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The Roles of Wnt Signaling in Early Mouse Development and Embryonic Stem Cells

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ABSTRACT

The Wnt family of secreted signaling molecules is conserved throughout the animal kingdom. Wnt signaling plays critical roles during embryonic development and mutations leading to the overactivation of the Wnt pathway have been linked to cancer. Wnt signals are transduced intracellularly by the Frizzled family of receptors. Moreover, proteoglycans and the co-receptors LRP5 and -6 participate in the transmission of Wnt signals, whereas a series of secreted antagonists can block Wnt signaling directly (i.e. Frzb and Sfrps) or indirectly (i.e. Dkks). Some of the biochemical interactions of the Wnts with their receptors and antagonists have recently been characterized, permitting further elucidation of how these proteins function *in vivo*. Expression pattern analyses in mouse embryos have shown that Wnt genes are active during most, if not all, developmental processes and gene inactivation has uncovered some of their key roles throughout mouse embryogenesis. Importantly, knock-out and overexpression studies have proven the importance of Wnt signaling during mesoderm, neurectoderm and body axis formation. With their ability to differentiate into all adult cell types *in vitro*, mouse embryonic stem (ES) cells have been used to mimic the developing embryo. In this ES cell system, it has recently been shown that Wnt signals contribute to mesoderm induction and neural inhibition. Here we will provide an overview of the Wnt signaling pathway and its roles during mouse embryonic development, focusing on gastrulation. Functional studies in the mouse, including gene ablation and overexpression experiments, will be reviewed. Finally, we will discuss the latest reports on the application of ES cells to study the Wnt pathway during development.

Keywords: Dkk, Frizzled, LRP, mesoderm, neural, pluripotency, Sfrp
Abbreviations: **APC**, adenomatous polyposis coli; **aVE**, anterior visceral endoderm; **CRD**, cysteine-rich domain; **Dkk**, Dickkopf; **dpc**, days post coitum; **dVE**, distal visceral endoderm; **EGO**, early gastrula organizer; **ES**, embryonic stem; **Fzd**, Frizzled; **GSK3β**, glycogen synthase kinase 3β; **LIF**, leukemia inhibitory factor; **LRP**, low-density lipoprotein-related receptor protein; **PCP**, planar cell polarity; **Sfrp**, secreted frizzled related protein; **Tcf/Lef**, T cell factor/lymphoid enhancing factor

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INTRODUCTION

More than 20 years ago, *int-1* was identified by its oncogenic properties when ectopically activated by the mouse mammary tumor virus (Nusse and Varmus 1982; Nusse and Varmus 1992). Later renamed *Wnt1*, it was one of the first oncogenes 'discovered' that was activated in tumors by a provirus. Since then, Wnt homologs have been shown to be conserved throughout the animal kingdom, from hydra, worms and sea urchin to humans, with 19 members identi-

Received: 6 March, 2007. Accepted: 25 April, 2007.

Special Feature

fied in mouse.

Wnt proteins, which can activate at least three different signaling pathways, are transduced intracellularly by the Frizzled (Fzd; also known as Fz) family of receptors. Moreover, proteoglycans and the co-receptors low-density lipoprotein-related receptor proteins 5 and -6 (LRP5/6) participate in the transmission of these signals. Modulation of Wnt signaling by secreted molecules can occur either directly, by interacting with Wnt proteins, or indirectly, by binding the LRP co-receptors. The specific interactions between the 19 Wnts, 10 Fzds and 2 LRPs appear to define the exact signaling pathway that will be activated.

During mouse embryonic development the Wnt pathway is implicated in the establishment of the basic body plan, including the formation of the anterior-posterior axis and gastrulation. In adult tissues, Wnt signaling plays roles in the self-renewal of the gut, epidermis and bone, among other tissues. More recently, studies have begun to focus on the role of Wnts in the differentiation of embryonic stem (ES) cells. This review concentrates on the extracellular players of the Wnt pathway, their characteristics, biochemical interactions and downstream signaling potentials, as well as their functions in mouse development. In addition, since ES cells can be used to model the embryo, the influence of Wnt signals on their proliferation and differentiation is also described. In order to concentrate on these topics in a clear manner, and because not all facets of Wnt signaling can be described here, the reader is referred to reviews that are cited within the text, and also to the Wnt homepage (www.stanford.edu/~rnusse/wntwindow.html).

THE WNT SIGNALING PATHWAY

The Wnt family

The Wnt genes encode for secreted proteins of 350-400 amino acids in length with 22 conserved cysteines. Post-translational modifications include the attachment of a palmitate to the first conserved cysteine as well as N-linked glycosylation (Willert *et al*. 2003; Mikels and Nusse 2006b; Coudreuse and Korswagen 2007). Whereas glycosylation appears to be essential for Wnt secretion, the lipid modification is necessary for Wnt activity.

Acting as a morphogen, Wingless, the *Drosophila* Wnt1 ortholog, has been shown to spread within the imaginal discs, signaling to distant cells (Cadigan *et al*. 1998; Marois *et al*. 2006). It is proposed that mammalian Wnt proteins also diffuse to create a signaling gradient, but the exact mechanism is yet to be elucidated (Nakaya *et al*. 2005).

Conservation of Wnts throughout the animal kingdom suggests a great functional importance, but characterization of the 19 mouse Wnt proteins has been hampered by difficulties in purifying active Wnt protein. In cell culture overexpression experiments, misfolded protein tends to accumulate in the endoplasmic reticulum. Moreover, Wnt proteins are extremely insoluble, due to the lipid modification, and remain tightly associated with the cell membrane or extracellular matrix (Papkoff and Schryver 1990; Papkoff 1994; Burrus and McMahon 1995). In spite of this, Wnt3a and -5a have now been successfully purified from conditioned medium, a paramount step for the further characterization of the biological activities of these growth factors (Willert *et al*. 2003).

Canonical and noncanonical Wnt signaling

Intracellular transduction of Wnt signals can activate at least three pathways, of which the canonical, or β-Catenin, pathway is the best characterized and is depicted in **Fig. 1** (also reviewed in Miller 2002; Logan and Nusse 2004; Gordon and Nusse 2006). Canonical Wnt signals are initiated by the binding of a Wnt to a Fzd receptor and LRP co-receptor to form a ternary complex (**Fig. 1**, right panel). The activated Fzd recruits the cytoplasmic protein Dishevelled (Dvl) to the cell membrane where Dvl is subsequently phosphorylated. The cytoplasmic tail of the LRP co-receptor is also phosphorylated, allowing LRP to interact with Axin. The phosphorylated Dvl inhibits glycogen synthase kinase 3β (GSK3β), which in turn allows for the cytoplasmic and nuclear accumulation of β-Catenin. In the nucleus, β-Catenin forms a complex with T cell factors or lymphoid enhancing factors (Tcf/Lef) to activate the transcription of Wnt target genes. In the absence of Wnt signals, Axin acts as a scaffold to adenomatous polyposis coli (APC), GSK3β and β-Catenin, as well as casein kinase 1 alpha (CK1α). Within this scaffold β-Catenin is first phosphorylated by CK1α and then by GSK3β. Subsequently, β-Catenin is ubiquitinated by β-TrCP and degraded. In absence of β-Catenin or when Wnt signaling is blocked, Groucho binds to Tcf/Lef, repressing target gene expression (**Fig. 1**, left panel). In the absence of Wnt signaling, the β-Catenin that is not degraded can be found at the cell membrane with α-Catenin, where it functions to connect cadherins to the actin cytoskeleton in adherence junctions. Wnts typically activating the canonical pathway include Wnt1, -2, -2b, -3, -3a, -6, -7b, -8a and -8b. As will be described in more detail below, canonical Wnt signaling is necessary for anterior-posterior axis formation and gastrulation in the mouse embryo.

Two noncanonical Wnt pathways also exist: the Wnt/Ca^{2+} and Wnt/planar cell polarity (PCP) pathways. The Wnt/Ca^{2+} pathway is implicated in the regulation of cell migration, as seen in *Xenopus* axis formation and gastrulation, and leads to the intracellular release of calcium ions and activation of calcium and calmodulin-dependent enzymes such as protein kinase II and protein kinase C (reviewed in Kuhl 2002). The Wnt/PCP pathway functions through small GTPases, including RhoA and Rac, to activate kinase effectors such as Jun-N-terminal-kinase and regulate cell polarity and movement (reviewed in Seifert and Mlodzik 2007). Extensively studied in the polarization of the *Drosophila* wing and eye, the Wnt/PCP pathway also plays a role in vertebrate patterning, including, for example, the convergent extension movements during gastrulation (reviewed in Barrow 2006). The importance of the Wnt/Ca^{2+} and Wnt/PCP pathways during mouse development has only recently begun to be investigated and concerns mostly the action of Wnt4, -5a and -11.

Wnt receptors and co-receptors

The Fzd receptors transduce Wnt signals into the cell. Ten Fzd genes exist in mouse and encode for seven-pass transmembrane proteins with a large extracellular domain that contains seven conserved cysteine residues, called the cysteine-rich domain (CRD), which binds Wnts with high affinity (Bhanot *et al*. 1996; Wang *et al*. 1996; Hsieh *et al*. 1999b; Dann *et al*. 2001; reviewed in Huang and Klein 2004). Fzd1, -2, -4, -5, -7, -8 and -10 also contain a cytosolic motif for the binding of proteins with a PDZ domain such as Dvl (Kay and Kehoe 2004). During embryonic development, Wnts and Fzds are expressed in overlapping domains, for example in the central nervous system, somites and hair follicle (Borello *et al*. 1999; Guo *et al*. 2004; Nunnally and Parr 2004; Kemp *et al*. 2005).

In addition to the Fzd receptors, two co-receptors, LRP5 and -6, participate in binding and transducing Wnt signals (reviewed in He *et al*. 2004). The LRP co-receptors are single-pass transmembrane proteins that have up to now been shown to be required for activation of the canonical Wnt pathway. Evidence suggests that the specific Wnt-Fzd or LRP-Wnt-Fzd interaction may dictate which signaling pathway is activated, however, with 19 Wnts, 10 Fzds and 2 LRPs, much has yet to be clarified (He *et al*. 1997; Sheldahl *et al*. 1999; Mikels and Nusse 2006).

Here we describe that the transmission of Wnt signals takes place at the cell membrane, however, recent studies indicate that the internalization of activated Fzd may be important for signaling. In the *Drosophila* embryo, Vincent and colleagues show that Wingless signaling is restricted

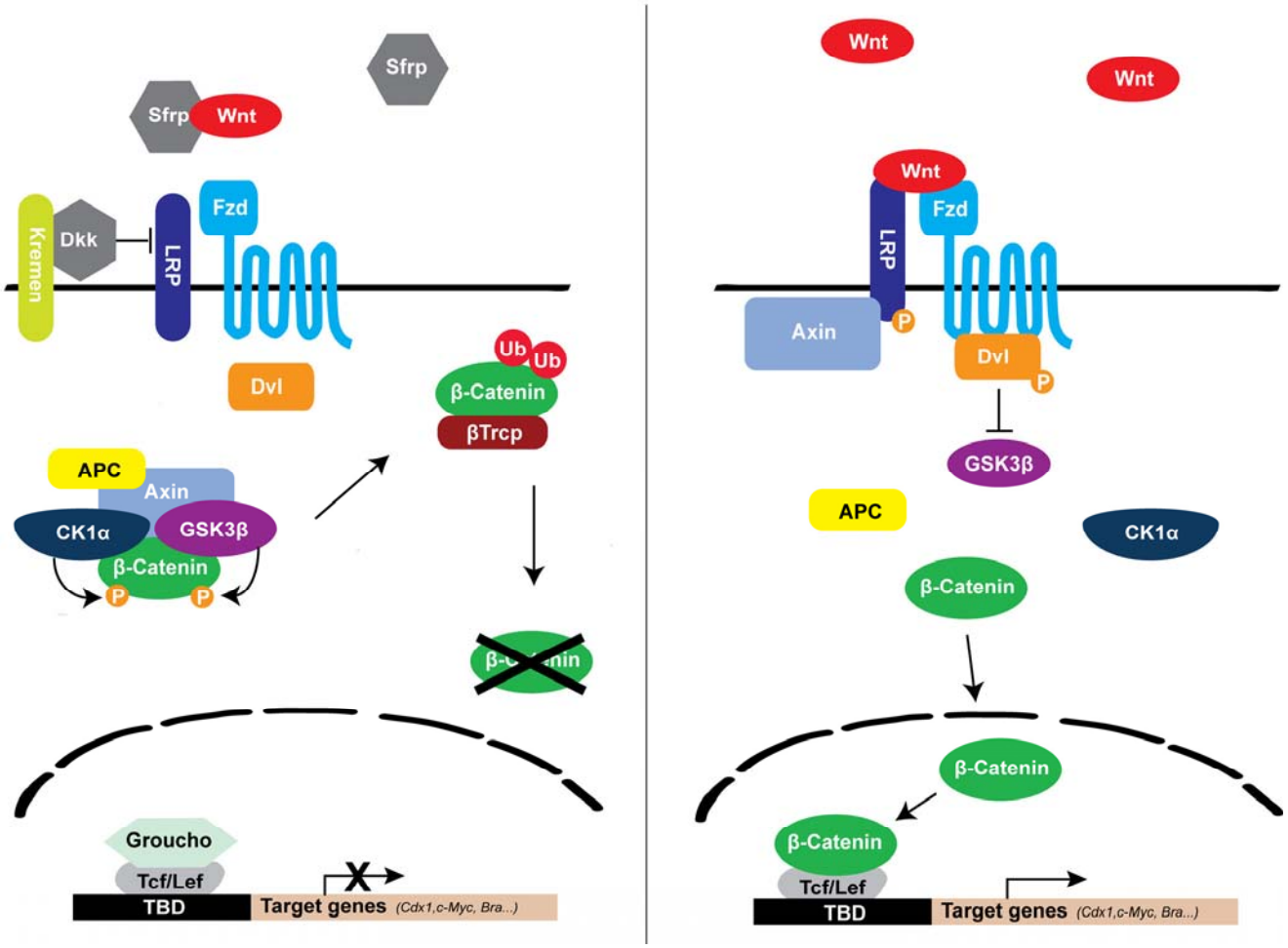

Fig. 1 Canonical Wnt signaling pathway in its active and inactive forms. The left panel depicts the lack of Wnt signaling due to antagonism by Sfrp or Dkk1, leading to the degradation of β-Catenin, while the right panel shows active Wnt signals being transmitted through Fzd and LRP to stabilize β-Catenin and permit target gene activation. TBD refers to the Tcf-binding domain.

by endocytosis, modulating its extracellular distribution to produce a morphogen gradient (Dubois *et al.* 2001; reviewed in Seto and Bellen 2004). Furthermore, this endocytosis is suggested to be receptor-mediated by frizzled2. In mammalian *in vitro* models, it has been shown that Fzd4 endocytosis is dependent on Wnt5a, while Wnt3a is necessary for LRP6 and Fzd5 internalization (Chen *et al.* 2003; Yamamoto *et al.* 2006). Although the signaling role of Fzd4 internalization has not yet been characterized, the internalization of the Fzd5-Wnt3a-LRP6 complex is necessary for Wnt3a-induced β-Catenin accumulation in the human cell line HeLaS3 (Yamamoto *et al.* 2006). Another role for the endocytosis of Fzd and LRP could include receptor down-regulation.

Although most Wnt signaling described involves the activation of Fzds, alternate Wnt receptors, as well as co-receptors, also exist. Ror2, a member of the receptor tyrosine kinase family, has an extracellular CRD similar to that of Fzds, and has been shown to mediate noncanonical Wnt5a signals by activating the Wnt/PCP pathway and/or inhibiting the β-Catenin pathway (Oishi *et al.* 2003). Ryk, on the other hand, acts as a co-receptor, and may form a ternary complex with Wnt1 and Fzd8 to activate the canonical Wnt pathway (Lu *et al.* 2004b).

Receptor-ligand interactions

As mentioned, the particular Wnt-Fzd or Fzd-Wnt-LRP interaction may be key in determining which Wnt pathway is activated. Because the CRDs of Fzds are implicated in Wnt-binding, a secreted form of this domain has been generated and commercialized, and often these Fzd CRDs are used in cell culture experiments to characterize their binding properties. Our lab has recently shown that the CRDs of Fzd5, -7 and -8 can block Wnt3a-induced activity in L cells. Addition of 0.5 nM Wnt3a alone to L cell cultures leads to the intracellular accumulation of β-Catenin, which can be quantified and indicates the activation of canonical signaling. With the addition of the Fzd CRDs to the culture medium, Wnt3a activity is reduced at half maximal inhibitory concentrations (IC50) of 2.0, 2.2 and 1.2 nM for Fzd5, -7 and -8, respectively (Kemp *et al.* in press). These results indicate that these three Fzds have the ability to bind Wnt3a through their CRD and may transduce its canonical signals *in vivo*.

Although quantification of β-Catenin accumulation serves as an indicator of canonical Wnt signaling, it does not confirm the translocation of β-Catenin to the nucleus and transcription of canonical Wnt target genes (Mikels and Nusse 2006a). Instead, canonical signaling can be evaluated using Tcf-reporter assays, which directly indicate the activation of the canonical Wnt pathway. Key examples of reported Wnt interactions and activities with their receptors are listed in **Table 1**. Studies to obtain these results often involve the overexpression of one or more of the molecules of interest and therefore caution must be taken when interpreting the data. It must also be noted that although Wnts, such as Wnt3a and -5a, may bind and signal through different Fzds, their activity *in vivo* is modulated by many factors such as the presence of antagonists, other receptors and molecules of the extra-cellular matrix. This is best demonstrated by Wnt5a activity, which is dependent on the expression of Fzd4 or Fzd4 with LRP5 (Mikels and Nusse 2006a). Wnt5a can induce β-Catenin accumulation in 293 cells transfected with Fzd4, but can also activate a Tcf-reporter if LRP5 is cotransfected with Fzd4. Moreover, Fzd6, -7 and

Table 1 Selected properties known for Fzd1-10 and LRP5 and -6. The known interactions or binding of Fzds, or the Fzd CRDs, and LRPs with their Wnt and non-Wnt ligands are presented. Inhibitory activities of the Fzd CRDs are listed as half maximal inhibitory concentrations (IC_{50}) with 0.5 nM Wnt3a (from Kemp *et al*. in press). Fzds and LRPs have not been shown to bind directly to each other, therefore, when listed under "Interacts with", note that the interaction is Wnt-dependent. Binding is demonstrated by either coimmunoprecipiation (Co-IP), or a dissociation constant (K_d). LRP6 is able to enhance Wnt- or Rspondin-induced Tcf-reporter activity in 293 cells, which express several Fzds, including *Fzd1* and -7 (Wang *et al*. 2005).

Gene Name	Interacts with	Activity	References
Fzd1	Wnt7b with LRP5	Induces β-Catenin accumulation and activates Tcf-reporter	Wang *et al*. 2005
	Wnt1,-3,-3a	Activates Tcf-reporter	Gazit *et al*. 1999
	Wnt3a,-5a	Binds (Co-IP)	Gazit *et al*. 1999
	Overexpression	Antagonizes Wnt3a-induced Tcf-reporter activity	Roman-Roman *et al*. 2004
Fzd2	Wnt3a	Activates Tcf-reporter	Liu *et al*. 2005
Fzd3	Wnt4	Axon guidance	Lyuksyotuvoa *et al*. 2003
Fzd4	Wnt5a	Induces β-Catenin accumulation	Mikels *et al*. 2006
	Wnt5a with LRP5	Activates Tcf-reporter	Mikels *et al*. 2006
	Wnt5a with Ror2	Antagonizes Wnt3a-induced Tcf-reporter activity	Mikels *et al*. 2006
	Wnt5a	Endocytosis of Wnt-Fzd	Chen *et al*. 2003
	Norrin	Binds, K_d = 3-4 nM	Xu *et al*. 2004
	Norrin with LRP5/6	Activates Tcf-reporter	Xu *et al*. 2004
Fzd5	Wnt3a	Endocytosis of Wnt-Fzd	Yamamoto *et al*. 2006
	Wnt3a with LRP6	Synergized endocytosis of LRP-Wnt-Fzd	Yamamoto *et al*. 2006
	Wnt5a	Endocytosis of Wnt-Fzd	Kurayoshi *et al*. 2006
Fzd5 CRD	Wnt3a	Antagonizes Wnt3a-induced β-Catenin accumulation, IC_{50} = 2.0 nM	Kemp *et al*. in press
	Wnt5a	Binds, K_d = 5 nM	Kurayoshi *et al*. 2006
Fzd6 CRD	Wnt4	Binds (Co-IP)	Lyons *et al*. 2004; Golan *et al*. 2004
Fzd7	Wnt3a	Activates Tcf-reporter	Liu *et al*. 2005
Fzd7 CRD	Wnt3a	Antagonizes Wnt3a-induced β-Catenin accumulation, IC_{50} = 2.2 nM	Kemp *et al*. in press
Fzd8	Wnt3a	Activates Tcf-reporter	Liu *et al*. 2005
Fzd8 CRD	Rspondin1,-3	Binds (Co-IP)	Nam *et al*. 2006
	Wnt3a	Antagonizes Wnt3a-induced β-Catenin accumulation, IC_{50} = 1.2 nM	Kemp *et al*. in press
	Wnt1	Binds (Co-IP)	Lu *et al*. 2004
	Ryk	Binds (Co-IP)	Lu *et al*. 2004
Fzd9	Wnt2	Activates Tcf-reporter	Karasawa *et al*. 2002
Fzd10	Wnt7b with LRP5	Induces β-Catenin accumulation and activates Tcf-reporter	Wang *et al*. 2005
LRP5	Wnt1	Binds (Co-IP)	Kato *et al*. 2002
	Wnt4	Binds (Co-IP)	Kato *et al*. 2002
LRP6	Wnt3a	Binds (Co-IP)	Liu *et al*. 2003
	Wnt3a	Endocytosis of Wnt-LRP and activates Tcf-reporter in 293 cells	Yamamoto *et al*. 2006
	Wnt3a with Fzd5	Endocytosis of LRP-Wnt-Fzd and Fzd5 enhances activation of Tcf-reporter	Yamamoto *et al*. 2006
	LRP6	Formation of a homodimer	Liu *et al*. 2003
	Rspondin1,-3	Binds (Co-IP)	Nam *et al*. 2006
	Rspondin1,-2,-3,-4	LRP6 enhances activation of Tcf-reporter in 293 cells	Nam *et al*. 2006

-8, as well as LRP6, cannot transduce the Wnt5a-mediated canonical Wnt signaling in the same system.

Wnt activity modulators

Two known mechanisms exist extracellularly to antagonize Wnt signaling. Frzb and its four homologs, forming the secreted Frizzled related protein (Sfrp; also written sFRP) family, as well as Wnt inhibiting factor 1 (Wif1), are able to bind Wnts directly to inhibit their interaction with, and thus activation of, the Fzd receptors (Leyns *et al*. 1997; Hsieh *et al*. 1999a; Jones and Jomary 2002; Kawano and Kypta 2003). The second mechanism involves an antagonist binding to an LRP co-receptor, leading to their cellular internalization and prevention of Wnt signaling. Dickkopf1 (Dkk1) and Wise can antagonize Wnt signals in such a way (Brott and Sokol 2002; Itasaki *et al*. 2003; Niehrs 2006).

The five members of the Sfrp family are divided into two subgroups based on their amino acid sequence similarity, which may also relate to their function (Wawrzak *et al*. 2007). They all have an N-terminus CRD similar to that of Fzds and a C-terminus Netrin-like domain (Lin *et al*. 1997; reviewed in Jones and Jomary 2002). Members of the Sfrp family bind to Wnts, but this does not always result in antagonism of Wnt signals. A *Drosophila* cell assay demonstrated that low concentrations of Sfrp1 (20 ng/ml to 500 ng/ml) could in fact augment Wingless-induced accumulation of Armadillo, the *Drosophila* β-Catenin ortholog (Uren *et al*. 2000). In addition, Sfrps may also interact with Fzds, as for example, Sfrp1 can bind Fzd2 to mediate axon guidance (Bafico *et al*. 1999; Rodriguez *et al*. 2005a).

The Dkk family consists of four members, whose genes encode for secreted glycoproteins (reviewed in Niehrs 2006). Dkks share two conserved cysteine-rich domains, of which the C-terminal one binds to LRP6 and is necessary and sufficient for antagonizing canonical Wnt signals. Moreover, Dkk2 can activate Wnt/β-Catenin signaling *in vitro* depending on the cellular context. Dkk3, however, is a functionally divergent member of the Dkk family and may not modulate Wnt activity at all. Dkk1 can block Wnt signaling by forming a ternary complex with LRP6 and a Kremen (Krm) co-receptor (Mao *et al*. 2002). This complex (LRP6-Dkk1-Krm) is subsequently endocytosed, depleting the supply of LRP6 from the membrane and thus preventing LRP6-mediated Wnt signaling.

Binding properties of Wnt modulators

To shed light on their biological roles, many studies have been performed to determine the interactions of Sfrp and Dkk family members with Wnts, or the LRP or Kremen receptors. **Table 2** summarizes the known molecular interactions of the Wnt modulators. Using Surface Plasmon Resonance, we have shown that purified Sfrps can directly bind to Wnt3a and -5a (Wawrzak *et al*. 2007). Sfrp1 and -2 bind to Wnt3a or -5a with affinities in the nanomolar range, while Frzb and Sfrp4 can only bind to Wnt3a. In addition, two Wnt3a binding sites possibly exist for Sfrp1, -2 and -4 (note the two K_d values in **Table 2**). Furthermore, Sfrp1 and -2 can antagonize Wnt3a-induced β-Catenin accumulation in a dose dependent manner, whereas Frzb and Sfrp4 have no effect on Wnt3a signaling (Galli *et al*. 2006; Wawrzak *et al*. 2007). These results support the idea that the members of the Sfrp family may form two functional sub-

Table 2 Selected properties known for Sfrps and Dkks. The known dissociation constants (K_d) of Sfrps and Dkks with Wnts or receptors (LRPs, or Kremens) are listed. Often the antagonist has two K_d values listed, indicating that two Wnt or Kremen molecules may bind. Interactions determined by coimmunoprecipitation (Co-IP) are also noted. Inhibitory activities of the Wnt antagonists are listed as half maximal inhibitory concentrations (IC_{50}) with 0.5 nM Wnt3a for Sfrp1 and -2. The IC_{50} for Dkk1 with LRP6 was determined by transfection of 293 cells with *Wnt3a*. Dkk activities were determined in 293 cells transfected or not with Wnt, Fzd and LRP.

Gene name	Interacts with	Affinity	Activity	References
Sfrp1	Wnt1	-	Antagonizes β-Catenin accumulation and Tcf-reporter activity	Bafico *et al*. 1999
	Wnt2	-	Antagonizes β-Catenin accumulation	Bafico *et al*. 1999
	Wnt3a	K_{d1} = 11.2 nM K_{d2} = 85.9 nM	Antagonizes β-Catenin accumulation IC_{50} = 8.5 nM	Galli *et al*. 2006; Wawrzak *et al*. 2007
	Wnt5a	K_d = 3.7 nM	-	Wawrzak *et al*. 2007
	Fzd2	-	Axon guidance	Rodriguez *et al*. 2005
	Fzd6 CRD	Binds (Co-IP)	-	Bafico *et al*. 1999
Sfrp2	Wnt3a	K_{d1} = 4.1 nM K_{d2} = 26.8 nM	Antagonizes β-Catenin accumulation IC_{50} = 2.5 nM	Galli *et al*. 2006; Wawrzak *et al*. 2007
	Wnt5a	K_d = 5.1 nM	-	Wawrzak *et al*. 2007
	Wnt4	Binds (Co-IP)	-	Lescher *et al*. 1998
Frzb	Wnt3a	K_d = 7.9 nM	Does not affect Wnt3a signaling	Galli *et al*. 2006; Wawrzak *et al*. 2007
	Wnt5a	Does not bind	-	Wawrzak *et al*. 2007
Sfrp4	Wnt3a	K_{d1} = 8 nM K_{d2} = 38.6 nM	Does not affect Wnt3a signaling	Wawrzak *et al*. 2007
	Wnt5a	Does not bind	-	Wawrzak *et al*. 2007
Dkk1	LRP6	K_d = 0.3-0.5 nM	Antagonizes Wnt3a-induced Tcf-reporter activity IC_{50} = 0.3-1 nM	He *et al*. 2004; Bafico *et al*. 2001
	Kremen1	K_{d1} = 0.3 nM K_{d2} = 2.6 nM	Synergistic antagonism of Tcf-reporter activity with exogenous *Wnt1* and *Fzd8*	Mao *et al*. 2002
	Kremen2	K_d = 0.28 nM	Synergistic antagonism of Tcf-reporter activity with exogenous *Wnt1* and *Fzd8*	Mao *et al*. 2002
	Kremen2 and LRP6	-	Endocytosis of Kremen-Dkk-LRP	Mao *et al*. 2002
Dkk2	LRP6	K_d = 10 nM	Activates Tcf-reporter without exogenous Wnt or Fzd	Mao and Niehrs 2003; Niehrs 2006
	Kremen1	K_{d1} = 0.36 nM K_{d2} = 3.0 nM	-	Mao *et al.* 2002
	Kremen2	K_d = 0.35 nM	Antagonizes Tcf-reporter with exogenous *LRP6*, *Wnt1* and *Fzd8*	Mao and Niehrs 2003
Dkk4	LRP6	Binds	-	Niehrs 2006
	Kremen2	-	Antagonizes Tcf-reporter with *Wnt1* and *Fzd8*	Mao and Niehrs 2003

groups, one with Frzb and Sfrp4 and the other with Sfrp1, Sfrp2 and possibly Sfrp5.

Alternative Wnt pathway activators

As our knowledge of the Wnt pathways grows, so does the complexity, as new molecules are identified and shown to participate in Wnt signaling. While studying the defects of mice lacking *Fzd4*, Xu *et al*. (2004) looked to *Norrin*, in which gene targeting resulted in similar vascular phenotypes as *Fzd4* mutants. They demonstrate that transfection of 293 cells with *Norrin*, *Fzd4* and *LRP5* or *-6* can highly activate a canonical Wnt reporter. These results identify Norrin, with no sequence similarties to Wnts, as a novel ligand for canonical Wnt signaling, and suggest that other ligands may exist for the Wnt receptors and LRP co-receptors. Indeed, members of the Cristin/R-spondin family of secreted proteins were more recently shown to interact with Fzd8 and LRP6 and to activate β-Catenin-dependent gene transcription (Nam *et al*. 2006). The cooperation of Wnt and non-Wnt ligands may modulate β-Catenin activation or may lead to the transcriptional activation of different β-Catenin dependent genes.

ROLES OF WNTS DURING DEVELOPMENT

Overview of early mouse embryonic development

To better understand the roles of Wnt signaling during development, a brief description of how the primary body axes of the mouse embryo are formed is presented. **Fig. 2** gives the reader a visual view of mouse development, including some basic cellular movements.

Three and a half days after fertilization of the oocyte a blastocyst is formed. The blastocyst consists of two cell types, the trophectoderm and the inner cell mass, and a blastocoel cavity. While the trophectoderm, the outer cell layer, gives rise to extraembryonic tissues, the pluripotent inner cell mass is the source of all embryonic as well as some extraembryonic tissues. The location of the inner cell mass defines the embryonic-abembryonic axis, which will later become the respective proximal-distal axis (reviewed in Zernicka-Goetz 2002).

When the blastocyst begins to implant into uterine tissue, at about 4.5 days post coitum (dpc), the primitive endoderm has formed on the surface of the inner cell mass facing the blastocoel cavity, and the dorsal-ventral axis of the embryo is already apparent. The cells of the inner cell mass are now considered epiblast and divide, expanding into the blastocoel cavity, to form a cup-like structure, called the egg cylinder, with a central proamniotic cavity. The primitive endoderm differentiates to form parietal endoderm and visceral endoderm, of which the visceral endoderm directly opposes the epiblast or embryonic ectoderm. The proximal-distal axis of the embryo at this point is defined in relation to the extraembryonic ectoderm.

At 5.5 dpc, the distal cells of the visceral endoderm, better known as the distal visceral endoderm (dVE), change shape and subsequently actively migrate to a more proximal location to become the anterior visceral endoderm (aVE), which marks the prospective anterior side of the embryo and thus allows the identification of the anterior-posterior axis (reviewed in Beddington and Robertson 1999). Gastrulation then commences at about 6.5 dpc as the epiblast forms the primitive streak opposite to the aVE. In the primitive streak, epiblast cells ingress between the epiblast and visceral endoderm and undergo an epithelial-mesenchyme transition to become mesoderm (Tam *et al*. 2006). A region of the posterior embryo at this stage has been shown to have organizing properties capable of axis induction when transplanted, similar to the Spemann organizer in frog, and is known as the early gastrula organizer (EGO) (Tam

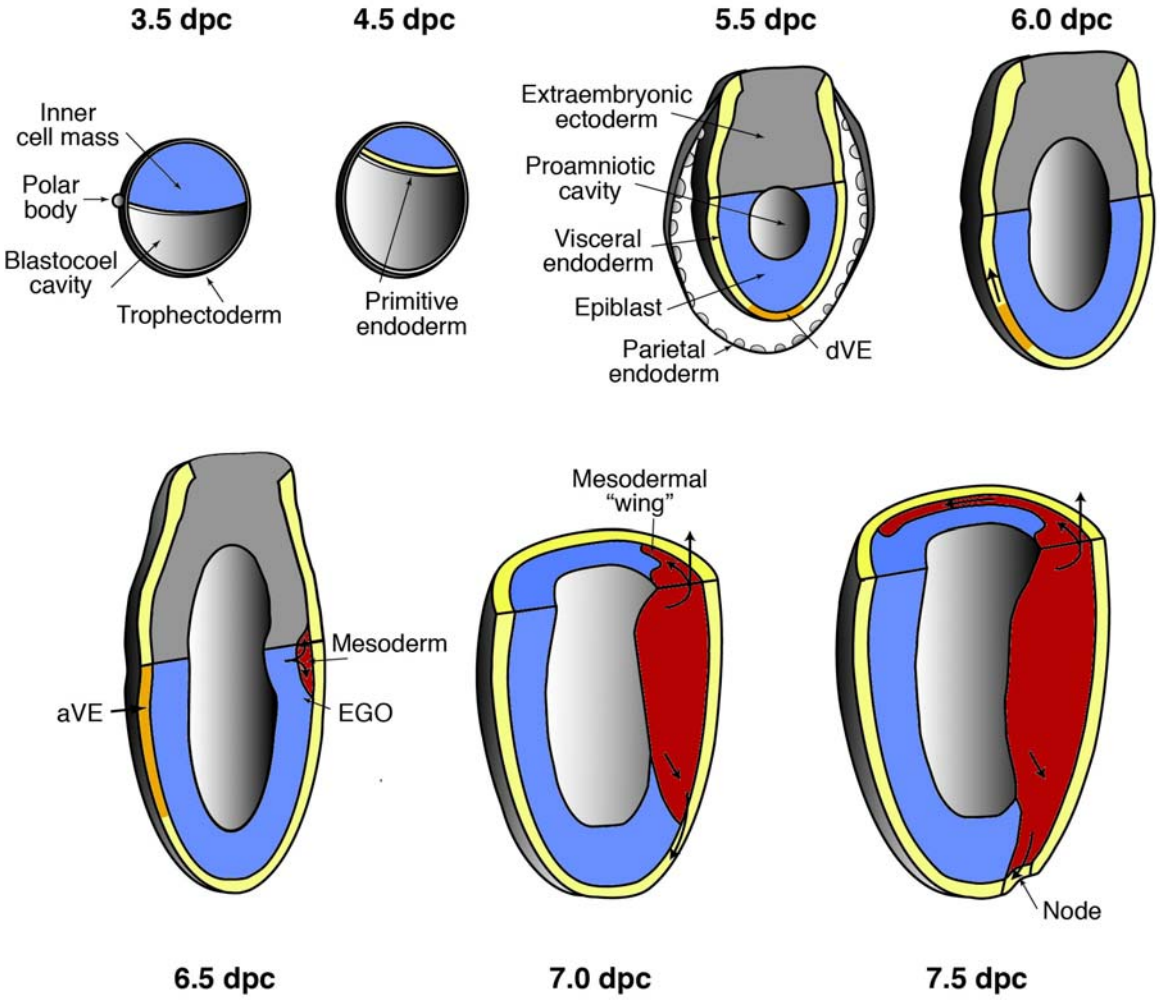

Fig. 2 Early mouse embryonic development. As described in the text, the blastocyst at 3.5 dpc consists of two tissues: the inner cell mass and trophectoderm. At 4.5 dpc the primitive endoderm is formed and the embryo starts to implant into the uterine wall. By 5.5 dpc, the embryo is cup-shaped and is often described according to the location of the extraembryonic ectoderm, and thus embryonic regions are located proximal or distal to this tissue. Extraembryonic and embryonic regions are separated by a line. The distal-most cells of the visceral endoderm (dVE) change shape at 5.5 dpc and begin their proximal migration. The parietal endoderm is also depicted at this stage but is removed from later stages for simplification. Gastrulation commences at 6.5 dpc with the formation of mesoderm and the primitive streak. At this stage, the anterior visceral endoderm (aVE) marks the anterior of the embryo and the mesoderm at the opposite side of the embryo, marks the posterior. The cells just anterior to the mesoderm have organizing properties and this region is called the early gastrula organizer (EGO). The extraembryonic region of the embryo is not depicted at 7.0 and 7.5 dpc to allow better visualization of mesoderm migration. By 7.5 dpc, the mesodermal wings have almost met at the anterior midline, and the node has formed at the tip of the embryo.

and Steiner 1999). The secondary axis that is induced, however, is truncated anteriorly, and grafting experiments show that both anterior embryonic tissues and the EGO are required for complete neural axis formation (Tam and Steiner 1999). As gastrulation continues the primitive streak elongates distally and mesoderm moves proximally into the extraembryonic region. The mesoderm also migrates laterally to form "wings" while the ingression of cells through the anterior primitive streak leads to the displacement of aVE by definitive endoderm. The mesodermal "wings" eventually meet at the anterior midline and form cardiogenic mesoderm.

At 7.5 dpc the primitive streak has reached the distal tip of the embryo and the node is formed. Cells coming from the node differentiate to become axial mesoderm and gut endoderm. The node also has axis-inducing, or organizing, properties similar to the EGO. Moreover, when grafted alone the ectopic axis also lacks anterior-most tissues (Beddington 1994). At 8.5 dpc the mouse embryo rotates and organogenesis commences.

Wnt signaling in embryonic axis formation

Many components of the Wnt pathway, including Wnts themselves, are already expressed at the blastocyst stage of development, but no evidence as yet shows that the pathway is active or necessary before 5.5 dpc (Lloyd *et al*. 2003; Kemp *et al*. 2005). Studies of embryos mutant for *Apc*, leading to the constitutive activation of β-Catenin, suggest, however, that β-Catenin at the perimplantation stage must be tightly regulated for proper patterning of the visceral endoderm and epiblast (Chazaud and Rossant 2006). Additionally, overexpression of *Wnt8a*, a canonical Wnt, throughout the epiblast of the pregastrula embryo can prevent the proximal migration of the dVE (Kimura-Yoshida *et al*. 2005).

Normally, the dVE at 5.5 dpc can be characterized by the restricted expression of genes such as *Hhex*, *Otx2*, and *Cer1*. In embryos lacking β-*Catenin* the dVE is formed but it fails to move proximally and the embryo never acquires an anterior-posterior axis. In fact, although *Cer1* expression is detected in the dVE, *Hex* is not, suggesting β-*Catenin* also plays a role in the molecular patterning of the dVE (Huelsken *et al*. 2000). In addition to the dVE defects, β-*Catenin* null embryos fail to form mesoderm (Haegel *et al*. 1995). Moreover, both dVE migration and mesoderm formation are dependent on β-*Catenin* expression specifically in the epiblast (Haegel *et al*. 1995; Huelsken *et al*. 2000).

Table 3 Knock-out and transgenic mouse models. Misregulation of canonical Wnt as well as Nodal signaling leads to embryonic axis defects. While upregulation of β-Catenin signaling or Nodal signaling (Cer1$^{-/-}$;Lefty1$^{-/-}$) often leads to the duplication of axial structures, downregulation usually results in no anterior-posterior (AP) axis forming. In both cases the mesoderm is either expanded or absent, respectively. Defects in the formation, patterning or migration of the distal visceral endoderm (dVE) are also observed in the mutants. The *APC^{min}* mutant is a result of a spontaneous mutation leading to the production of a truncated protein that lacks all β-Catenin binding domains. The *Axin* mutant was produced by a transgenic insertional allele resulting in the disruption of major mRNA production. In the *APC* hypomoph, *APC* expression is attenuated by as much as 80%. Mutation of *Nodal* results in embryos lacking the dVE and mesoderm, and Cripto is a Nodal co-receptor, while Cer1 and Lefty1 are two Nodal antagonists.

Gene	Mesoderm	dVE	Axis	Reference
Upregulation of β-Catenin signaling				
APC$^{min/min}$	Expanded	Absent	No AP axis	Chazaud and Rossant 2006
Cwnt8c Tg	Expanded	-	Duplication with anterior truncation	Pöpperl *et al*. 1997
Axin$^{Tg1/Tg1}$	-	-	Duplication with anterior truncation	Zeng *et al*. 1997
APC Hypomorph	-	-	Duplication with anterior truncation	Ishikawa *et al*. 2003
Activated β-Catenin	Expanded	Absent	No AP axis	Kemler *et al*. 2004
Downregulation of β-Catenin signaling				
β-Catenin$^{-/-}$	Absent	No migration	No AP axis	Haegel *et al*. 1995; Huelsken *et al*. 2000
Wnt3$^{-/-}$	Absent	OK migration	AP not patterned	Liu *et al*. 1999
Lrp5$^{-/-}$;Lrp6$^{-/-}$	Absent	OK migration	AP not patterned	Kelly *et al*. 2004
Nodal Signaling				
Nodal$^{-/-}$	Absent	Absent	No AP axis	Varlet *et al*. 1997
Cripto$^{-/-}$	Extra-embryonic only	No migration	Lacks posterior structures	Ding *et al*. 1998
Cer1$^{-/-}$;Lefty1$^{-/-}$	Expansion	Expansion	Duplication of primitive streak	Perea-Gomez *et al*. 2002

One clue to the molecular basis of dVE migration comes from studies of *Dkk1*. Before the movement of the dVE, *Dkk1* is expressed in the proximal visceral endoderm and subsequently in the future aVE (Kimura-Yoshida *et al*. 2005). In an attempt to clarify the mechanism behind dVE migration, protein-soaked beads where used in whole-embryo cultures to show that Dkk1 acts as an attractive guidance cue for the dVE, whereas in contrast, Wnt3a can act as a repulsive cue (Kimura-Yoshida *et al*. 2005). These results intimate that Wnt/β-Catenin antagonism is necessary in the proximal visceral endoderm to guide the migration of the dVE. Although *Dkk1* null embryos gastrulate, other Wnt antagonists are present in the aVE at this early stage of development, including *Sfrp1* and *-5* (Mukhopadhyay *et al*. 2001; Finley *et al*. 2003; Kemp *et al*. 2005). Embryos mutant for *Sfrp1*, *Sfrp5* or *Dkk1;Sfrp5*, however, do not have defects in the aVE, but it is possible that the double (*Sfrp1;Sfrp5*) or triple (*Dkk1;Sfrp1;Sfrp5*) knock-out would (Leaf *et al*. 2006; Satoh *et al*. 2006). Furthermore, signals from the extraembryonic region are necessary for the proper patterning and migration of the dVE (Rodriguez *et al*. 2005b; Richardson *et al*. 2006). *Wnt7b* is expressed in this region and could therefore also participate in guiding dVE migration (Kemp *et al*. in press). The expression of *Fzd5* and *-8* in the dVE also suggests a role for Wnt signaling in dVE migration (Lu *et al*. 2004a; Kemp *et al*. in press).

As the dVE migrates to its anterior position, *Wnt3* is expressed in the posterior visceral endoderm (Rivera-Perez and Magnuson 2005). *Wnt3* expression then expands to include the posterior epiblast, which is where the primitive streak will form. *Wnt2b*, *-5a*, *-8a* along with *Fzd10*, are expressed in this posterior domain as gastrulation is initiated, possibly further defining the anterior-posterior axis of the embryo (Bouillet *et al*. 1996; Zakin *et al*. 1998; Yamaguchi *et al*. 1999; Kemp *et al*. 2005). Identification of active canonical Wnt signaling in the forming primitive streak has been demonstrated using a transgenic reporter mouse line. In fact several mouse lines have been generated that carry a Wnt reporter transgene, typically containing multiple Tcf binding sites upstream of the β-*galactosidase* gene (Maretto *et al*. 2003; Merrill *et al*. 2004). In the resulting transgenic embryos, β-galactosidase activity is detected at 6.0 dpc in the posterior proximal epiblast, similar to the expression pattern of *Wnt3*, and subsequently becomes localized in the primitive streak. Gene inactivation of *Wnt3* results in embryos that fail to generate mesoderm, however, the aVE is correctly patterned (Liu *et al*. 1999). This, together with the β-*Catenin* null phenotype and the localized activity of the Tcf reporter in transgenic embryos, suggests that Wnt3 is the signal activating β-Catenin for mesoderm formation.

Evidence indicates up to now that canonical Wnt signaling is dependent on the LRP co-receptors. Although individual mutation of *LRP5* or *-6* does not result in early gastrula phenotypes, double *LRP5;LRP6* knock-out embryos fail to form mesoderm, similar to *Wnt3* deficient embryos (Kelly *et al*. 2004). The anterior expression of *Hhex* and *Cer1* transcripts in *LRP5;LRP6* mutants indicates initial aVE patterning. These results suggest that the dVE migration, although dependent on β-Catenin, may be independent of a canonical Wnt ligand. However, the possibility of a specific role for canonical Wnt signaling, independent of LRP5 or -6, in the patterning and migration of the dVE cannot be dismissed.

The role of Wnt signals in mesoderm induction has also been studied by ectopically activating the canonical Wnt pathway in the pregastrula embryo. In a gain-of-function transgenic mouse model, misexpression of chick *wnt8c* (*Cwnt8c*), the ortholog of *Wnt8a* in mouse, results in the partial duplication of axial structures (Popperl *et al*. 1997). Formation of the ectopic axis was caused by the duplication of the primitive streak during gastrulation as determined by the expanded expression of *Brachyury*, a mesodermal marker. Mutation of *Axin*, a negative regulator of β-Catenin, leads to axial duplications, further supporting the role of canonical Wnt signaling in the formation of mesoderm (Gluecksohn-Schoenheimer 1949; Zeng *et al*. 1997). These experiments also demonstrate the importance of tightly regulating Wnt and/or β-Catenin signaling for proper anterior-posterior patterning of the embryo.

To summarize the results above, **Table 3** reviews the different knock-out and transgenic models that result in dVE, aVE or mesodermal defects. In addition to members of the Wnt signaling pathway, components of the Nodal pathway are shown, as they are also important for the formation of these gastrula regions.

Just as Wnt signaling is necessary for mesoderm formation, inhibition of Wnt signaling appears to be equally as important for anterior neural development. Grafting experiments show that anterior patterning is dependent on the interaction of the EGO with anterior tissues, including the aVE (Tam and Steiner 1999). The lack of anterior-most tissues when canonical Wnt signaling is upregulated, in the *Cwnt8c* transgenic for example, supports the idea that Wnt signals need to be down-regulated for anterior development. The expression of Wnt antagonists in the aVE, including *Dkk1*, *Sfrp1* and *Sfrp5*, further suggests that suppression of posterior signals is necessary for development of the ante-

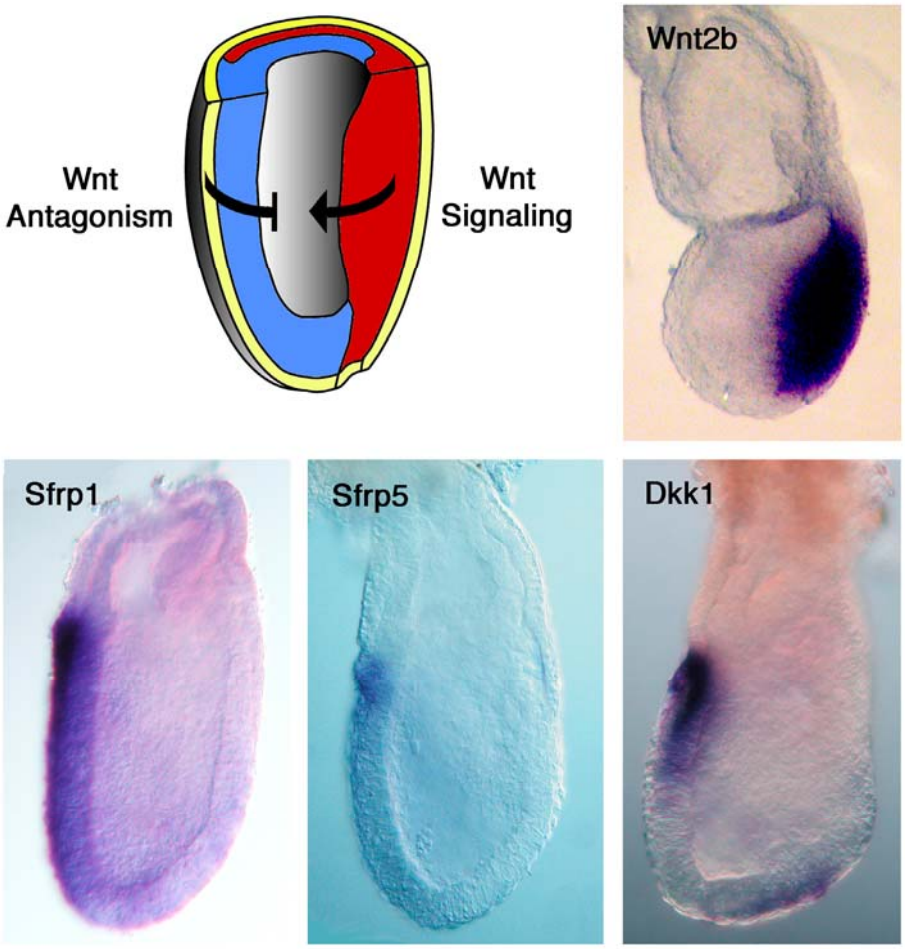

Fig. 3 A model for anterior-posterior patterning in the mouse embryo. In this model, posteriorizing Wnt signals, such as Wnt2b, are inhibited by antagonists, such as Sfrp1 and -5 and Dkk1, in the anterior region of the embryo to promote proper patterning of the anterior-posterior axis.

rior neurectoderm. Moreover, embryos lacking *Dkk1* fail to form head structures anterior to the midbrain (Glinka *et al*. 1998; Mukhopadhyay *et al*. 2001). A model for anterior-posterior patterning, requiring anteriorly expressed Wnt antagonists and posteriorly expressed Wnts, is shown in **Fig. 3**. In this model the posteriorizing signals of Wnt2b and -3 are counteracted by Sfrps and Dkk1 to promote anterior development.

Other Wnt functions in the embryo

As the embryo continues to develop, the Wnt pathway becomes important in many different processes. In the central nervous system, for example, at least seven Wnts are expressed in discrete regions of the neural tube (Parr *et al*. 1993). Studies show that Wnt1 and Wnt3a play redundant and proliferative roles dependent on β-Catenin in the central nervous system, however their role in patterning has yet to be elucidated (McMahon and Bradley 1990; Takada *et al*. 1994; Ikeya *et al*. 1997). More surprising roles for Wnts, such as Wnt4, -7a and -7b, in the central nervous system include axon remodeling and guidance (Hall *et al*. 2000; Lyuksyutova *et al*. 2003; reviewed in Zou 2004; Rosso *et al*. 2005). Recent studies show that axon guidance by Wnts can be mediated by Fzd and/or Ryk, a Wnt coreceptor (Lyuksyutova *et al*. 2003; Lu *et al*. 2004b). Whereas Fzd receptors act as an attractive cue, Ryk acts as a repulsive one (Schmitt *et al*. 2006).

The overlapping expression patterns of Wnts, their receptors and modulators in somites, osteoblasts, and cartilage, to name only a few tissues, indicate that Wnt has many diverse roles in development (Parr *et al*. 1993; Borello *et al*. 1999; Glass *et al*. 2005; Spater *et al*. 2006). Gene targeting experiments have also revealed the dependence on Wnt signaling for the proper formation of organs such as the lung and kidneys (Stark *et al*. 1994; Parr *et al*. 2001; Shu *et al*. 2002). More recently, noncanonical roles of Wnts during development have also been revealed. Interestingly, mutation of *Fzd6* leads to defects in hair orientation, producing whorls of hair on the head and feet (Guo *et al*. 2004). Hair patterning is dependent on PCP signaling cues and the *Fzd6* mutant phenotype strikingly resembles the wing hair defects seen in *Drosophila frizzled* mutants. Moreover, Fzd6 is functionally redundant with Fzd3, and double mutant embryos fail to undergo neural tube closure, a process dependent on convergent extension movements, also PCP-dependent (Wang *et al*. 2006).

WNTS AND ES CELL DIFFERENTIATION

Overview of embryonic stem cells

Mouse embryonic stem (ES) cells were derived from the inner cell mass of a blastocyst for the first time in 1981 (Evans and Kaufman 1981; Martin 1981). Human ES cells, however, were not obtained until 1998 (Thomson *et al*. 1998). These cells have two important characteristics: they can be propagated unlimitedly *in vitro* and they are pluripotent, meaning that they can be differentiated *in vitro* and *in vivo* to give rise to almost any cell type. When ES cells are injected into a pre-implantation embryo, they can participate again in development and can contribute to all fetal lineages (Beddington and Robertson 1989; reviewed in Smith 2001). Moreover, when injected into adult mice, ES cells can form teratomas, or benign tumors, which contain cell types derived from all three embryonic germ layers (endoderm, ectoderm and mesoderm).

Since the derivation of ES cells, many researchers have investigated the molecular basis of their pluripotency and self-renewal. Initially, the isolated cells needed to be cultured on a layer of fibroblasts feeder cells to maintain their undifferentiated state. Later it was discovered that a secreted factor, leukemia inhibitory factor (LIF), could substitute for the presence of these feeder cells (Williams *et al*.

1988). Intracellular factors, such as Oct3/4 (also known as Pou5f1) and Nanog, have also been shown to be involved in the maintenance of pluripotency (Niwa *et al*. 2000; Mitsui *et al*. 2003).

Differentiation of ES cells can be carried out *in vitro* as mono-adherent cultures or as embryoid bodies, which are tight clumps of ES cells that spontaneously form when plated on a non-adherent substrate. In both cases the cells are cultured in the desired medium to which different factors of interest can be added.

Wnts play roles in several aspects of stem cell biology that often reflect their roles in embryonic development, including mesoderm differentiation, inhibition of neural induction as well as neural patterning. Moreover, Wnts have also been implicated in the maintenance of ES cell pluripotency. Difficulties in obtaining active Wnt proteins have hindered studies of Wnt signaling, nevertheless, the availability of purified Wnt3a has been exploited as a representative of canonical Wnt signaling in the study of ES cell biology.

Wnts maintain pluripotency

Although no functional evidence exists, the expression of several Wnts and components of the Wnt pathway in the blastocyst indicate that Wnts possibly play a role in self-renewal and the maintenance of pluripotency in the early embryo or in the implantation of the blastocyst (Mohamed *et al*. 2004; Kemp *et al*. 2005). Several lines of research demonstrate that the Wnt pathway can contribute to maintaining ES cells in their undifferentiated state and are described below.

While LIF can sustain pluripotency in mouse ES cells, it is not sufficient for human ES cells. Wnt3a or BIO, a pharmacological inhibitor of GSK3βthat induces the upregulation of canonical Wnt signaling, can participate in maintaining the self-renewal of both human and mouse ES cells in the absence of LIF (Sato *et al*. 2004; Dravid *et al*. 2005; Ogawa *et al*. 2006; Singla *et al*. 2006; Miyabayashi *et al*. 2007). Moreover, mouse ES cells cultured in the presence of BIO and subsequently injected into mice can induce teratomas, and when these cells are injected in mouse blastocysts, chimeric mice can be generated (Sato *et al*. 2004). These results demonstrate a role for canonical Wnt signaling in maintaining pluripotency of both human and mouse ES cells.

Other studies provide evidence for the activity of LIF to influence the canonical Wnt pathway. *Myc*, a transcriptional target of canonical Wnt signaling, is expressed at high levels in mouse ES cells cultured with LIF. Upon LIF withdrawal, *Myc* mRNA levels decrease and Myc protein is degraded. It has been shown that maintained expression of *Myc* makes self-renewal LIF-independent, while a dominant negative form of *Myc* promotes differentiation (Cartwright *et al*. 2005). More recently, it has been reported that LIF can enhance the levels of nuclear β-catenin in ES cells (Takao *et al*. 2007). In addition, the expression of an activated mutant form of β-*catenin* can maintain the expression of self-renewal markers in ES cells, even in the absence of LIF. Together, these reports suggest that the LIF and Wnt signaling pathways may cooperate to promote pluripotency.

Even though the canonical Wnt pathway has been shown to contribute to ES cell pluripotency, several lines of evidence suggest that it may be redundant with other signaling pathways. ES cells that are homozygous mutant for β-*Catenin*, can be maintained in culture, and can contribute to the generation of chimeric embryos when injected in low numbers (Huelsken *et al*. 2000). Thus, β-*Catenin* is not essential for the maintenance of pluripotency. Moreover, Wnts are not the only signaling factors involved in maintaining the pluripotency of mouse and human ES cells (Dravid *et al*. 2005). More specifically, addition of BMP, bFGF, Activin and/or Nodal to mouse and human ES cell cultures can, similarly to Wnt3a or BIO, maintain the ES cells in their undifferentiated state (Ying *et al*. 2003a; James *et al*. 2005; Vallier *et al*. 2005; Xu *et al*. 2005). Thus, the exact role of Wnt signaling in ES cell pluripotency and its relationship to other signaling pathways has yet to be established but is still under avid investigation.

Wnt signals promote mesodermal differentiation

The importance of Wnt signaling for embryonic mesoderm induction is reflected in ES cell mesodermal differentiation. Using embryoid bodies as a model of the embryo, Keller and his coworkers have shown that Wnt3a cooperates with TGFβ signaling to induce mesoderm, identified by *Brachyury* expression, in differentiating embryoid bodies (Gadue *et al*. 2006). While these experiments involve the addition of growth factors to a serum-free culture medium to induce mesoderm, when embryoid bodies are cultured in serum-containing medium, spontaneous mesoderm induction occurs. Dkk1 has been shown to antagonize spontaneous mesoderm formation, while BMP4, a member of the TGFβ superfamily, can rescue the induction of mesoderm (Lindsley *et al*. 2006). Both studies confirm that Wnt and TGFβ pathways cooperate in mesoderm differentiation of ES cells.

Further confirming the importance of Wnt signaling in mesoderm differentiation, our lab has also recently shown that attenuation of Wnt signaling leads to a reduction in spontaneous mesoderm formation in embryoid bodyies (Kemp *et al*. in press; Wawrzak *et al*. 2007). More specifically, Dkk1, Sfrp2 and the secreted CRDs of Fzd5, -7 and -8 could all individually antagonize *Brachyury* expression.

In summary, ES cell differentiation to mesoderm is dependent on canonical Wnt signals. Moreover, in the embryoid body model of embryonic development both Wnt and TGFβ signals are necessary for induction of mesoderm.

Wnts inhibit neural differentiation

The mechanism of neural induction in the embryo has been described in *Xenopus* by the neural "default" model (reviewed in Hemmati-Brivanlou and Melton 1997; Stern 2005). In this model, the lack of epidermal, endodermal or mesodermal inducing signals leads to the formation of neural tissue. Furthermore, when Bmp4 is present, embryonic ectoderm will form epidermis, but when Bmp4 antagonists are present, neural differentiation can occur. Possibly a similar mechanism can be extrapolated for Wnts and their antagonists. Neural induction may also occur by default when the embryonic ectoderm is devoid of mesoderm-inducing Wnt signals due, for example, to the presence of Wnt antagonists. The *Dkk1* mutant, which has no forebrain, is an excellent example of how modulation of Wnt signaling is key for neural induction (Mukhopadhyay *et al*. 2001).

The best-known method for neural differentiation of ES cells is the treatment of embryoid bodies with retinoic acid (Bain *et al*. 1995; Fraichard *et al*. 1995). In 2002, Smith and his coworkers identified *Sfrp2* as being highly expressed in retinoic acid-treated embryoid bodies (Aubert *et al*. 2002). ES cells transfected with *Sfrp2* differentiate into neural precursors even in the absence of retinoic acid. In contrast, if *Wnt1* transfected ES cells are treated with retinoic acid, the number of neural differentiated cells is strongly reduced (Aubert *et al*. 2002). In a similar study it has been shown that *Dkk1* expression is also strongly upregulated in retinoic acid-treated embryoid bodies. Addition of recombinant Dkk1 to embryoid bodies cultured without retinoic acid induces the expression of neural markers, while knock-down of *Dkk1* using siRNA in retinoic acid-treated embryoid bodies largely reduces the expression of neural markers (Verani *et al*. 2007). Together these reports show that in this retinoic acid-induced method of neural differentiation, the Wnt pathway is blocked.

It has also been described that in serum-free media neural differentiation can occur spontaneously (Watanabe *et al*. 2005; Verani *et al*. 2007). In a special serum-free medium and without going through the formation of embryoid

bodies, spontaneous neural induction was shown to be very efficient. In this so-called 'mono-adherent culture' system more than 60% of the cells become positive for the neural marker *Sox1* by the fourth day of differentiation (Ying *et al*. 2003b). However, even in serum-free medium the level of neural conversion can be further elevated by the addition of Dkk1 in combination with LeftyA, a Nodal inhibitor (Watanabe *et al*. 2005). In our own research, we have also shown that the Wnt antagonist Dkk1, as well as the secreted CRDs of Fzd5, -7 and -8, can individually increase neural induction in differentiating monolayer cultures in serum-containing medium (Kemp *et al*. in press).

These results are all in favor of the neural default model in ES cells and are reflective of embryonic development. Neural induction requires the absence by antagonism of mesoderm inducers such as Nodal and Wnts.

WNTS AND DISEASES

Wnt signaling is not only important during embryogenesis, but also plays key roles in the self-renewing tissues of the adult. As in ES cells, adult stem cells require a proper balance of Wnt signals to maintain their proliferative and multipotent character, but also to control cell fate. The skin and gut epithelium are examples of highly regenerating tissues and both contain stem cell compartments that are responsible for maintaining homeostasis (Marshman *et al*. 2002; Niemann and Watt 2002). Research indicates that the canonical Wnt pathway is crucial in regulating these adult stem cells, and mutations within this pathway often lead to a disruption of homeostasis followed by pathological conditions such as cancer (reviewed in Reya and Clevers 2005; Clevers 2006). A well-studied example of this is the upregulation of canonical Wnt signals due to the mutation of APC, a cause of up to 80% of sporadic colorectal carcinomas (Schneikert and Behrens 2007). Because aberrant Wnt signaling has been found to be the cause of many cancers, the canonical pathway has become an important focus of study in this field.

CONCLUDING REMARKS

Since Wnt1 was first discovered in 1982, much has been revealed about canonical Wnt signaling and additional noncanonical pathways have emerged (Nusse and Varmus). It has also been shown that Wnts undergo several posttranslational modifications, necessary for proper secretion and signaling activity. The 19 Wnts signal by binding to the Fzd receptors alone or together with LRP co-receptors, and various Wnt-Fzd or LRP-Wnt-Fzd combinations, of which there are numerous possibilities, can lead to the transduction of different signaling activities. The discovery of non-Wnt ligands that can bind Fzd and LRP to signal through β-Catenin suggests that other unidentified proteins may also interact with components of the Wnt pathway.

Wnt signaling can be modulated either by Sfrps, which directly bind Wnts to prevent interactions with Fzds, or Dkks, which bind to LRPs and Kremens to prevent canonical Wnt signaling through LRP. Moreover, non-antagonizing roles for the Wnt modulators include activating the canonical Wnt pathway and promoting axon guidance.

Studies have revealed that Wnts have many functions, sometimes redundant, in many different tissues of the developing embryo. During early embryonic development, canonical Wnt signals are necessary for dVE patterning and migration as well as mesoderm formation, while inhibition of Wnt is important for the development of anterior structures. Most of these functions can be reflected in ES cell biology, where Wnt signaling is necessary for mesoderm induction and Wnt antagonism is necessary for neural differentiation.

Initially, studies of Wnt signaling were hampered by the difficulties in obtaining purified and active Wnt proteins. With the purification of active Wnt3a and -5a, new research possibilities have become possible, of which many have already been exploited. In particular, the availability of Wnt5a should lead to the further establishment of methods for the qualification of noncanonical signaling, which can then be transferred from cell culture and applied to the embryo. The identification of the necessary posttranslational modifications to Wnt3a and -5a will hopefully facilitate the purification of other Wnts, and the unknown roles of other Wnts may then be investigated.

Little research has demonstrated the morphogenic effect of Wnt proteins in the mouse. One study shows that Wnt3a is able to signal to cells at least 15-20 cell diameters from its expresion domain in the mouse embryo (Nakaya *et al*. 2005). In comparison, *Drosophila* Wingless can spread and act up to 25 cell diameters away in the wing imaginal discs (Zecca *et al*. 1996). As technological advances are made, researchers may be able to visualize the movements of Wnts and determine whether or not a morphogenic signaling gradient is indeed formed in the mouse.

In the embryo, the molecular mechanism controlling the migration of the dVE and thus forming the anterior-posterior axis of the mouse embryo has yet to be determined. This migration is dependent on β-Catenin, but as other molecules are also necessary, such as Cripto, the cooperation of Wnt/β-Catenin signaling with other molecular effectors, such as the TGFβ-related protein Nodal or Bmp, is most likely essential (Morkel *et al*. 2003; reviewed in Srinivas 2006).

Although many extracellular components of the Wnt pathway have been identified, new molecules continue to be discovered that activate or antagonize Wnt signaling, creating a stimulating challenge for investigators. Thus, for now, the complete repertoire of molecules involved in the Wnt pathways, along with all of their biochemical interactions, remains a mystery. In addition, much investigation has yet to be done before we will fully comprehend all the functions of Wnts in the growth and patterning of the developing embryo. Indeed, the Wnt field is an exciting place to be.

ACKNOWLEDGEMENTS

The laboratory of L.L. is supported by grants from the European Commission (FP6-NEST 12930), the Federal Government of Belgium (IUAP V-35) and the Fund for Scientific Research-Flanders, Belgium (G.0485.06). M. H. is an aspirant of FWO (Belgium).

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