thus more research is urgently needed to ascertain the toxicologic profile of the compound and its benefit-to-risk ratio (Burgos-Moron et al., 2010).

Sclerostin is an endogenous antagonist of the WNT cascade and is expressed practically exclusively by osteocytes. Sclerostin binds LRP5/6 (Li et al., 2005) and disrupts the LRP5/6-FZD complex formation. In fact, it binds more strongly to LRP6, whereas antisclerostin antibodies can reverse the sclerostin-mediated LRP6 inhibition (van Dinther et al., 2013) and prevent noxious effects on the bones (Tian et al., 2011).

Because phosphorylation of LRP6 is an essential element of WNT-mediated activation, interaction with the LRP5/6 coreceptor is another major point where pharmacological interventions could act on to influence the signaling cascade. Salinomycin is a potassium ionophore compound that was shown to possess WNT antagonistic activity. Salinomycin was found to inhibit the WNT1/FZD<sub>5</sub>/LRP6 and WNT3/FZD<sub>5</sub>/LRP6 complexes, to robustly reduce LRP6 protein levels, and to induce degradation of LRP6 in chronic lymphocytic

TABLE 5  
Interventions in  $\beta$ -catenin and gene transcription

Intervention	Use	Proposed target	Effect	Affinity	Reference
<i>1. Targeting of the TCF/LEF Transcription Factors</i>					
iCRT3, iCRT5 and iCRT14	In vitro	$\beta$ -catenin-TCF/LEF complex	Interference with the $\beta$ -catenin/TCF interaction $\rightarrow$ Inhibition of WNT Signaling	IC <sub>50</sub> : 8.2 nM (iCRT3); IC <sub>50</sub> : 18.7 nM (iCRT5); IC <sub>50</sub> : 40.3 nM (iCRT14) - in STF16 luciferase assay	Gonsalves et al. (2011), Griffiths et al. (2015)
Thiazole and oxazole	In vitro	$\beta$ -catenin-TCF/LEF complex	Interference with the $\beta$ -catenin/TCF4 interaction $\rightarrow$ Inhibition of WNT Signaling	n/a	Narayanan et al. (2012)
15-oxospiramylactone (NC043)	In vitro	$\beta$ -catenin-TCF/LEF complex	Interference with the $\beta$ -catenin/TCF4 interaction $\rightarrow$ Inhibition of WNT Signaling	IC <sub>50</sub> : 0.9–6.9 $\mu$ M (depending on the cell type)	Wang et al. (2011b)
Henryin	In vitro	$\beta$ -catenin-TCF/LEF complex	Interference with the $\beta$ -catenin/TCF4 interaction $\rightarrow$ Inhibition of WNT Signaling	IC <sub>50</sub> : 0.6 $\pm$ 0.02 $\mu$ M (ST-luciferase assay)	Li et al. (2013c)
11 $\alpha$ ,12 $\alpha$ -epoxy leukamenin E (EPL E)	In vitro	$\beta$ -catenin-TCF/LEF complex	Interference with the $\beta$ -catenin/TCF4 interaction $\rightarrow$ Inhibition of WNT Signaling	n/a	Ye et al. (2015)
LF3	In silico, in vitro and in vivo (mice)	$\beta$ -catenin-TCF/LEF complex	Interference with the $\beta$ -catenin/TCF4 interaction $\rightarrow$ Inhibition of WNT Signaling	IC <sub>50</sub> : 2.4–4.0 mM (HeLa cells); IC <sub>50</sub> : 22.2 $\pm$ 4.9 mM (HEK293 TOPFlash assay)	Fang et al. (2016)
BC-21	In silico and in vitro	$\beta$ -catenin-TCF/LEF complex	Interference with the $\beta$ -catenin/TCF4 interaction $\rightarrow$ Inhibition of WNT Signaling	IC <sub>50</sub> : 5 $\mu$ M ( $\beta$ -catenin-TCF4 inhibition assay)	Tian et al. (2012)
BC-23 (monotherapy or with radiation therapy)	In silico and in vitro	$\beta$ -catenin-TCF/LEF complex	Interference with the $\beta$ -catenin/TCF4 interaction $\rightarrow$ Inhibition of WNT Signaling	IC <sub>50</sub> : 5 $\mu$ M ( $\beta$ -catenin-TCF4 inhibition assay)	Zhang et al. (2016a)
PNU-74654	In vitro	$\beta$ -catenin-TCF/LEF complex	Interference with the $\beta$ -catenin/TCF interaction $\rightarrow$ Inhibition of WNT Signaling	IC <sub>50</sub> : 117.2–5026.0 $\mu$ M (depending on time and cell line used in the assay)	Leal et al. (2015)
PKF115-584	In vitro and in vivo (Xenopus embryos) In vitro and in vivo (mice)	$\beta$ -catenin-TCF/LEF complex	Interference with the $\beta$ -catenin/TCF4 interaction, TCF binding with DNA, $\beta$ -catenin/APC complex formation and antagonism of exogenous $\beta$ -catenin $\rightarrow$ Inhibition of WNT Signaling	IC <sub>50</sub> : 3.2 $\mu$ M ( $\beta$ -catenin - TCF4 inhibition assay) IC <sub>50</sub> : 0.31–2.05 $\mu$ M (sphere forming assay, range depending on cell type)	Lepourcelet et al. (2004), Hallett et al. (2012)
CGP049090	In vitro and in vivo (Xenopus embryos)	$\beta$ -catenin-TCF/LEF complex	Interference with the $\beta$ -catenin/TCF4 interaction, TCF binding with DNA, $\beta$ -catenin/APC complex formation and antagonism of exogenous $\beta$ -catenin $\rightarrow$ Inhibition of WNT Signaling	IC <sub>50</sub> : 8.7 $\mu$ M ( $\beta$ -catenin-TCF4 inhibition assay)	Lepourcelet et al. (2004)
PKF222-815	In vitro and in vivo (Xenopus embryos)	$\beta$ -catenin-TCF/LEF complex	Interference with the $\beta$ -catenin/TCF4 interaction and antagonism of exogenous $\beta$ -catenin $\rightarrow$ Inhibition of WNT Signaling	IC <sub>50</sub> : 4.1 $\mu$ M ( $\beta$ -catenin - TCF4 inhibition assay)	Lepourcelet et al. (2004)
PKF118–310	In vitro and in vivo (mice)	$\beta$ -catenin-TCF/LEF complex	Interference with the $\beta$ -catenin/TCF4 interaction and antagonism of exogenous $\beta$ -catenin $\rightarrow$ Inhibition of WNT Signaling	IC <sub>50</sub> : 0.8 $\mu$ M ( $\beta$ -catenin-TCF4 inhibition assay)	Lepourcelet et al. (2004)
	In vitro and in vivo (mice)			IC <sub>50</sub> : 0.54–1.54 $\mu$ M (sphere forming assay, range depending on cell type)	Hallett et al. (2012)

(continued)

TABLE 5 —Continued

Intervention	Use	Proposed target	Effect	Affinity	Reference
Daxx	In vitro	$\beta$ -catenin-TCF/LEF complex	Binding to TCF4 $\rightarrow$ Potentiation of $\beta$ -catenin/TCF4-mediated transcriptional activation $\rightarrow$ Activation of WNT Signaling	n/a	Huang and Shih (2009)
Quercetin	In vitro	$\beta$ -catenin-TCF/LEF complex	Interference with the $\beta$ -catenin/TCF4 interaction $\rightarrow$ Inhibition of WNT Signaling	n/a	Park et al. (2005)
Isoquercitrin	In vitro and in vivo (Xenopus embryos)	$\beta$ -catenin-TCF/LEF complex	Interference with the $\beta$ -catenin/TCF4 interaction $\rightarrow$ Inhibition of WNT Signaling	n/a	Amado et al. (2014)
6,7-dihydroxycoumarin (Aesculetin)	In vitro and in vivo (Xenopus embryos and mice)	$\beta$ -catenin-TCF/LEF complex	Interference with the $\beta$ -catenin/TCF4 interaction $\rightarrow$ Inhibition of WNT Signaling	n/a	Lee et al. (2013)
Curcumin	In vitro	$\beta$ -catenin-TCF/LEF complex	Interference with the $\beta$ -catenin/TCF4 interaction $\rightarrow$ Inhibition of WNT Signaling	n/a	Jaiswal et al., 2002
	In vitro	$\beta$ -catenin-TCF/LEF complex	Decrease of TCF4, CBP and p300 protein levels $\rightarrow$ Interference with the $\beta$ -catenin/TCF4 interaction $\rightarrow$ Inhibition of WNT Signaling	n/a	Teiten et al. (2011)
Aspirin	In vitro	$\beta$ -catenin-TCF gene transcription	Suppression of gene transcription of $\beta$ -catenin/TCF4 $\rightarrow$ Inhibition of WNT Signaling	n/a	Dihlmann et al. (2001)
Indomethacin	In vitro	$\beta$ -catenin-TCF gene transcription	Suppression of gene transcription of $\beta$ -catenin/TCF4 $\rightarrow$ Inhibition of WNT Signaling	n/a	Dihlmann et al. (2001)
Celecoxib and 2,5-dimethyl-celecoxib	In vivo (mice)	$\beta$ -catenin and TCF4 expression	Suppression of $\beta$ -catenin and TCF4 expression (not clear if the complex formation itself is affected) $\rightarrow$ Inhibition of TCF-mediated transcription $\rightarrow$ Inhibition of WNT Signaling	n/a	Fan et al. (2011), Egashira et al. (2017)
Sulindac	In vitro and in vivo (mice)	TCF gene transcription	Suppression of gene transcription of TCF $\rightarrow$ Inhibition of WNT Signaling	IC <sub>50</sub> : 1.8–33.9 $\mu$ M (for COX-1/2 and cGMP)	Tinsley et al. (2010)
	In vitro	$\beta$ -catenin and TCF4 expression	Suppression of gene transcription of TCF and $\beta$ -catenin synthesis $\rightarrow$ Inhibition of WNT Signaling	IC <sub>50</sub> : 75–83 $\mu$ M (following 72 hour treatment of various human cancer cell lines)	Li et al. (2013)
ICAT	In vitro and in vivo (Xenopus embryos)	$\beta$ -catenin-TCF/LEF complex	Interference with the $\beta$ -catenin/TCF4 interaction $\rightarrow$ Inhibition of WNT Signaling	n/a	Tago et al. (2000)
	In vitro	$\beta$ -catenin-TCF/LEF complex	Interference with the $\beta$ -catenin/TCF/LEF interaction $\rightarrow$ Inhibition of WNT Signaling	n/a	Daniels and Weis (2002)
			<i>2. Targeting of CBP</i>		
ICG-001 (PRI-724)	In vitro, in vivo (mice) and CT	CBP	Disruption of the $\beta$ -catenin/CBP interaction $\rightarrow$ Inhibition of WNT Signaling	IC <sub>50</sub> : 3 $\mu$ M (TOPFlash assay)	Emami et al. (2004), Henderson et al. (2010), El-Khoueiry et al. (2013), Sasaki et al. (2013)
PMED-1	In silico and in vitro	CBP	Disruption of the $\beta$ -catenin/CBP interaction $\rightarrow$ Inhibition of WNT Signaling	IC <sub>50</sub> : 4.87–32 $\mu$ M (depending on cell type in the TOPFlash assay)	Delgado et al. (2014)

(continued)

TABLE 5 —Continued

Intervention	Use	Proposed target	Effect	Affinity	Reference
Curcumin	In vitro	CBP	Decrease of TCF4, CBP and p300 protein levels → Interference with the $\beta$ -catenin/TCF4 interaction → Inhibition of WNT Signaling	n/a	Teiten et al. (2011)
			<i>3. Targeting of p300</i>		
ICAT	In vitro	p300	Inhibition of the $\beta$ -catenin/Lef1/p300 interaction → Inhibition of WNT Signaling	n/a	Daniels and Weis (2002)
ICAT-61 (helical domain of ICAT)	In vitro	p300	Disruption of the $\beta$ -catenin/p300 interaction (but no interference with the binding of TCF/LEF) → Inhibition of WNT Signaling	n/a	Daniels and Weis (2002)
Windorphen (WD)	In vitro and in vivo (Zebrafish embryos)	p300	Disruption of the $\beta$ -catenin/p300 interaction → Inhibition of WNT Signaling	IC <sub>50</sub> : 4.2 $\mu$ M (p300); IC <sub>50</sub> : 1.5 $\mu$ M (WNT3a-inducible TOPFlash assay)	Hao et al. (2013)
IQ1	In vitro and in vivo (mice)	p300	Disruption of the $\beta$ -catenin/p300 interaction → Amplification of the $\beta$ -catenin/TCF4 interaction → Activation of WNT Signaling (paradox?)	n/a	Sasaki and Kahn (2014)
Bisdemethoxycurcumin (BDMC) and demethoxycurcumin (DMC)	In vitro	p300	Downregulation of p300 (without effects on TCF4 or $\beta$ -catenin levels) → Inhibition of WNT Signaling	n/a	Ryu et al. (2008)
Curcumin	In vitro	p300	Decrease of TCF4, CBP and p300 protein levels → Interference with the $\beta$ -catenin/TCF4 interaction → Inhibition of WNT Signaling	n/a	Teiten et al. (2011)
			<i>4. Targeting of BCL9</i>		
Carnosic acid (CA)	In silico and in vitro	$\beta$ -catenin/BCL9 complex	Interference with $\beta$ -catenin/BCL9 interaction and degradation of $\beta$ -catenin → Inhibition of WNT Signaling	n/a	de la Roche et al. (2012)
	In silico and in vitro	$\beta$ -catenin/BCL9 complex	Interference with $\beta$ -catenin/BCL9 interaction → Inhibition of WNT Signaling	IC <sub>50</sub> : 8.7 $\pm$ 1.5–20 $\pm$ 0.18 $\mu$ M (depending on cell line)	Hoggard et al. (2015)
LATS Large Tumor Suppressor (LATS) 2	In vitro and in vivo (mice)	$\beta$ -catenin/BCL9 complex	Interference with $\beta$ -catenin/BCL9 interaction → Inhibition of WNT Signaling	n/a	Li et al. (2013)
Compound 11	In silico and in vitro	$\beta$ -catenin/BCL9 complex	Interference with $\beta$ -catenin/BCL9 interaction → Inhibition of WNT Signaling	IC <sub>50</sub> : 22 $\pm$ 4.0–26 $\pm$ 6.6 $\mu$ M (depending on cell line)	Wisniewski et al. (2016)
SAH-BCL9	In vitro and in vivo (mice)	$\beta$ -catenin/BCL9 complex	Interference with $\beta$ -catenin/BCL9 interaction → Inhibition of WNT Signaling	IC <sub>50</sub> : 135–810 nM (depending on assay)	Takada et al. (2012)

leukemia cells (Lu et al., 2011a). Similar results were demonstrated by Lu and Li (2014), which also implicated mTORC1 and a activation of GSK3 $\beta$  in an LRP6-mediated cross-talking mechanism, leading to antitumor effects in prostate and breast cancer.

Leptin has been shown to possess WNT signaling-mediating capabilities. This weight-regulating

hormone could induce the phosphorylation of LRP6 in *Leptin* KO mice (which show an attenuated LRP6 expression), whereas DKK1 had the opposite effect. Furthermore, leptin was shown to affect GSK3 $\beta$  levels as well as axin2 expression, implying that it possesses an activator role in the signaling (Benzler et al., 2013).

Silibinin, is a naturally occurring compound that exhibits *in vitro* and *in vivo* antitumor effects. Silibinin attenuated the endogenous LRP6 expression at the transcription level and blocks its phosphorylation in a variety of prostate and breast cancer cell lines (Lu et al., 2012). Some research groups have provided evidence for the potential of anti-LRP6 antibodies as blockers (Ettenberg et al., 2010; Gong et al., 2010) or inducers (Gong et al., 2010) of WNT binding via LRP6-mediated mechanisms. Lastly, a 28-amino acid peptide mimicking the LRP5/6 domain was described to interfere with the LRP5/6 and N-cadherin complex in osteoblasts. The peptide led to an activation of WNT signaling and its effects were dependent on N-cadherin (Hay et al., 2012).

The utilization of endogenous mediators of the WNT signaling pathway is also another means of interfering with the cascade. DKK1 is a natural inhibitor of the cascade (Mao et al., 2001), whereas sFRP isoforms demonstrate variable activities (see *section IV.2*) (Shi et al., 2007). DKK1 and DKK2 bind LRP6 at domains different to the ones required for the WNT/FZD complex formation (Mao et al., 2001). Neutralizing the effects of DKK1 by a polyclonal antibody was shown to inhibit tumor cell growth and cell invasion *in vitro*; the treatment was found to be relative safe (Sato et al., 2010b). A humanized monoclonal antibody against DKK1 called BHQ880 had beneficial effects in a mouse myeloma model, where it exhibited not only antitumor effects but also promoted bone formation (Fulciniti et al., 2009). Thus far, BHQ880 has been investigated in three clinical trials in myeloma patients (<https://clinicaltrials.gov/ct2/results?term=BHQ880+&Search=Search>). The trials have been completed; however, the drug is not yet available. It was reported that patients have been tolerating the treatment and that the antibody has caused meaningful improvements in the quality of their lives (Iyer et al., 2014).

Furthermore, active immunization with a DKK1 vaccine showed impressive results in boosting of T-cell production and protecting mice from myeloma or treating mice that were suffering from a myeloma tumor. The beneficial effects of the active vaccine could be further improved by the addition of B7H1-blocking or OX40-agonist mAbs.

sFRP manipulations can also regulate WNT signaling. Iminooxothiazolidines (compound 1–34) are reported to be potential inhibitors of SFRP1 and to promote osteogenesis (Shi et al., 2009). Following a detailed screening of approximately 440,000 compounds, Bodine et al. (2009) also described an inhibitor of sFRP1 called WAY-316606 that led to increased bone formation; the same group proposed the use of several other similar inhibitory compounds (Moore et al., 2010).

### C. Intracellular Compartment

*1. Interventions Targeting Dishevelled.* The first intracellular step following the successful binding of

WNT and FZD is the activation of the DVL proteins, which acts downstream and phosphorylates the intracellular domain of LRP5/6. The PDZ domain of DVL is of paramount importance for the transduction of the signal, because it was shown to interact directly with seventh transmembrane helix of FZD (Wong et al., 2003). Compound 3289–8625 could suppress *in vitro* luciferase activity by targeting the PDZ domain of DVL when the system is activated by WNT3A. Compound 3289–8625 can readily penetrate membranes (*in vitro* and of *Xenopus*) and showed a suppressive effect on  $\beta$ -catenin levels in prostate cancer cells (PC-3), which led to an attenuation of the cells' proliferation (Grandy et al., 2009). In addition, the organic compound NSC668036 was identified within the National Cancer Institute's small-molecular library to functionally interact with PDZ. NSC668036 was proven to counteract WNT signaling with the additional advantages of high solubility, stability, and simplicity of its molecule, making it a great candidate to produce analogs from with improved properties (Shan et al., 2005). Shan et al. (2012) also developed several compounds, with the most potent one being J01-017a; this compound demonstrated improved binding affinity for the PDZ. Additionally, another organic compound known by the short name FJ9 was proposed as a PDZ inhibitor and an antitumor molecule (Fujii et al., 2007). However, it was shown to be toxic at concentrations normally employed to inhibit WNT signaling (Zhang et al., 2009b), limiting its use. CXXC-type zinc finger protein 5 (CXXC5) was shown to interact with the PDZ domain of DVL and thus operate as a negative feedback mechanism of the WNT signaling pathway. Small inhibitors that can block the interaction between DVL and CXXC5 led to an activation of WNT signaling; Kim et al. propose three inhibitors, the peptide PolyR-DBM (Kim et al., 2015), KY-02372, and KY-02061 (Kim et al., 2016), which could be regarded as candidates of orally available antiosteoporosis therapy. Lastly, a brief communication by Zhang et al. (2009b) reported a PDZ peptide called pen-N3.

*2. Interventions Targeting Axin.* The scaffolding protein axin is, as previously shown, the rate-limiting factor of the destruction complex and it is regulated by tankyrases (TNK). TNKs are a class of nuclear proteins that belong to the big family of Poly (ADP-ribose) polymerases (PARP). TNKs play instrumental roles in the organization of telomere length, the regulation of the spindle assembly checkpoint, as well as the control of glucose uptake in the presence of insulin. Furthermore, it was found that the TNKs may exert regulatory control over WNT signaling by controlling the destruction of  $\beta$ -catenin and appear to confer inhibitory effects on several tumor types by reducing telomere length (Lehtio et al., 2013). Intriguingly, TNK inhibitors are active even in the absence of APC, thus they can be of great interest in APC mutation-related diseases that

are accompanied by hyperactivated WNT signaling pathway (Huang et al., 2009; Waaler et al., 2012). Several groups have attempted to investigate the role of TNKs in the regulation of WNT signaling. Huang et al. (2009) published a seminal paper demonstrating for the first time that a small molecule named XAV939 can have profound effects on WNT signaling. XAV939 induced the activity of the destruction complex and thus led to the phosphorylation of  $\beta$ -catenin, which renders the latter inactive. Axin2 ubiquitination was suppressed and axin1/2 (protein but not mRNA) levels were substantially induced, mediating a robust increase in the axin/GSK3 $\beta$  complex formation that phosphorylates  $\beta$ -catenin leading to its degradation. Interestingly, XAV939 was shown to suppress proliferation and myofibroblast transdifferentiation, while inducing the differentiation of bone marrow-derived mesenchymal stem cells (Wang et al., 2014a). Lastly, the cardiovascular potential of XAV939 (as well as IWRs and IWP) is high, taking reports into account showing important effects of the compound on promotion of cardiogenesis in human embryonic stem cells (Willems et al., 2011) and human induced pluripotent stem cells (Kadari et al., 2015). Moreover, the inhibitors of WNT response (IWRs) are also important tools in this class. Chen et al. (2009a) propose two small compounds named IWR-1 and IWR-2 that promote  $\beta$ -catenin degradation. The authors showed that this is achieved by promoting stabilization of axin, and not via changes in GSK3 $\beta$  or APC,  $\beta$ -catenin/axin-2 binding affinity, or the interaction of axin with other WNT signaling components. Inhibition of the WNT cascade in keloid-derived skin fibroblasts by IWR-1 led to changes in the expression levels of MMPs (MMP-1, MMP-3, and MMP-13) (Zhou et al., 2017), this going hand-in-hand with the aforementioned findings of Wang et al. (2014a) on lung myofibroblasts.

Combined treatment with several agents targeting WNT signaling is gaining more and more interest. XAV939 can have profound effects on tumor resistance, for example in colorectal cancer (Wu et al., 2016b) in combinational regimens with chemotherapy (e.g., alongside 5-fluorouracil and cisplatin). Quite recently, Ono et al. (2014) investigated a triple cocktail therapy with XAV939, ICAT (a compound targeting the  $\beta$ -catenin/p300 complex), and niclosamide (targeting the cascade at several levels) in human uterine leiomyoma cells. The combination exhibited antitumor effects *in vitro* by disrupting proliferation of cancer cells.

E7449, another TNK inhibitor, was shown to be slightly less potent compared with XAV939, but to target other players (such as PARPs) and to work in parallel in conferring antitumor effects (McGonigle et al., 2015). WXL-8, a nitro-substituted derivative of XAV939, has been shown to possess improved antiproliferative activity in hepatic cancer cells, both *in vivo* and *in vitro*, whereas it led to stabilization of both axin isoforms and a

complete removal of  $\beta$ -catenin from within the nucleus (Ma et al., 2015). Similar results have been demonstrated with other novel TNK inhibitors, such as JW67, JW74 (Waaler et al., 2011), and JW55 (Waaler et al., 2012). Nathubhai et al. (2017) showed that when compounds 13 and 14 are compared with XAV939, they exhibit 1) higher potency (approximately 10 $\times$ ) since they can both display strong antiproliferative activity in DLD-1 colon cancer cells at low concentrations (at which XAV939 is inactive) and 2) isoform selectivity that could imply fewer off-target effects. The highly potent WNT inhibitor kinase inhibitor 4 (WIKI4), a new tool in the TNK inhibitory arsenal, was recently proposed as another potential target. WIKI4 regulates axin degradation, actually by inhibiting axin's ubiquitination, in a variety of cell lines and it is positioned as a potential WNT inhibitory candidate for further investigation (James et al., 2012). G007-LK was shown to inhibit WNT signaling and plays a particularly important role in pathologies where the decisive factor is an APC mutation (e.g., in many human cancers). The compound exhibits a 50% attenuation of APC mutation-related WNT signaling (Lau et al., 2013); however, gastrointestinal tract toxicity might be a restricting factor in G007-LK's wider use.

AZ1366 is another TNK inhibitor that produces antitumor effects, in combination with an EGFR inhibitor, in non-small cell lung cancer cells, mediated via effects on axin. The compound induced the stabilization of axin1 and thus the upregulation of  $\beta$ -catenin target genes (c-myc, axin2, survivin), but loss of axin1 rendered AZ1366 ineffective, implying that axin1 is required for the action of the molecule (Scarborough et al., 2017). This finding was challenged by Quackenbush et al. (2016) in a mouse colorectal model. They showed that when the TNK inhibitor was coadministered with irinotecanin, on the one hand axin2 levels were induced and the treatment led to attenuation of tumor growth; however, there was no change in  $\beta$ -catenin or c-myc levels. This finding makes the association between WNT inhibition and the anti-tumor effects exhibited by AZ1366 improbable and it seems that other non-WNT-related TNK-mediated actions might be involved (Quackenbush et al., 2016).

PORCN IWP inhibitors were extensively discussed in section A of this chapter. Nonetheless, IWRs might also be acting via axin2 stabilization (Chen et al., 2009a). Lastly, NVP-TNKS656 (Shultz et al., 2013) and compounds 9 and 25 (Johannes et al., 2015) were described to have favorable pharmacokinetic properties that could be used in further preclinical investigations (the first two for oral and the latter for intravenous administration).

There is also a number of compounds that target axin without belonging to the TNK inhibitor class. The group of Salerno has repeatedly shown that the inhibitor SEN461 can stabilize axin1, leading to suppressed



$\beta$ -catenin activity. The findings of the authors have been translated to an antitumor effect of SEN461 against glioblastoma (De Robertis et al., 2013) and sarcoma (De Robertis et al., 2014). An induction of levels of axin1 (but not axin2) is correlated with the mode of action of tigecycline, which has been shown to confer beneficial effects in cervical cancer when given in combination with paclitaxel in a mouse xenograft model (Li et al., 2015). Nevertheless, an effect of the tigecycline-mediated antitumor activity on other signaling pathways (such as PI3K/Akt) cannot be excluded (Zhong et al., 2016). Lastly, SKL2001 is a compound selected by screening of approximately 270,000 molecules and was shown to possess agonist properties. These are mediated mainly via a disruption of the axin/ $\beta$ -catenin interaction by competing with axin for binding to  $\beta$ -catenin, leading to activation of the WNT cascade. Interestingly, SKL2001 does not affect the phosphorylation status of GSK3 $\beta$  (Gwak et al., 2012).

**3. Interventions Targeting CK1.** Pyrvinium is an anthelmintic drug that was shown to allosterically activate CK1 $\alpha$ . Thorne et al. (2010) identified CK1 $\alpha$  as the main target of pyrvinium, albeit effects were shown toward all CK1 isoforms and also secondarily on pygopus. Saraswati et al. (2010) confirmed the CK1 $\alpha$  involvement of pyrvinium treatment in vitro and showed that the compound can inhibit WNT signaling and confer beneficial effects following ischemia in a mouse MI model. Nevertheless, a high number (approximately 59%) of intracardially injected mice demonstrated sudden death without an etiology for the pyrvinium-mediated cardiac toxicity being evident. Venerando et al. (2013) challenged the CK1 $\alpha$  theory and proposed an alternative mechanism of pyrvinium's actions via an effect on PI3K/Akt, whereas Harada et al. (2012) indicated a STAT3-mediated mechanism. Furthermore, it is possible that other CK1 isoforms (other than the CK1 $\alpha$ ) could be targeted to attenuate the destruction complex or act at other components upstream in the signaling cascade (Del Valle-Perez et al., 2011). CKI-7 blocked CK (as well as DVL but not  $\beta$ -catenin) in *Xenopus* (Peters et al., 1999). Kim et al. (2010b) demonstrated that inhibiting CK1 $\epsilon$  with the specific inhibitory compound IC261 led to a suppression of  $\beta$ -catenin transcription. A study with IC261 and selective inhibitors for CK1 $\delta/\epsilon$  (PF670462) and CK1 $\epsilon$  (PF4800567) implied that they could all inhibit the signaling pathway; however, IC261 might also possess activity elsewhere, such as inhibition of microtubule polymerization leading to halting of mitosis (Cheong et al., 2011). More recently, Laco et al. (2015) showed that the p38 MAPK inhibitor SB203580 might be inhibiting CK1 $\delta/\epsilon$ , which was proposed to play a crucial role in mediating changes in pluripotent stem cells differentiating to cardiomyocytes. SR-3029 (a potent CK1 $\delta/\epsilon$  inhibitor) robustly inhibited CK1 $\delta$  and was very effective against several forms of human breast cancer

(in vivo data) (Rosenberg et al., 2015). CK1 $\gamma$  targeting does not appear to affect the  $\beta$ -catenin destruction complex. Nevertheless, CK1 $\gamma$  is associated with LRP6, thus its pharmacological targeting could lead to suppression of WNT signaling (Davidson et al., 2005).

**4. Interventions Activating GSK3 $\beta$ .** GSK3 $\beta$  plays a key role in many cellular functions and in the mechanisms controlling cell cycle, gene expression, protein translation, apoptosis, etc. Furthermore, GSK3 $\beta$  plays a prominent role in the regulation of several signaling pathways, such as PI3K/mTOR, WNT, MAPK, etc. (Phukan et al., 2010).

Curcumin is a naturally occurring compound, broadly used in Indian cuisine. It has been associated with antioxidant, anti-inflammatory, as well as antitumor activities. Its anticancer effects have been extensively studied in a wide range of human cancer cell lines and it is noteworthy that at the point of the preparation of this review paper, 583 papers on "curcumin in cancer" were identified in PubMed. This compound affects various transcription factors, receptors, kinases, inflammatory cells, microRNAs, and different signaling pathways, including the Wnt signaling pathway. It generally exhibits its anti-WNT signaling pathway effects at the level of transcription, by blockade of STAT, NF- $\kappa$ B, and AP-1-related transcription factors (Kasi et al., 2016). However, Choi et al. (2010) showed that GSK3 $\beta$  might also be implicated. The theory proposed by the authors was that curcumin affects Akt expression, which inhibits phosphorylation of GSK3 $\beta$ , thereby inducing the destruction complex to reduce  $\beta$ -catenin's stabilization and translocation into the nucleus. Nevertheless, this mode of action of curcumin is not very well understood, and more studies are required to reveal its actual effects on GSK3 $\beta$  (Choi et al., 2010). Interestingly, although the orchestrated collaboration of GSK3 $\beta$ , axin, CK1 $\alpha$ , and APC have been shown to be decisive for the formation of the destructive complex, Ohishi et al. (2015) attempted to challenge the theory. They showed that an alkaloid compound called 9-hydroxycanthin-6-one attenuated WNT/ $\beta$ -catenin signaling by activating GSK3 $\beta$ , an effect that is not dependent on CK1 $\alpha$  phosphorylation. All-trans retinoic acid (ATRA) promoted GSK3 $\beta$ , leading to a disruption of  $\beta$ -catenin levels in hepatic cancer stem cells and demonstrates maximum efficacy when coadministered with docetaxel (making tumor cells more sensitive to chemotherapy) (Zhu et al., 2015). Surprisingly, the nonsteroidal anti-inflammatory *S*-ibuprofen induces the phosphorylated form of GSK3 $\beta$ , which in turn suppresses NF- $\kappa$ B and the  $\beta$ -catenin target genes in cancer cells (Greenspan et al., 2011).

**5. Interventions Inhibiting GSK3 $\beta$ .** It was only approximately 20 years ago that Klein and Melton (1996) reported GSK3 $\beta$  to be a target of LiCl, the well-established drug treatment of bipolar disorder. What is more fascinating is that LiCl does not generally inhibits

other kinases (e.g., MAPK, ERK, etc.), although cross-talking mechanisms between GSK3, Akt/mTOR, and HIF-1 $\alpha$  do exist (Mottet et al., 2003). It is remarkable that in many WNT-related assays, LiCl is regarded as a positive control for the activation of WNT/ $\beta$ -catenin signaling. The association between LiCl and the  $\beta$ -catenin cascade is further strengthened by the findings of Wexler et al. (2008). They showed that LiCl can stimulate the WNT cascade and induce proliferation in adult hippocampal progenitor cells, but inhibition of  $\beta$ -catenin by RNAi counteracts the effects of LiCl. Valproic acid, another agent routinely used in modern psychopharmacology, has also been shown to target GSK3 $\beta$  (Chen et al., 1999). Human neuroblastoma SH-SY5Y cells were treated with valproic acid, which led to a robust inhibition of GSK3 $\beta$  and an increase in levels of  $\beta$ -catenin both in cytosol and in the nucleus. A report by Hall et al. (2002) suggested that valproate inhibits GSK3 $\beta$  indirectly via an unknown mechanism in developing neurons, in contrast to LiCl, which has been shown to act directly in developing neurons. Nevertheless, Ryves and Harwood (2001) implied that the ability of valproate (and LiCl) to exhibit inhibitory effects on GSK3 $\beta$  depends largely on the cell type or particular areas of the brain, a finding showing that our understanding of the effects of mood stabilizers on the GSK3 $\beta$  of the mammalian brain are far from complete.

6-Bromindirubin-3'-oxime (BIO) is a specific and competitive inhibitor of GSK3 $\beta$  that has been shown to exert important effects on the differentiation and viability of cells, thus having an important role to play in regenerative medicine strategies. BIO was shown to induce neovascularization both in vitro and in vivo (Kohler et al., 2014). BIO activates the WNT cascade and, when combined with other factors, it improves survival and maintains the undifferentiated status of porcine embryonic germ cells (Wen et al., 2010), as well as of embryonic stem cells (Sato et al., 2004). In addition, the boosting effect it exerts on  $\beta$ -catenin activity led to a robust amplification of the proliferation of both adult and neonatal cardiomyocytes (Tseng et al., 2006), a very interesting finding in the pursuit for novel treatments of heart disease (e.g., myocardial infarction). In contrast, BIO is found to exert rather noxious effects on human mesenchymal stem cells, because it was shown to inhibit the cell cycle and thus reduce viability of human mesenchymal stem cells preparations (both in vitro and in a mouse model) (Krause et al., 2010). A novel bifunctional compound IBU-PO (taking its name from the diptych ibuprofen and octyl-pyridostigmine of which it consists) is also another compound targeting GSK3 $\beta$ . It is a combination of a nonsteroidal anti-inflammatory drug and a cholinesterase inhibitor and has been used in Alzheimer's amyloid-B-peptide (A $\beta$ ) in neuronal in vitro cultures as models of Alzheimer's disease. IBU-PO was shown to inhibit GSK3 $\beta$  by inducing its phosphorylation at Ser<sup>9</sup> and to stabilize

$\beta$ -catenin; the consequent activation of the WNT signaling leads to a restoration of A $\beta$ -mediated neurotoxicity (Farias et al., 2005). Curcumin appears to be an all-around player, acting on several levels of the WNT cascade, and it was described to inhibit GSK3 $\beta$  and axin in 3T3-L1 cells, leading to an inactivation of the destruction complex, translocation of  $\beta$ -catenin to the nucleus and activation of the WNT cascade (Ahn et al., 2010), suppressing eventually adipogenesis. SB-216763 and SB-415286 are two molecules that have been described by Coghlan et al. (2000) to possess anti-GSK3 properties (inhibiting GSK3 $\alpha$  and - $\beta$  with comparable potency). In addition, the authors showed that the selectivity of the two compounds is quite remarkable, because they show minimal activity toward a long list of kinases (including AMPK, CKII, JNK, MAPK, PKA, PKC, etc.). Kenpaullone and SB-216763 were demonstrated to possess the strongest inhibitory effect on GSK3 $\beta$  in human neural progenitors, in contrast with LiCl, valproic acid, and indirubin-3-monoxime (Lange et al., 2011).

CHIR 99021 is a small aminopyrimidine derivative that inhibits both GSK3 isoforms; however, it appears to be quite selective for GSK3 without affecting other kinase types. Inhibition of GSK3 $\beta$  leads to the observed induction of  $\beta$ -catenin stabilization in 3T3-L1 preadipocytes. The activation of WNT signaling is the responsible factor for an inhibition of adipogenesis (Bennett et al., 2002). Similarly, the stabilization of  $\beta$ -catenin (and especially the induction of c-myc expression) following CHIR 99021 treatment, plays a major role in the maintenance of self-renewal of embryonic stem cells (Ye et al., 2012) and the reprogramming of pluripotent stem cells in combination with other factors (Hou et al., 2013). It should be noted that a comparative in vitro study employing mouse embryonic stem cells, showed that CHIR 99021 demonstrated the highest potency and the lowest toxicity in various concentrations tested (Naujok et al., 2014). The novel GSK3 inhibitor CG0009 phosphorylates GSK3 $\beta$  at Ser<sup>9</sup> and dephosphorylates it at Tyr<sup>216</sup> (both actions leading to inhibition of GSK3 $\beta$ ). When several breast cancer cell lines are treated with CG0009, WNT target genes (e.g., c-myc) are upregulated but cyclin D1 levels are paradoxically suppressed, probably due to cancer cell death (mediated via CG0009), and breast cancer cell growth is robustly attenuated (Kim et al., 2013). Lastly, a peptide called L803-mts was suggested as a potent GSK3 $\beta$  inhibitor. In vitro data showed that it is rather specific for GSK3 (Plotkin et al., 2003), whereas in vivo investigation from the same group revealed an interesting GSK3 $\beta$ -mediated antidepressive-like action, which warrants further investigation (Kaidanovich-Beilin et al., 2004).

#### D. $\beta$ -Catenin and Gene Transcription

Once  $\beta$ -catenin translocates into the nucleus, it forms complexes with several TCF/LEF transcription factors,

as well as coactivators (namely p300, CBP, BCL9, Pygo, and others) (Cadigan and Waterman, 2012). Following this crucial step, it drives the transcription of a long list of  $\beta$ -catenin target genes, including *AXIN2*, *CD44*, *CCND1* (cyclin-D1), *MMP2/9*, *MYC*, *VEGF*, and others (Herbst et al., 2014). The fact that the  $\beta$ -catenin transcriptional complex plays a key role in a wide range of pathologies (especially in cancer), explains the strong pursuit of new compound intervening with its action.

*1. Targeting of the T-cell Factor/Lymphoid Enhancer Factor Transcription Factors.* The  $\beta$ -catenin responsive transcription (CRT) is the critical step prior to the activation of the target genes and is very often the culprit for an abnormally functioning WNT signaling. Thus, inhibitors of CRT (iCRT) might pose as interesting tools in the hunt for strategies to inhibit WNT signaling. Following screening of approximately 15,000 compounds, Gonsalves et al. (2011) identified iCRT3, iCRT5, and iCRT14, which interfere with the  $\beta$ -catenin/TCF interaction, inhibit the transcription of WNT target genes, and eradicate cancer cells. Two remarkable findings of the authors were that these three iCRTs: 1) were specifically deleterious to human colon tumor biopsy cultures and colorectal cancer cell lines, both demonstrating an abnormally activated WNT signaling pathway and 2) did not appear to have  $\beta$ -catenin-independent WNT signaling pathway effects. One group has confirmed the WNT-mediated antitumor effect of iCRT3 in acute myeloid leukemia (Griffiths et al., 2015), while others have proposed iCRT5 as a stimulator of T-cell proliferation mediating a beneficial response in dendritic cells without off-target effects (Kafer et al., 2016). In addition, Narayanan et al. (2012) have managed to show as well that (at least some) iCRTs, such as thiazole and oxazole, mediate their effects via  $\beta$ -catenin/TCF4 interaction and not due to effects on DKK. Quite recently, the diterpenoids NC043 (Wang et al., 2011b), henryin (Li et al., 2013c), and EPLE (Ye et al., 2015) were shown to impair the  $\beta$ -catenin/TCF association and consequently possess antitumorigenic properties against colon cancer cells (both in vitro and in vivo studies). LF3 is a robust inhibitor of the aforementioned interaction and it led to strong antitumor effects (Fang et al., 2016). Lastly, the compounds code-named BC-21 (Tian et al., 2012) and BC-23 (Zhang et al., 2016a) were proposed as novel inhibitors of the  $\beta$ -catenin/TCF4 interaction, as shown by studies where they were jointly used with radiation therapy to lung cancer (BC-23) or given as monotherapy to HCT116 colon cancer cells (BC-21). PNU-74654 is another compound that has been shown to interact with the  $\beta$ -catenin and TCF/LEF complex formation. In vitro experiments demonstrate that PNU-74654 suppresses adrenal steroid synthesis and induces tumor cell apoptosis by attenuating  $\beta$ -catenin synthesis and accumulation in the nucleus, as well as reducing gene transcription (Leal et al., 2015). Furthermore, PNU-

74654 exhibited cell type selectivity and it was effective against human adrenocortical cell lines but not against human nonadrenal cell lines or mouse adrenal cell lines. The potent compounds PKF115-584 and CGP049090 have been proposed to possess robust WNT inhibitory effects with minimal toxicity. Lepourcelet et al. (2004) showed that these molecules can block the  $\beta$ -catenin/TCF transcriptional complex and antagonize exogenous  $\beta$ -catenin when the latter is injected in embryos of *Xenopus*, as well as attenuating expression of cyclin D1 and c-myc to cause a halt in the growing of colon cancer cells. As these authors demonstrated, PKF115-584, PKF222-815, and CGP049090 (all of fungal origin) completely inhibited the  $\beta$ -catenin/TCF4 interaction (Lepourcelet et al., 2004), whereas the first two were also found to prevent the binding of TCF proteins to DNA. The selectivity of these compounds is a matter of further investigation, since PKF115-584 and CGP049090 were also found to inhibit the  $\beta$ -catenin/APC complex formation. In addition, PKF115-584 and CGP049090 appeared to be associated with effects on inflammatory components such as tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) and interleukin 1 $\beta$  (IL-1 $\beta$ ), also key players in cancer as well as other pathologic conditions (Landman et al., 2013). In addition, the PKF118-310 compound elicited deleterious effects on breast tumor initiating cells and halted their growth in vivo (Hallett et al., 2012). On the other hand, Huang and Shih (2009) reported the compound Daxx to amplify the  $\beta$ -catenin/TCF interaction, thus producing inducing effects on WNT signaling pathway.

Several naturally occurring compounds have been associated with the regulation of  $\beta$ -catenin/TCF interaction. The polyphenol flavonoid quercetin and its derivative, isoquercitrin, have received ample interest. There is evidence for quercetin-mediated antitumor activity from in vitro studies (Matsukawa et al., 1997), as well as studies involving cancer patients not responding to standard therapies (Ferry et al., 1996). Park et al. (2005) used immunoprecipitation studies and demonstrated that quercetin can interfere with the  $\beta$ -catenin/TCF4 complex formation in the nucleus. This led to blockade of the WNT signaling pathway in SW480 colon cancer cells, as demonstrated by a suppression of c-myc expression. Isoquercitrin was also found to inhibit the signaling at the level of TCF and to possess antitumor activity against three different colon cancer cell lines, with the additional benefit of lower noncancerous cell toxicity in comparison with the parent molecule quercetin (Amado et al., 2014). Aesculetin, which is a derivative of the anticoagulant coumarin, could also disrupt the  $\beta$ -catenin/TCF transcriptional complex formation by binding directly to specific residues of  $\beta$ -catenin (Asn<sup>387</sup>, Gly<sup>307</sup>, Lys<sup>312</sup>, and Lys<sup>345</sup>). Thus, aesculetin robustly suppresses the expression of c-myc and cyclin D1, switching the signaling off and conferring antiproliferative and antitumor effects on three

different cell types of colon cancer (HCT116, HCT15, and DLD1) (Lee et al., 2013). Curcumin is a naturally occurring compound, already mentioned previously (*section XI.C.4*) The main mode by which curcumin confers its inhibitory effect on WNT signaling pathway is by affecting the  $\beta$ -catenin/TCF4 complex formation, and this has been shown in a wide range of cancer cell lines (Jaiswal et al., 2002). Interestingly, apart from TCF4, curcumin was also shown to act at two other levels of the CRT, namely at CBP and p300, leading to suppression of WNT target genes transcription (Teiten et al., 2011) and an inhibition of androgen-dependent prostate cancer cells. What makes curcumin more intriguing as a compound is that it exhibits its effects in part via affecting the WNT signaling/androgen cross-talking. Curcumin inhibits the expression of the androgen receptor (AR) and contributes to the apoptosis of human endometrial carcinoma cells, a phenomenon that appears to be WNT-mediated (since WNT3A can reverse the effect) (Feng et al., 2014). Furthermore, another fascinating point is that according to Teiten et al. (2011), curcumin only has an antiproliferative and antitumor effect against AR-dependent prostate cancers lines, whereas AR-independent ones do not respond to curcumin. In contrast, curcumin is shown to activate the WNT/ $\beta$ -catenin signaling pathway and stimulate neurogenesis in neural stem cells (Tiwari et al., 2016) or reverse the effects of dexamethasone on osteoporosis in a rat model (Chen et al., 2016), which increases the notion for a compound that behaves differently according to the cell type or the environment established around or by the cell types themselves.

Lastly, nonsteroidal anti-inflammatory drugs have been suggested as potentially attractive agents interfering with the transcriptional activity of  $\beta$ -catenin. Aspirin and indomethacin suppress gene transcription of  $\beta$ -catenin/TCF4, without altering the expression of TCF4 itself. The most probable explanation for the observed effects is that aspirin and indomethacin affect the phosphorylation status of  $\beta$ -catenin and TCF4, leading to inactivation of the complex and subsequently to a robust inhibition of target gene transcription (Dihlmann et al., 2001). Celecoxib and its analog 2,5-dimethyl-celecoxib (the latter lacking anti-COX-2 activity) were shown to inhibit TCF7L2 (TCF4) expression in the myocardium in a cardiomyopathy mouse model (Fan et al., 2011) and in human colon cancer (Egashira et al., 2017). Both studies demonstrated that the WNT inhibitory activity of these molecules is not dependent on COX-2 inhibition; however, further investigation is warranted to reveal the precise mode of action in preventing WNT target gene transcription. Lastly, sulindac (a COX-1/2 inhibitor), was also shown to possess antitumor activity by suppressing TCF transcriptional activity (Tinsley et al., 2010; Li et al., 2013b) as well as  $\beta$ -catenin synthesis (Li et al., 2013b), although other investigators have provided data that

sulindac might also be mediating its effects via DVL (Lee et al., 2009).

2. *Targeting of CREB Binding Protein.* ICG-001 (also known as PRI-724), is a small compound that has been extensively used in basic research and has already been tested in Ib and IIa clinical trials. It is a potent specific inhibitor of the  $\beta$ -catenin/CBP interaction, acting by binding to the N terminus of CBP and leading to suppression of growth-related gene expression. Interestingly, ICG-001 kills mouse xenograft models of colon cancer and colon tumor cells but leaves healthy colon cells unaffected (Emami et al., 2004). ICG-001 acts as a WNT pathway antagonist and robustly prevents fibrosis or reverses its development, while improving animal survival in various pathologies (possibly via effects on [myo]fibroblasts) such as idiopathic pulmonary fibrosis (Henderson et al., 2010). Similar beneficial effects of the ICG-001 were demonstrated in drug-resistant acute lymphoblastic leukemia (Gang et al., 2014), urological fibrosis (Zhang et al., 2015a), pancreatic adenocarcinoma (Arensman et al., 2014), multiple myeloma (Grigson et al., 2015), hepatic fibrosis (Osawa et al., 2015), ECM-related lung remodeling (Koopmans et al., 2016), skin fibrosis (Beyer et al., 2013), and quite recently following ischemia in an MI rat model (Sasaki et al., 2013); please refer to *section VIII.A* for details. In addition, reports from a Phase Ia clinical trial are showing that ICG-001 is, toxicologically, safe in an acceptable level in patients with advanced solid tumors (El-Khoueiry et al., 2013). It should be noted here that combinational therapy of ICG-001 with other compounds (e.g., PKF118 discussed above) is feasible and can produce beneficial effects. Nevertheless, Ayadi et al. (2015), showed that it is possible for these compounds (ICG-001 and PKF118) to possess off-target side effects that might not be directly associated with WNT signaling, and thus making the deeper investigation of the field more urgent than ever. Lastly, the team of Delgado et al. (2014) performed a meticulous search for compounds possessing a structural similarity to ICG-001, examining a database of almost 3.5 million compounds. Following filtering, they eventually narrowed their search to one compound, PMED-1. This compound indeed targets the  $\beta$ -catenin/CBP complex (just like ICG-001) and was successful in limiting hepatocellular carcinoma but was not deleterious to healthy human hepatocytes. Furthermore, the shorter half-life and fewer off-target effects compared with the parent molecule can be regarded as major advantages but its hydrophobicity is an important disadvantage for its further studying in vivo (Delgado et al., 2014).

3. *Targeting of p300.* A peptide compound called ICAT was first shown to associate with  $\beta$ -catenin in both cytoplasm and nucleus and to disrupt the formation of  $\beta$ -catenin/TCF4 complex in vitro and in vivo (Tago et al., 2000). Daniels and Weis (2002) took it one step further, showing that the full-length peptide

(81 amino acids long) inhibited the  $\beta$ -catenin/LEF1/p300 interaction, whereas its helical domain (ICAT-61) can itself disrupt the binding with p300 but not interfere with the binding of TCF/LEF, proving that ICAT actually possesses two regions leading to antagonism of the pathway. ICAT has been tested as a WNT signaling negative regulator in various studies, encompassing glioma tumors (Zhang et al., 2015b), osteogenesis by stem cells (Kim et al., 2008), and the development of T-cells (Pongracz et al., 2006). Quite recently, a molecule wittily named windorphen (WD) was described (Hao et al., 2013). Studies using zebrafish embryos showed that WD acts downstream of the destruction complex and the level of  $\beta$ -catenin translocation into the nucleus, leading to profound structural effects on the zebrafish embryo. Furthermore, it appears that WD is selective in targeting the interaction of  $\beta$ -catenin with p300, although it has no effect whatsoever in the  $\beta$ -catenin/CBP association (Hao et al., 2013), exactly the opposite effect demonstrated by ICG-001 (Emami et al., 2004). These interesting WNT inhibitory results were extrapolated to cancer. The authors demonstrated with *in vitro* studies (using cancer cells) that WD's inhibitory effects on WNT signaling lead to the dramatic apoptosis of human colon adenocarcinoma SW480 and RKO cells, as well as of prostate cancer DU145/PC3 cells (which all show an exaggerated WNT signaling activation), but not of human lung cancer H460 cells (which are not associated with WNT signaling activation abnormalities) (Hao et al., 2013). IQ1 is a molecule that is suggested to mediate the  $\beta$ -catenin/p300 coactivator interaction, during lung morphogenesis in mice. Interestingly, Sasaki and Kahn (2014) demonstrated that pharmacological inhibition of the  $\beta$ -catenin/p300 interaction by IQ1 actually amplifies the  $\beta$ -catenin/TCF interaction, thus inducing the signaling pathway in the lungs of mouse embryos, leading to proximalization of the lung epithelium. It is interesting to add here that although curcumin is regarded chiefly to act at the  $\beta$ -catenin/TCF level (see above), certain of its derivatives, namely bisdemethoxycurcumin and demethoxycurcumin, have been shown to possess p300-mediating activity instead. Ryu et al. (2008) provided evidence for the involvement of bisdemethoxycurcumin and demethoxycurcumin in the downregulation of p300 without any effect on TCF4 or in the levels of  $\beta$ -catenin, demonstrating for the first time that the class of curcumin-related compounds might also act at this level.

**4. Targeting of BCL9.** Antagonism of the  $\beta$ -catenin/BCL9 protein-protein interaction (PPI) complex formation is a very attractive strategy for inhibiting the WNT signaling pathway, for several reasons: 1) BCL9 mediates  $\beta$ -catenin gene transcription of several pathologic conditions, 2) the aforementioned complex is robustly expressed in tumors but not in healthy cells, and 3) eliminating BCL9/ $\beta$ -catenin interaction does not appear to have off-target effects (Takada et al., 2012). de la

Roche et al. (2012) showed that the  $\beta$ -catenin molecule possesses a weak point, which can be exploited by carnosic acid to degrade  $\beta$ -catenin in an  $\alpha$ -helix H1-dependent fashion. Carnosic acid was selected after screening a library of a total of more than 45,000 compounds and was found to disrupt binding of  $\beta$ -catenin to BCL9 and lead to degradation of free (unphosphorylated)  $\beta$ -catenin, thus blocking the WNT signaling pathway. Mammalian LATS large tumor suppressor (LATS) 2, is a kinase that plays major roles in the Hippo/YAP signaling pathway (Hoa et al., 2016), mediating especially functions relevant to cell proliferation and oncogenesis. LATS2 is downregulated in various colorectal cancers but studies with immunoprecipitation showed that it directly binds to  $\beta$ -catenin and disrupts the complexing of the latter with BCL9. Thus, compounds that could target LATS2 could pose as attractive tools for the blockade of WNT signaling and the halting of tumors such as colorectal cancer (Li et al., 2013a). Wisniewski et al. (2016) proposed several small compound inhibitors, attached to piperazine scaffolds (which can form salt bridge interactions with  $\beta$ -catenin) that show  $\beta$ -catenin/BCL9 disrupting capabilities. The most promising compound was found to be compound 11, as it exhibits a surprising selectivity and is also more hydrophilic than the other compounds, while being very effective in halting growth of colorectal cell lines (such as SW480 and HCT116). In the same context, Hoggard et al. (2015) designed several selective compounds attached to a scaffold mimicking the binding of the side chains of an  $\alpha$ -helix. The orchestrated action of the inhibitors along with the scaffold lead to selective and robust  $\beta$ -catenin/BCL9 inhibition, as demonstrated by studies using HEK293, SW480 (colorectal cancer), and MDA-MB-231 (breast cancer) cell lines. Lastly, Takada et al. (2012) designed a stabilized  $\alpha$ -helix of BCL9 called SAH-BCL9, which is shown to selectively disrupt the  $\beta$ -catenin/BCL9 interaction, inhibit WNT target gene transcription, and possess antitumor activities, as well as effects on angiogenesis and metastasis, without toxic off-target effects, as observed from *in vitro* and *in vivo* mouse studies.

### *E. Conclusions and Future Directions*

In the last decade, there has been a remarkable growth in the number of compounds targeting the Wnt signaling pathway. Based on the mutations identified in different components of the WNT/ $\beta$ -catenin pathway in multiple cancers, the majority of these compounds is designed to aim at this branch of the WNT signaling cascade. In cardiovascular conditions, however, alterations in the regulation of the pathway rather than mutations in its components are likely to contribute to the disease process. Therefore, for cardiovascular conditions the preferential sites of intervention in the pathway would be more upstream, at the level of WNT proteins or their interaction with the receptor complex.

Fortunately, the number of compounds allowing upstream intervention is increasing and several of them have been tested, particularly in models of MI. As discussed in detail in *section VIII*, interventions in PORCN (WNT-974 and GNF-6231) or at the level of the receptor complex (UM206) showed beneficial effects on infarct healing. Moreover, also interventions aiming at the  $\beta$ -catenin signaling branch (BIO, ICG-001, and XAV939) improved the wound healing following MI, suggesting an involvement of  $\beta$ -catenin-mediated signaling; however, this does not exclude the involvement of non- $\beta$ -catenin-mediated signaling in infarct healing. Unfortunately, pharmacological interventions have been only sparsely tested in other cardiovascular conditions, so it is recommended to perform drug interventions at different levels of the pathway in these models in the near future, preferably in a single experiment.

A major concern for the global inhibition of WNT signaling is the occurrence of side effects. Several rapidly dividing cells, e.g., in intestinal epithelium and skin, rely on WNT signaling, and an intervention therefore could disturb their normal proliferation, causing, e.g., diarrhea. Surprisingly, such side effects have rarely been observed where these drugs were administered. A potential explanation for this could be that the amount of WNT signaling needed by these epithelial cells is limited, allowing a partial blockade to be without harmful side effects on these cells. Obviously, further research will be needed on this topic before these compounds can be successfully applied in patients. Moreover, these studies should go hand in hand with the development and validation of biomarkers (such as circulating DKK) for WNT signaling activity, to obtain a quick surrogate end point for the effectiveness of the therapy in individual patients.

Recently, several registered drugs and food supplements have been shown to act as inhibitors of the WNT pathway. An obvious advantage of these compounds is that they can be readily applied in man, allowing a rapid translation of preclinical findings to patients. However, a downside is that the dose needed to block WNT signaling usually is quite high, which may give rise to side effects. This problem could be addressed by administering several different drugs at a lower dose. These drugs can be chosen to target the pathway at different levels, which can have an additive (or even synergistic) effect and result in sufficient inhibition of WNT signaling, without the occurrence of severe side effects. On the other hand, the newly developed drugs that target Wnt signaling in a more selective way should be tested and optimized for selectivity, toxicity, and pharmacokinetic profile to develop them toward use in the clinic.

## **XII. Future Perspectives**

In the last decade, a large number of studies have been published on the characteristics of WNT signaling

in general and its role in cardiovascular diseases in particular. In this review, we focused on studies on the role of WNT signaling in cardiovascular conditions as diverse as atherosclerosis, cardiac hypertrophy, and arrhythmias. However, potential common denominators in the pathologic mechanisms underlying these diseases appear to be processes such as cell proliferation and differentiation, inflammation and fibrosis, and the role for WNT signaling in these processes is well established. This helps to explain the regulatory role that WNT signaling plays in these diverse cardiovascular conditions.

Most of the studies presented in this review describe the effect of an intervention in WNT signaling in a cardiovascular disease model. The results are sometimes contradictory with other studies, making it hard to draw firm conclusions regarding the potential therapeutic benefit of the intervention. However, a couple of remarks have to be made. First, in many studies, genetic interventions and deletions of a specific gene in particular have been used. Since the WNT pathway has many homologs at different levels in the signaling cascade, this may give rise to redundancy and adaptation of the organism to the absence of the specific gene. The large number of regulatory mechanisms that can control the signaling activity can further influence the outcomes. Moreover, many gene deletion experiments with little or no phenotype are likely to never have been published in the first place, giving rise to a biased picture. This calls for interventions that can target the signaling pathway in a more general way. Second, experimental details such as age and genetic background of the animals, sex, and method of disease induction can be confounding the results. Third, the timing of the intervention can be critical for the outcome, as, e.g., described in *section IX* of this review, where inhibition of WNT signaling needs to be carefully timed to achieve optimal cardiomyocyte differentiation.

These considerations clearly indicate that the field is about to become ready for the next leap, where therapeutic interventions in WNT signaling are tested in clinically relevant models of cardiovascular disease or even in patients suffering from cardiovascular diseases. In the past, the major problem was that the options for pharmacological intervention in the pathway were limited or even absent due to the lack of suitable drugs. As can be deduced from *section XI*, this situation has changed drastically in the last years. Many experimental drugs are now available, targeting the pathway at multiple levels, and some of them are currently tested in man. Moreover, several registered drugs have been shown to target WNT signaling in diseases such as cancer, allowing the testing of the benefit of intervening in WNT signaling in cardiovascular conditions. It is highly likely that these kinds of experiments will teach us more about the feasibility and the benefit of targeting WNT signaling in cardiovascular diseases.

## Authorship Contributions

Wrote or contributed to the writing of the manuscript: Foulquier, Daskalopoulos, Lluri, Hermans, Deb, Blankesteyn.

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