

## The Role of Wnt Signalling in Angiogenesis

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### Abstract

Angiogenesis is a normal biological process wherein new blood vessels form from the growth of pre-existing blood vessels. Preventing angiogenesis in solid tumours by targeting pro-angiogenic factors including vascular endothelial growth factor (VEGF), angiopoietin-1 (Ang-1), basic fibroblast growth factor (bFGF), hepatocyte growth factor, and platelet-derived growth factor (PDGF) is currently under investigation for cancer treatment. Concurrently targeting the cell signalling pathways involved in the transcriptional and post-translational regulation of these factors may provide positive therapeutic results. One such pathway is the Wnt signalling pathway. Wnt was first discovered in mice infected with mouse mammary tumour virus, and has been crucial in improving our understanding of oncogenesis and development. In this review, we summarise molecular and cellular aspects of the importance of Wnt signalling to angiogenesis, including  $\beta$ -catenin-dependent mechanisms of angiogenic promotion, as well as the study of Wnt antagonists, such as the secreted frizzled-related protein family (SFRPs) which have been shown to inhibit angiogenesis. The growing understanding of the underlying complexity of the biochemical pathways mediating angiogenesis is critical to the identification of new molecular targets for therapeutic applications.

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### Angiogenesis and Cancer

Angiogenesis is the process whereby new vessels form by the budding and sprouting of pre-existing blood vessels into an avascular area.<sup>1-4</sup> Stages of angiogenic growth include the degradation of the basement membrane, migration and proliferation of endothelial cells to form a vessel sprout, tube formation, and recruitment of pericytes or smooth muscle cells to form a mature vessel.<sup>3,5-7</sup> Angiogenesis occurs during embryogenesis, menstruation, and pathological processes that include tumour growth.<sup>2,5,6</sup> Remodelling is common in angiogenesis and includes the growth and regression of vessels, which adapt to meet the demands of the local tissues.<sup>1</sup> Vessel diameter and thickness is dependent on the needs of local tissue, often beginning as a plexus of primitive tubes that is modified to form a more complex vascular network in the adult.<sup>1</sup> The control of apoptosis is critical for the

formation and maintenance of the vasculature.<sup>3</sup> An increase in endothelial apoptosis occurs during remodelling and/or regression of the vascular network.<sup>3</sup> It is important for vessel formation and maintenance to be tightly regulated, otherwise pathologies may arise, such as the development of tumours.

There are many angiogenic growth factors that prevent the apoptosis of endothelial cells and stimulate proliferation to optimise vessel growth.<sup>3</sup> Growth factors that regulate angiogenesis include vascular endothelial growth factor (VEGF), angiopoietin-1 (Ang-1), basic fibroblast growth factor (bFGF), hepatocyte growth factor, and platelet-derived growth factor (PDGF).<sup>1,3,7,8</sup> These angiogenic factors and their respective receptors have been implicated in the progression of many cancers, and have been extensively researched as potential therapeutic targets.<sup>9</sup> Pro-angiogenic factors also

promote the sprouting of capillaries toward the tumour that is secreting the growth factor, therefore vascularising the region. Anti-angiogenic factors include thrombospondin-1, angiostatin, endostatin, and tumstatin.<sup>5,7,8</sup> and they suppress vascular cell recruitment and growth of the vasculature.

The formation and remodelling of vessels are prominent processes in a variety of pathological states.<sup>3</sup> There is a shift towards pro-angiogenic activities during tumour growth that is mediated by factors such as VEGF, which is produced and secreted by the tumour cells.<sup>8,10,11</sup> Excessive vessel growth contributes to tumour progression and, therefore, tumour growth is angiogenesis-dependent.<sup>3,7,8,11</sup> Angiogenesis is necessary for a tumour to grow beyond 1–2 mm<sup>3</sup> in size and for metastasis to occur.<sup>5,7,8</sup> Without a new and functional vasculature being present, a tumour’s ability to grow and proliferate would be limited.<sup>12</sup> The demand for oxygen is met by transcription of hypoxia-inducible genes for proteins that turn on the angiogenic switch to stimulate the production of more VEGF.<sup>5</sup> The new vasculature also aids in the removal of waste products and facilitates metastasis.<sup>7</sup> The process of angiogenesis in tumours is shown in Figure 1. There are many signalling pathways implicated in the control of angiogenesis, including Wnt signalling.<sup>13</sup>

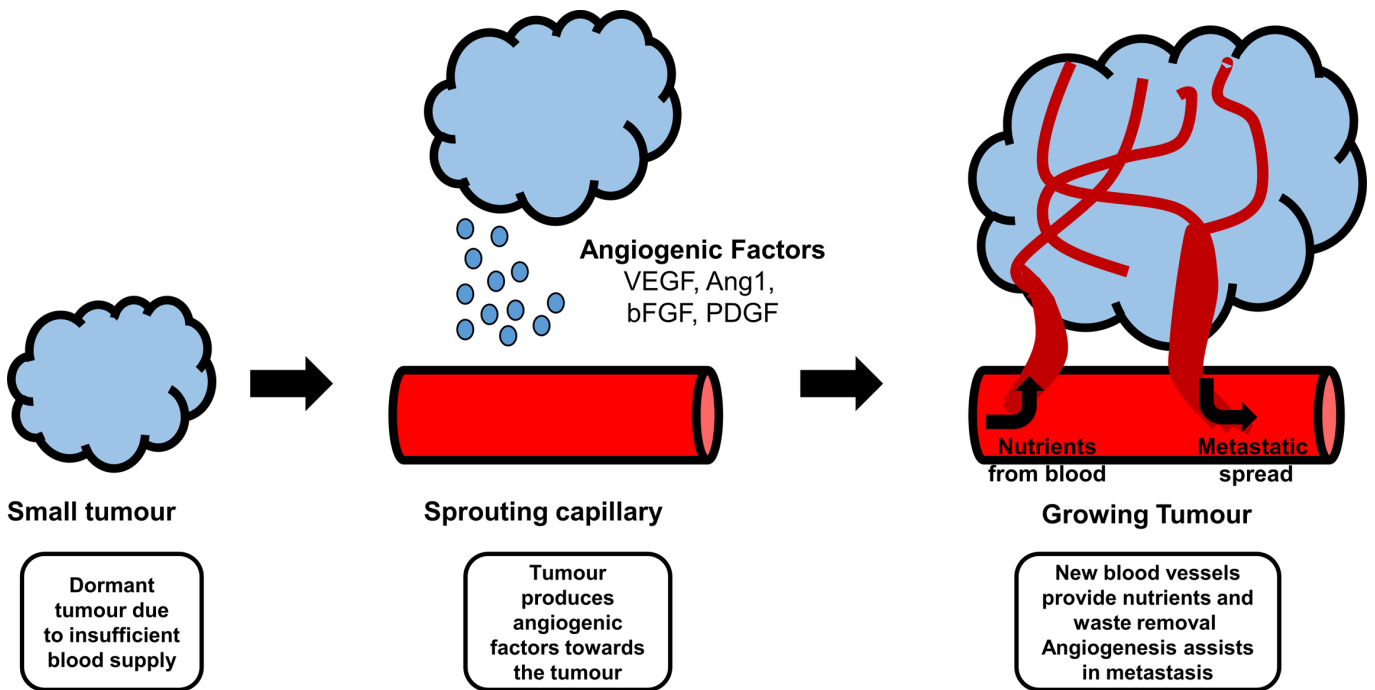
**Wnt Signalling**

Wnts are potent angiogenic factors, and their signalling pathways play an important role in normal development and in

the pathogenesis of a variety of diseases, including cancer.<sup>3,14,15</sup> Wnt pathways are involved in cellular proliferation, survival, differentiation, migration, and apoptosis.<sup>3,16-18</sup> The development of a number of organ systems depends on Wnt signalling, including the kidney, female reproductive tract, and placenta.<sup>3</sup> The Wnt pathway plays an important role in angiogenesis and vessel remodelling.<sup>3,16</sup>

Wnt proteins are a family of secreted cysteine-rich glycoproteins that are highly conserved among species.<sup>2,3,19,20</sup> There are 19 Wnt genes that have been identified in humans.<sup>3,19</sup> The majority of human Wnt genes contain four coding exons, with exon 1 containing the initiator methionine,<sup>19</sup> and exert their effects through modulating cellular events at the post-translational and transcriptional level.<sup>21</sup> Wnt proteins transduce cellular signals by binding to a range of cell-surface co-receptor molecules: the transmembrane Frizzled (Fzd) receptors and members of the lipoprotein receptor-related proteins 5 or 6 (LRP5/6)<sup>3,19,20</sup> are perhaps the most important of these. LRP5/6 are made up of an ectodomain with four β-propeller repeats, each of which is interspersed with a single EGF repeat, culminating in three low-density lipoprotein receptor-A (LDLR-A) domains. The cytoplasmic tail contains PPP[S/T]PxS motifs that are phosphorylated upon Wnt ligand stimulation.<sup>22</sup>

The majority of Wnt proteins share about 35% amino acid sequence identity, with an increased sequence identity if they



**Figure 1.** A schematic diagram of tumour angiogenesis.

are members of a subgroup.<sup>19</sup> Human Wnt proteins are very similar in size, ranging in molecular weight from 39 kDa (Wnt7a) to 46 kDa (Wnt10a).<sup>19</sup> Wnt proteins are modified with palmitoleate lipids by Porcupine, a Wnt-specific acyltransferase, at a conserved serine (in Wnt3a, at S209).<sup>23</sup> This modification facilitates both secretion and the interaction with Fzd,<sup>24-26</sup> although it is unknown if lipidation facilitates interaction with LRP co-receptors; recent structural evidence suggests that the Wnt lipid facilitates dimerisation of Fzd CRDs, which may imply it does not facilitate co-receptor interaction.<sup>27,28</sup> The structure of Wnt proteins has been recently revealed (Figure 2), as complexed with the cysteine-rich domain (CRD) of a Fzd receptor.<sup>29</sup> Wnt proteins feature 23 or 24 highly conserved cysteine residues, the majority of which were revealed in the crystal structure to form an intricate network of disulphide bonds to stabilise the unusual fold of the Wnt protein; the disulphide bond network is critical for Wnt signalling.<sup>30</sup> The CRD utilises two binding sites to bind Wnt; a hydrophobic site to bind the Wnt lipid (often referred to as the Wnt 'thumb'; formed within the N-terminal domain), and a region on the opposite face of the CRD binding a moderately conserved, cysteine-rich, two-stranded  $\beta$ -sheet (often referred to as the Wnt 'index finger'; formed within the C-terminal domain). Some Wnts have been shown to display selectivity for specific Fzds.<sup>31</sup> Specific regions of Wnts and Fzd CRDs frequently involved in mediating Wnt-Frizzled interactions have recently been proposed computationally.<sup>32</sup>

There are at least three Wnt signalling pathways: the canonical Wnt/ $\beta$ -catenin pathway, and the two non-canonical pathways: the Wnt/calcium (Wnt/ $\text{Ca}^{2+}$ ) pathway, and the Wnt/planar cell polarity pathway (Wnt/PCP).<sup>19</sup> In addition, there are endogenous Wnt antagonists, including the secreted Frizzled-related proteins (SFRP1-5), the Dickkopf (DKK) family of proteins, the Wnt inhibitory-factors (WIFs), and Cerebus.<sup>3</sup>

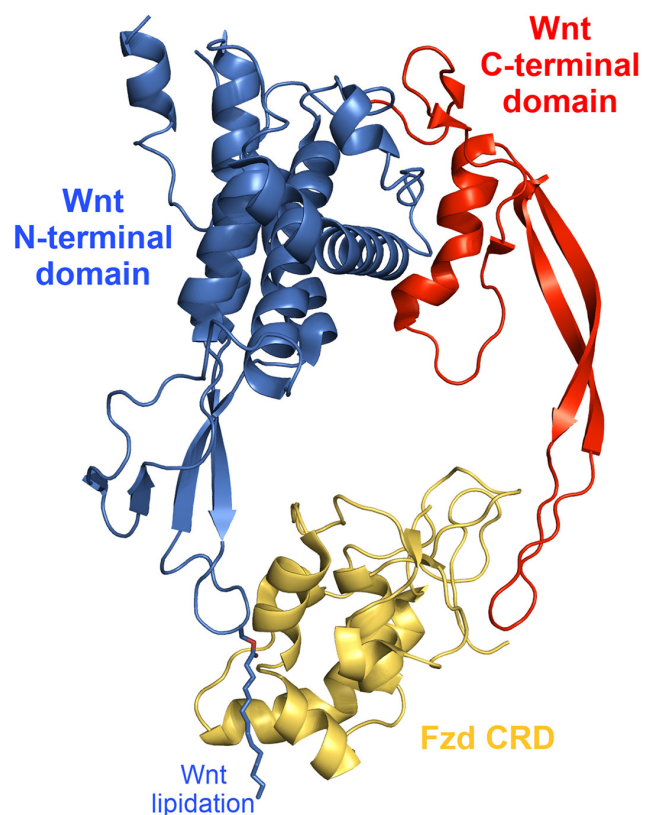
#### Wnt/ $\beta$ -catenin Signalling Pathway

The canonical Wnt/ $\beta$ -catenin signalling pathway targets the key cellular regulatory molecule  $\beta$ -catenin in signalling (Figure 3).<sup>33</sup> Signalling through a  $\beta$ -catenin-dependent mechanism relies on the post-translational modification status of  $\beta$ -catenin. This is regulated through the cytoplasmic destruction complex, comprising glycogen synthase kinase-3 $\beta$  (GSK3 $\beta$ ), Axin, Casein kinase1/2 (CK1/2), Protein phosphatase 2A (PP2A), and adenomatous polyposis coli (APC).<sup>33</sup> In the absence of Wnt signalling, cytosolic  $\beta$ -catenin is phosphorylated by CK1 at Thr41, and GSK3 $\beta$  at Ser33 and Ser37.<sup>34</sup> Phosphorylated  $\beta$ -catenin is then primed for ubiquitination by the E3 ligase,  $\beta$ -TrCP, and targeted for proteosomal degradation.<sup>34</sup> Wnt binding to Fzd recruits co-receptor LRP5/6, which results in the activation of the cytoplasmic phosphoprotein Dishevelled (Dvl).<sup>35,36</sup> These

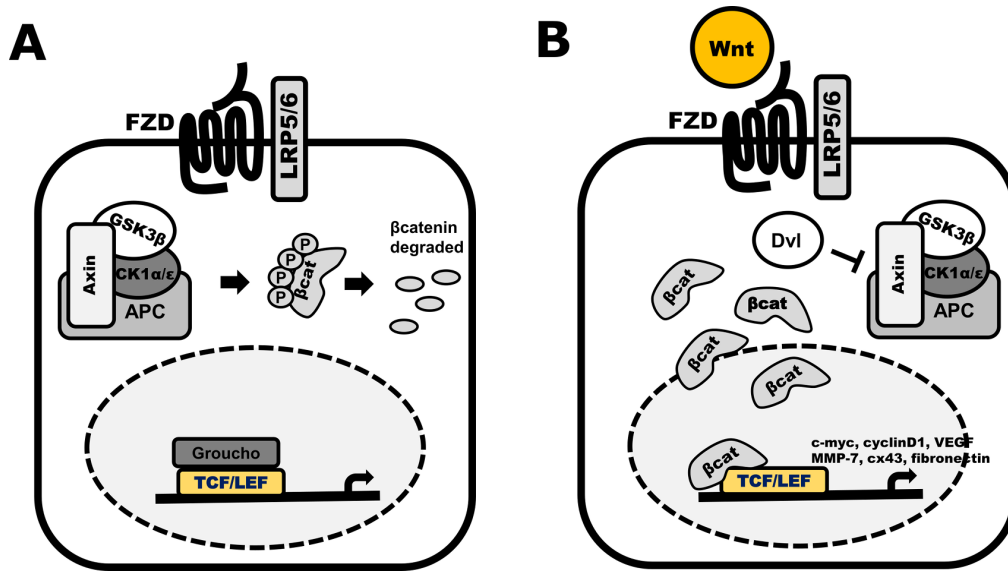
events lead to the inhibition of GSK3 $\beta$ , thereby promoting the accumulation of unphosphorylated (or active)  $\beta$ -catenin and its subsequent translocation to the nucleus, where it binds to a variety of transcription factors, including T-cell factor/lymphocyte enhancing factor (TCF/LEF)<sup>37</sup> and forkhead box (in particular, the FOXO subtypes)<sup>38</sup> family proteins. TCF/LEF are able to bind and facilitate the transcription of a number of Wnt target genes involved in cell proliferation, Wnt signal transduction and vascular growth, including CCND1 (cyclin D1),<sup>39</sup> AXIN2,<sup>40</sup> and VEGF.<sup>41</sup>

#### Wnt/ $\text{Ca}^{2+}$ Signalling Pathway

In the Wnt/ $\text{Ca}^{2+}$  pathway, the activation of Fzd stimulates the release of calcium ( $\text{Ca}^{2+}$ ).<sup>3</sup> The Wnt/ $\text{Ca}^{2+}$  pathway mobilises intracellular  $\text{Ca}^{2+}$  to activate a pertussis toxin-



**Figure 2.** Crystal structure complex of the Xenopus Wnt8 with the mouse Fzd8 cysteine-rich domain (PDB 4F0A). The N-terminal domain (blue) contains the Wnt 'thumb', featuring lipid modification and is bound by a site generally conserved among Frizzled cysteine-rich domains. The C-terminal domain (red) contains the Wnt 'index finger', a moderately conserved, cysteine-rich, two-stranded  $\beta$ -sheet, which is bound by a site on the opposite face of Frizzled cysteine-rich domains to that bound by the thumb.



**Figure 3.** Schematics of the Wnt/β-catenin signalling pathway. A) The inactive canonical pathway (OFF state). Without Wnt ligand stimulation through the FZD-LRP5/6 receptor complex, no signal is transduced through Dvl. This results in stabilisation of the destruction complex consisting of Axin, APC, GSK3 and CK1, resulting in phosphorylation of β-catenin and subsequent degradation. B) Active canonical Wnt signalling (ON state). Stimulation by a Wnt ligand activates the FZD receptor and LRP5/6 co-receptors. A signal is transduced through Dvl leading to destabilisation of the destruction complex. Non-phosphorylated (active) β-catenin translocates to the nucleus where it binds to TCF/LEF transcription factors, initiating the transcription of Wnt target genes.

sensitive subclass of heterotrimeric G protein, leading to an increase in intracellular Ca<sup>2+</sup> and subsequent activation of Ca<sup>2+</sup>/calmodulin-dependent protein kinase II (CaMKII) and protein kinase C (PKC).<sup>3,19,21</sup> After CaMKII activation of calcineurin (Cn), a serine/threonine phosphatase, Nuclear factor of activated T-cells (NFAT) transcription factors are activated through dephosphorylation and initiate transcription.<sup>42</sup> Calcium activation of PKC is able to initiate a variety of cellular responses.<sup>43</sup> Stimulation of the Wnt/Ca<sup>2+</sup> pathway has also been shown to antagonise Wnt/β-catenin signal transduction through Wnt5a-induced activation of the TAK1-NLK pathway.<sup>44</sup> NLK is able to negatively regulate TCF/LEF transcription through phosphorylation of TCF-4, which interferes with its ability to bind to DNA.<sup>45</sup> This demonstrates some of the complex cross-talk between Wnt signalling pathways.

**Wnt Planar Cell Polarity (PCP) Pathway**

The Wnt/PCP pathway regulates the morphogenesis of both vertebrates and invertebrates. Polarisation during cell division, migration, and cytoskeletal rearrangements are governed, in part, by the Fzd-Wnt/PCP pathway.<sup>46</sup> Regulation of this pathway is synchronised through Dvl upon Wnt activation. Signal transduction is then mediated through Rho activation of Rho-associated protein kinase (ROCK) or Rac activation of

the c-Jun N-terminal kinase (JNK) pathway, leading to actin polymerisation and changes in cell and tissue polarity.<sup>47,48</sup>

**Wnt and Angiogenesis**

Recent evidence suggests that both the canonical and non-canonical Wnt signalling pathways are implicated in angiogenesis in a variety of organs in both normal and pathological conditions.<sup>49-51</sup> Wnt signalling, both canonical and non-canonical, appears to be essential in vascular endothelial cells and functions through a variety of regulators, including R-spondin3<sup>52</sup> and Fzd7.<sup>53</sup> Transcriptional regulation of VEGF by Wnt/β-catenin signalling has been demonstrated, where seven TCF binding sites have been found in the VEGF gene promoter.<sup>54</sup> Furthermore, defects in APC resulting in constitutively active β-catenin and Wnt signalling can lead to over-expression of VEGF.<sup>54</sup> The β-catenin/TCF complex is also responsible for the transcriptional regulation of interleukin-8 and matrix metalloproteinases (MMPs), which are key drivers and regulators of angiogenesis.<sup>17,20</sup> More recent evidence also suggests that Wnt7a and Wnt7b, both canonical Wnt ligands, are able to activate β-catenin-mediated signalling through Gpr124.<sup>51,55</sup> The role of SFRP family proteins has been demonstrated to regulate angiogenesis via regulating both canonical and non-canonical Wnt signalling.

**VEGF**

VEGF is produced and secreted by mesenchymal and stromal cells in a paracrine manner.<sup>1,56</sup> It is a potent mitogen and chemoattractant for endothelial cells.<sup>1,5,10,56</sup> Mutations in the Wnt signalling pathway have resulted in up-regulation of VEGF expression.<sup>10</sup> VEGF induces neovascularisation of malignant cells and has an increased expression in tumour cells.<sup>5</sup> VEGF is a stimulator of endothelial proliferation, migration, and survival, and has critical roles in physiological and pathological angiogenesis.<sup>3,10</sup> Tyrosine kinases Flt-1 and Flk-1/KDR are receptors for VEGF that are expressed primarily by the endothelium.<sup>1</sup> Flk-1/KDR is the main receptor that allows for the mitogenic, angiogenic, and permeability-enhancing effects of VEGF.<sup>10</sup> VEGF is capable of inducing the expression of the anti-apoptotic protein B-cell leukaemia/lymphoma 2 (Bcl-2).<sup>10</sup> VEGF-activated endothelial cells have also been demonstrated to increase the expression of pro-angiogenic proteins, MMPs.<sup>7</sup> Inhibition of VEGF signalling inhibits both angiogenesis and the growth in tumour size.<sup>56</sup>

There are numerous small molecules targeting VEGF and VEGFR undergoing clinical trials, as well as the anti-VEGF monoclonal antibody, bevacizumab, which has been approved as a first line treatment for colorectal and non-small cell lung cancer.<sup>57</sup> Bevacizumab was originally approved by the FDA for combinatorial treatment with 5-fluorouracil-based chemotherapies for colon cancer.<sup>58</sup> Following its initial success, it was approved for use in glioblastoma multiforme,<sup>59</sup> ovarian carcinoma,<sup>60</sup> renal cell carcinoma,<sup>61</sup> cervical cancer<sup>62</sup> and HER2-negative breast cancer.<sup>63</sup> While initial reports of success were overwhelming, the use of bevacizumab was revoked for use in breast cancer, after improvement in progression-free survival was unable to be replicated in subsequent cohorts.<sup>64</sup> The effectiveness of bevacizumab has been questioned due to its toxicity profile and the high number of adverse reactions associated with its addition to treatment regimens.<sup>65,66</sup> Currently, there are no FDA-approved drugs for cancer that directly target the Wnt pathway.<sup>67</sup>

**MMPs**

MMPs create and maintain a microenvironment that facilitates the growth and angiogenesis of tumours by mediating the degradation and remodelling of the extracellular matrix (ECM), thereby facilitating the sprouting of new vessels.<sup>3,6,68,69</sup> Matrilysin (MMP-7) plays an important role in cancer metastasis and is expressed by endothelial cells of the vasculature adjacent to the MMP-7-producing tumour, thereby promoting angiogenesis by accelerating the migration of endothelial cells.<sup>68,69</sup> MMP-7 promotes cancer invasion and angiogenesis by proteolytic cleavage of ECM substrates.<sup>69</sup> MMP-7 promotes the secretion of soluble FasL, increasing apoptosis in surrounding cells by activating Fas.<sup>69</sup>

In response to Wnt signalling, MMP-7 is upregulated in colon cancer cells and can stimulate the proliferation of endothelial cells in culture.<sup>3</sup> MMP-7 is upregulated in invasive cancers. Some MMP inhibitors are thought to act as cancer metastasis suppressors and exhibit the ability to inhibit tumour angiogenesis.<sup>68</sup> Tissue inhibitors of metalloproteinases (TIMP) inhibit angiogenesis by downregulating the activity of MMPs.<sup>4,6,68</sup> It has also been demonstrated that TIMPs inhibit angiogenesis in an MMP-independent fashion by blocking signalling through  $\alpha 3 \beta 1$  integrins.<sup>70</sup>

**GPR124**

A recently discovered essential regulator of Wnt signalling and angiogenesis is the G-protein coupled receptor, Gpr124. It was initially linked to vascular development in colorectal tumour models.<sup>71</sup> It is now known that Gpr124 is crucial for angiogenesis and vascularisation in the central nervous system through direct regulation of canonical Wnt signalling. Gpr124 has been demonstrated to act as a receptor for Wnt7a and Wnt7b, and may enhance the formation of a Wnt signalosome, consisting of Wnt7a/b, Gpr124, Fzd4 and LRP5 (Figure 4).<sup>51</sup> Studies have indicated that Wnt7a and Wnt7b signal transduction through Gpr124 is dependent on leucine-rich repeats (LRRs) in the ectodomain of Gpr124 and an intracellular PDZ-binding motif.<sup>55</sup> Recent evidence also suggests that Gpr124 works synergistically with Reversion-inducing-cysteine-rich protein with kazal motifs (Reck) to co-activate Wnt7a and Wnt7b signalling.<sup>50</sup> The structure of Gpr124 is likely similar to the Leucine-rich repeat-containing G-protein coupled receptor family of GPCRs, which also feature LRRs in their ectodomain,<sup>72</sup> and can also activate Wnt signalling. The likely structure of Gpr124 suggests it may potentially be an LGR family member; however, it is currently unknown if Gpr124 interacts with the R-spondins, which are the major mediators for Wnt signalling via the LGRs.<sup>73</sup>

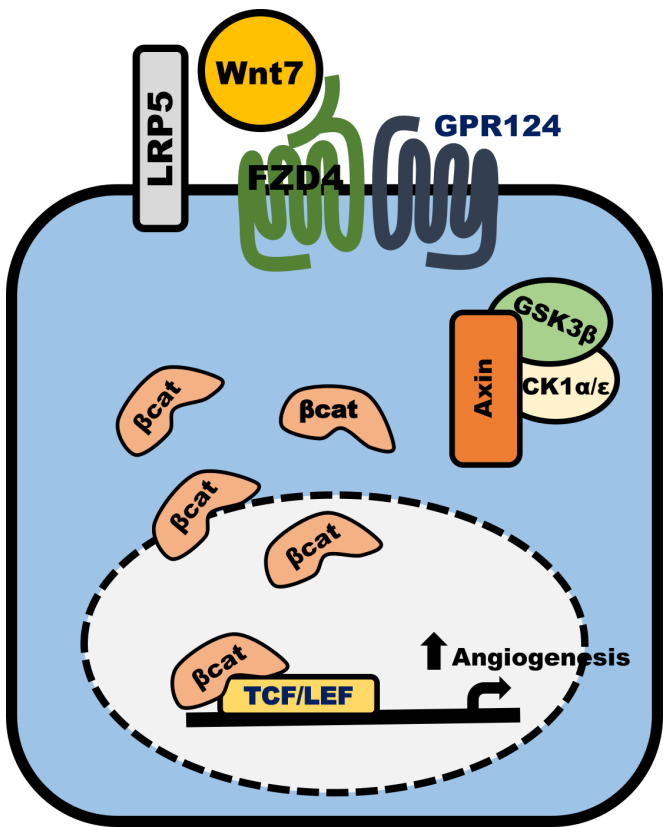
Gpr124<sup>-/-</sup> embryos feature forebrain and ventral spinal cord angiogenic defects, similar to those seen in embryos deficient in Wnt7a and Wnt7b. These angiogenic defects were dramatically more pronounced in Fzd4<sup>-/-</sup>;Gpr124<sup>-/-</sup> embryos, which exhibited vascular leakage through up-regulation of plasmalemma vesicle-associated membrane protein and downregulation of claudin5.<sup>51</sup> This phenotype was embryonically lethal by E10.5. The defects in angiogenesis and vasculature were able to be rescued through activating Wnt/ $\beta$ -catenin signalling by generating a Gpr124<sup>-/-</sup>;Ctnnb1<sup>fl<sup>ex</sup>3/+</sup> phenotype with constitutively active  $\beta$ -catenin. This study demonstrates the role of Gpr124 in the regulation of angiogenesis and vascularisation in the central nervous system directly through canonical Wnt signalling.<sup>51</sup>

**SFRPs**

Proteins of the SFRP family are soluble inhibitors of Wnt signalling;<sup>3,74</sup> they can also block endothelial cell proliferation.<sup>2</sup> SFRPs feature a two-domain structure, consisting of a CRD, similar to that found in Fzd receptors,<sup>2,3,74</sup> and a netrin-like domain (NLD), which likely bears structural similarity to the TIMP NLD.<sup>75</sup> SFRPs may act as soluble modulators competing with Fzd to bind the Wnt ligands.<sup>76</sup> SFRPs can also bind to Wnt proteins directly and antagonise Wnt function by preventing its interaction with Fzd receptors.<sup>2,19,74,77</sup> The SFRPs may also interact with Fzds, which might antagonise Wnt signalling through the formation of a non-functional complex with the Fzd receptors.<sup>19,77</sup>

Early studies provided evidence for an anti-angiogenic role of SFRP-1.<sup>78</sup> It was observed that bovine SFRP-1 was endogenously expressed in bovine endothelial cells and increased expression was associated with decreased proliferation rates.<sup>78</sup> SFRP-1 inhibiting angiogenesis is

logical as it likely antagonises Wnt signalling;<sup>79</sup> however, more recent research contradicts this, describing a pro-angiogenic role for SFRP-1.<sup>80</sup> Exposure to SFRP-1 activated the non-canonical Wnt signalling pathways, enhancing the velocity of endothelial cell spreading on laminin and collagen.<sup>81</sup> Interestingly, SFRP-1 treatment was initially associated with a decrease in proliferation and then, after an extended period of time (15 days), a pro-angiogenic response was seen. This effect coincided with the up-regulation of Rac1 and RhoGTPases, and is probably due to endothelial cell cytoskeletal rearrangement involving the PCP/Wnt signalling pathway.<sup>81</sup> Thus, it seems that endothelial cells have a biphasic response to SFRP-1: initial inhibition of the canonical pathway, followed by Rac-1 activation and angiogenesis promotion. Courtwright *et al.* discovered that SFRP-2 is pro-angiogenic. SFRP-2 was shown to increase endothelial cell migration and tube formation and protect against hypoxia-driven apoptosis in a dose-dependent fashion.<sup>82</sup> Currently, there is only one study that has reported the effects of SFRP-4 on different endothelial cell lines to assess its role in angiogenesis.<sup>16</sup> This study showed that SFRP-4 induced a significant reduction in endothelial cell migration. The authors suggest that SFRP-4 may be exerting its effects by inhibiting both proliferation and migration of endothelial cells. In the same study, the administration of SFRP-4 induced a significant reduction in the number of endothelial rings formed, a reduction in pseudopodia, and a loss of ring stability, all of which are important processes necessary for the initiation and development of angiogenesis. The effect of SFRP-4 on physiological and tumour-associated angiogenesis was also assessed using *in vivo* models, and they found there was a decrease in vascularisation and angiogenesis.



**Figure 4.** Wnt7/FZD4/GRP124/LRP5 signalling cascade. Activation of canonical Wnt/β-catenin signalling is initiated through Wnt7a/b binding to Fzd4. GPR124 and LRP5 act as co-receptors through which signal transduction is conveyed to destabilise the destruction complex (Axin, GSK3β, CK1α/ε). β-catenin is stabilised, allowing its translocation to the nucleus, where it can bind to TCF/LEF transcription factors, leading to vascularisation and angiogenesis.

**Stem Cells and Angiogenesis**

**Cancer Stem Cells and Angiogenesis**

Cancer stem cells (CSCs) are a subpopulation of cells within the heterogenous tumour niche which have demonstrated higher angiogenic and tumourigenic potential than non-stem-like cells.<sup>83</sup> CSCs derived from breast, colorectal, liver and glioblastoma tumours also demonstrate higher Wnt/β-catenin activity.<sup>84-87</sup> Increased Wnt activity may also partly be responsible for the elevated levels of VEGF seen in CSCs.<sup>88-90</sup>

The relationship between CSCs and angiogenesis has not yet been conclusively established, although CD133<sup>+</sup> CSCs from glioma cells show elevated expression of VEGF,<sup>91</sup> and when compared with CD133<sup>-</sup> glioma cells demonstrated increased tumour initiation with increased vascularity, necrosis, and haemorrhage. CD133 expression has been linked to Wnt/β-catenin signalling in glioma stem cells. Decreased expression of CD133 was seen after the addition of the Wnt antagonist, SFRP-4. This was demonstrated to be a Fzd-dependent mechanism.<sup>92</sup>

In CD133<sup>+</sup> glioma cells, CD31 staining showed increased vascular density and 10–20 fold up-regulated VEGF expression. Furthermore, bevacizumab treatment on CD133<sup>+</sup> glioma cells depleted the vascular endothelial cell migration and tube formation. Additionally, bevacizumab *in vivo* inhibited tumour progression and down-regulated VEGFR in a CD133<sup>+</sup> glioma cell-derived xenograft. Thus, targeting VEGFs may have direct implications on endothelial cells and tumour progression.<sup>91</sup> These studies in glioma CSCs have also been replicated *in vitro*.<sup>93,94</sup>

In glioma cells, CSCs also contribute to tumour vascular development, suggesting the role of CSCs in recruiting endothelial progenitor cells (EPC). CSCs stimulate pro-angiogenic factors, such as VEGF and SDF-1, suggesting that CSCs promote local angiogenesis and EPC mobilisation.<sup>95</sup>

CSCs express proteins that induce neovascular growth and establish an aberrant vascular niche. Glioblastoma CSCs express Nestin, a major intermediate filament protein, which is located in close proximity to CD34<sup>+</sup> capillaries, which are correlated to microvessel density.<sup>96</sup> Nestin has also been shown to positively regulate Wnt/ $\beta$ -catenin signalling to promote invasiveness through up-regulation of MMP-2, MMP-7 and VEGF.<sup>97</sup> The influence of vascular endothelial cells on CSCs was demonstrated when CSCs were co-cultured with endothelial cells. It was found that the interaction of endothelial cells with glioblastoma CSCs provides vasculogenic factors supporting self-renewal and differentiation capacity. Therapeutically targeting VEGF depleted the tumour vasculature and segregated the vascular niche of the CSCs.<sup>96</sup> However, further investigation showed a dual effect of DC101 (a VEGFR-2 inhibitor) in C6 rat glioma xenograft tumours. The treatment depleted the pro-angiogenic factors secreted by CSCs and decreased tumour angiogenesis.<sup>95</sup>

These studies highlight the importance of targeting CSCs to inhibit tumour angiogenesis, as well as the close relationship between CSCs and Wnt/ $\beta$ -catenin in promoting angiogenesis.

### **Mesenchymal Stem Cells in Angiogenesis**

Mesenchymal stem cells (MSCs) play a major role in postnatal angiogenesis, especially during tissue regeneration and repair. One of the mechanisms through which MSCs influence angiogenesis is through paracrine signalling via secreting various cytokines and growth factors, which can improve endothelial cell proliferation, migration, and functionality. For instance, when human amniotic or bone marrow-derived MSCs were co-cultured with human umbilical vein endothelial cells (HUVEC), the expression of platelet endothelial cell adhesion molecule and the vessel density were increased as compared to HUVEC monoculture.<sup>98</sup> The observed pro-

angiogenic effect was correlated to the increased activity of MMP-2 and MMP-9 in the co-culture as compared to the monoculture system.<sup>98</sup>

Secondly, MSCs can exploit their transdifferentiation potential into endothelial cells during vascular regeneration. In therapeutic angiogenesis, the benefits of using MSCs can arise from their transdifferentiation capabilities into either endothelial cells or smooth muscle cells.<sup>99</sup> The potential clinical applications of MSCs in vascular regeneration have been demonstrated through various *in vitro* and *in vivo* studies. One study on myocardial ischaemia showed that rat foetal heart MSCs improved cardiac function by transdifferentiating into endothelial cells and smooth muscle cells.<sup>100</sup> Similar results of improved cardiac function contributed by bone marrow-derived MSCs were reported in another *in vivo* study performed in swine animal models.<sup>101</sup> Furthermore, MSCs overexpressed with VEGF/Hepatocyte growth factor have been reported to play a role in promoting angiogenesis and cardiac function of porcine heart after myocardial infarction.<sup>102</sup>

### **Wnt Signalling on the Angiogenic Potential of MSCs**

Although the role of Wnt signalling in angiogenesis has been demonstrated, the role of the Wnt signalling pathway in the endothelial differentiation of MSCs has not been thoroughly studied. Wnt-inducing factors, such as Wnt1, VEGF, and the GSK3 $\beta$  inhibitor, CHIR99021, induced the vascular differentiation of MSCs derived from dental pulp-derived stem cells (DPSCs), as observed by an increased expression of VEGFR2 or Tie-2. On the other hand, the inhibition of Wnt signalling using the small molecule inhibitor JW67 or by silencing  $\beta$ -catenin inhibited the differentiation of DPSCs into endothelial cells.<sup>103</sup> VEGF is one of the key factors promoting the endothelial differentiation of MSCs. However, in MSCs it has been reported to act through the Rho/ROCK pathway, wherein inhibition of the Rho/ROCK pathway suppressed the endothelial differentiation of human and rat MSCs.<sup>104</sup>

Another study by Zhang *et al.* demonstrated the role of Wnt signalling in MSC-mediated angiogenesis. Exosomes from human umbilical cord derived-MSCs enhanced endothelial functionality in terms of the proliferation, migration, and tube-formation potentials of endothelial cells. Wnt4 was identified in these exosomes, which was responsible for these pro-angiogenic effects in endothelial cells. This was evidenced by the nuclear translocation of  $\beta$ -catenin in endothelial cells, thereby up-regulating canonical Wnt signalling. They also observed an increase of *in vivo* angiogenesis in the rat skin burn model, where the rats were subcutaneously injected with MSC-derived exosomes. The observed pro-angiogenic effect was reversed with the  $\beta$ -catenin inhibitor ICG-001 or with a Wnt4 knock-down in these MSCs, demonstrating the role of Wnt signalling in promoting MSC-mediated angiogenesis.<sup>105</sup>

### ***Wnt Antagonism on the Angiogenic Potential of MSCs***

Amongst the few studies that reported the effect of Wnt antagonists on the angiogenic potential of MSCs, one demonstrated the role of Dkk-1 in MSC-derived angiogenesis. It was reported that interleukin-1 $\beta$  (IL-1 $\beta$ ) enhanced the angiogenic potential of MSCs derived from bronchoalveolar lavage through up-regulation of miR-433, which in turn decreased the expression of the Wnt antagonist Dkk-1. To further confirm the role of the Wnt pathway in this, they also demonstrated that the exposure of MSCs to IL-1 $\beta$  as well as the over-expression of miR-433 in MSCs upregulated the expression of  $\beta$ -catenin.<sup>106</sup> The decrease in Dkk-1 expression during an up-regulated angiogenesis indicates the need to have an activated Wnt signalling state to promote the angiogenic potential of MSCs.

A study by Dufourcq *et al.* demonstrated that over-expression of SFRP-1 in MSCs resulted in better vessel stability and maturation. SFRP-1 over-expression enabled the pro-angiogenic effects through different mechanisms. One of these was by promoting an elongated morphology of MSCs, permitting an efficient integration and closer contact of MSCs around the vessel walls. Secondly, SFRP-1 also increased the extent of cellular contact and communication between MSCs and endothelial cells via  $\beta$ -catenin-dependent cell-cell junctions around the vessel wall. Additionally, SFRP-1 increased the expression of platelet-derived growth factor-BB and  $\alpha$ -smooth muscle actin in MSCs. All of these transformations induced by SFRP-1 over-expression in MSCs facilitated an enhanced stability, maturation, and organisation of the blood vessels.<sup>107</sup>

### **Conclusions**

Wnt signalling appears to have an important role in mediating angiogenesis, influencing the role of many known angiogenic factors, including VEGF. However, further studies are needed to fully elucidate the role of Wnt signalling and its interplay with other molecular pathways in mediating angiogenesis. The relevance of angiogenesis to normal biological function, as well as cancer growth and metastasis, represents a formidable challenge in developing therapeutics that are both safe and specific. Targeting VEGF and VEGFR directly to block angiogenesis has been clinically successful, although the effects are typically short-lived, highlighting the need for alternative therapeutics. The up-regulation of the Wnt signalling pathway in many types of cancers, particularly in CSCs, provides a potential opportunity for the development of a reasonably safe and specific anti-angiogenic. However, caution is needed to identify and validate appropriate targets in the Wnt signalling pathway for this purpose, due to the complexity of the pathway and the great multitude of proteins and biological processes under its control.

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