Review

Wnt signaling in limb organogenesis

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Secreted signaling molecules of the Wnt family have been found to play a central role in controlling embryonic development of a wide range of taxa from Hydra to humans. The most extensively studied Wnt signaling pathway is the canonical Wnt pathway, which controls gene expression by stabilizing β -catenin, and regulates a multitude of developmental processes. More recently, noncanonical Wnt pathways, which are β -catenin-independent, have been found to be important developmental regulators. Understanding the mechanisms of Wnt signaling is essential for the development of novel preventive and therapeutic approaches of human diseases. Limb development is a paradigm to study the principles of Wnt signaling in various developmental contexts. In the developing vertebrate limb, Wnt signaling has been shown to have important functions during limb bud initiation, limb outgrowth, early limb patterning, and later limb morphogenesis events. This review provides a brief overview on the diversity of Wnt-dependent signaling events during embryonic development of the vertebrate limb.

Introduction

At the limb initiation stage, lateral plate mesoderm at the fore and hind limb level is induced to grow and form limb buds. Ongoing proliferation of lateral plate mesenchymal cells in the presumptive limb fields concomitant with decreased proliferation in the flank leads to initial formation of the buds.¹ Shortly thereafter, cells from the lateral edges of the adjacent somites begin migrating into the limb to form limb muscles.²⁻⁴ Like limb muscles, nerves, pigment cells, and vasculature also originate outside the limb, while other limb tissues, including the mesenchyme, connective tissue, cartilage, bone and tendons, derive from the lateral plate mesoderm. In the distal ectoderm of the early limb bud, the formation of a local ectodermal thickening, the apical ectoderm ridge (AER), is induced by the underlying limb mesoderm. In all tetrapods, the AER forms at the distal tip of the ectodermal pocket, running along

the antero-posterior axis of the limb bud at the boundary of dorsal and ventral ectoderm, and is necessary for proximo-distal limb outgrowth.^{5,6} Three well-characterized signaling centres coordinate the overall limb patterning and growth along the three spatial axes: The AER directs the proximal-distal (P/D) limb outgrowth; the zone of polarizing activity (ZPA) in the posterior limb mesenchyme patterns the limb along the anterior-posterior (A/P) axis; and the non-AER limb ectoderm sets up the dorsal-ventral (D/V) polarity.^{7,8} Signals originating from these signaling centers are integrated to set up spatial coordinates to pre-pattern the limb mesenchyme, and finally the various cell types to be specified. During later limb morphogenesis, skeletal elements, muscles, tendons, blood vessels, etc., form and mature to make the limb a functional organ.^{9,10}

Wnt Signaling Pathways

Wnts are secreted proteins that control a multitude of diverse developmental processes, but also tumorigenesis.¹¹⁻¹⁵ They signal through the canonical, β -catenin dependent pathway, or at least two different noncanonical pathways (Fig. 1; refs. 16-18). Central to this pathway is the regulation of β -catenin activity, which depends on its protein abundance and nuclear localization. In the absence of Wnt, glycogen synthase kinase 3 (GSK-3) phosphorylates β -catenin, which allows the ubiquitin ligase complex to bind and tag β -catenin for proteasome-mediated degradation. In the presence of Wnts, Wnt ligand binds to its coreceptors Frizzled and LRP5/6, so that the cytoplasmic target of Frizzled, Dishevelled, is activated and suppresses GSK-3 activity. As a result, β -catenin is not phosphorylated and thus not degraded, resulting in accumulation of β -catenin in the cytoplasm which is translocated to the nucleus. There it binds to transcription factors of the LEF/TCF family and converts them from repressors to activators, which triggers downstream gene transcription.^{14,19} In this pathway, Arrow (LRP5/6) recruits Axin to the membrane and this interaction leads to Axin degradation upon canonical Wnt signaling. As a consequence, β -catenin is no longer bound by Axin, resulting in reduced β -catenin degradation and cytoplasmic sequestration. Thus, canonical Wnt signaling controls gene expression by allowing β -catenin to accumulate when Wnts are present and by reducing nuclear β -catenin levels when Wnts are absent.

The second pathway is the planar cell polarity pathway, in which Frizzled activates JNK and directs asymmetric cytoskeletal organization and coordinated polarization of cells within the plane of epithelial sheets.^{20,21} Both of these pathways utilise Dishevelled (Dsh): the β -catenin pathway is dependent on all three functional

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Fig 1. Three Wnt signaling pathways (modified from Huelsken and Behrens, 2002). (A) The Wnt/ β -catenin pathway. Wnt signal is transduced through the frizzled receptor (Fz) via dishevelled (Dsh), which represses the axin/glycogen synthase kinase-3 β (GSK3 β) complex, which induces the degradation of β -catenin. Accumulated Cytoplasmic β -catenin is translocated to the nucleus where it binds with Tcf/Lef, thus activating the transcription of its target gene. (B) The planar cell polarity (PCP) pathway activated in response to Wnt signaling signals via the small GTPases Rho and Cdc42 to c-Jun N-terminal kinase (JNK), acting primarily on the cytoskeleton (C) An alternative pathway stimulates the release of intracellular Ca²⁺, activating protein kinase C (PKC) and Ca²⁺/calmodulin-dependent kinase II (CamKII).

domains present in Dsh (DIX, PDZ and DEP) whilst the JNK pathway only requires the DEP domain. Dsh is connected via Daam1 to downstream effectors such as the small GTPase Rho and Rho-associated kinase (ROCK). The product of the Wnt target gene *naked* (Nkd) was recently identified as an antagonist for Wnt signaling that binds to Dsh and blocks β -catenin but stimulates the JNK pathway. This pathway is e.g. utilised in *Drosophila* planar cell polarity regulation and by Wnt11 in convergent extension movements during gastrulation. Thirdly, the Wnt/Ca²⁺ pathway lead to release of intracellular calcium, possibly via G-proteins.^{17,22} This pathway involves activation of phospholipase C (PLC) and protein kinase C (PKC). Elevated Ca²⁺ can activate the phosphatase calcineurin, which leads to dephosphorylation of the transcription factor NF-AT and its accumulation in the nucleus. In *Xenopus* embryos, NF-AT activity suppresses canonical Wnt signals during axis formation.^{23,24}

Wnt Signaling and Limb Bud Initiation

At the onset of limb development, the limb buds initially form as a result of an interplay between fibroblast growth factor (FGF) and Wnt signaling, both acting downstream of the T-box transcription factors Tbx5 and Tbx4 in the developing forelimb and hindlimb, respectively.²⁵⁻²⁷ In the chick limb bud, Wnt2b in the presumptive forelimb region and Wnt8c in the presumptive hindlimb region, both signal through β -catenin/TCF to maintain the expression of

FGF 10 in the lateral plate mesoderm (LPM) at the presumptive limb level (Fig. 2).²⁸ Implantation of cells that express β -catenin, which acts as a constitutively active molecule, into the flank region can ectopically induce *FGF10* expression and limb bud outgrowth. In contrast, misexpression of Axin, which is an antagonist of the β -catenin pathway, downregulates *FGF10* expression and affects limb outgrowth.²⁸

In the avian embryo, Kawakami and colleagues found that at the level of the presumptive forelimb, *Wnt2b* is expressed in both the IM and the LPM. Local application of cells expressing Wnt2b in the flank region induced *FGF10* expression and subsequently resulted in ectopic limb outgrowth. Further, ectopic application of FGF8 was found to induce *Wnt2b* expression prior to the induction of *FGF10*, indicating that Wnt2b is downstream of FGF8 but upstream of FGF10. Similarly in the hindlimb, Wnt-8c has been shown to mediate the FGF8/FGF10 regulatory loop. *Wnt-8c* is only expressed in the caudal LPM of the prospective hindlimb and precedes the expression of *FGF10* in this region. Moreover, Wnt8c, like Wnt2b, can induce the expression of *FGF10*, making Wnt8c a candidate mediator of limb bud initiation in the hindlimb region. Thus, the β -catenin pathway drives the initiation of limb bud formation in both fore and hind limb levels, albeit distinct Wnt molecules activate this pathway in the two limb fields. However, in the mouse embryo the expression patterns of *Wnt2b* and *Wnt8c* were not detected.²⁵ Therefore, it is not clear whether in mammals a Wnt ligand expressed

Fig 2. Model of early limb bud initiation in chick (modified from Kawakami et al., 2001). (A) Prior to limb initiation (stage 12), FGF8 (in grey) is expressed in the intermediate mesoderm (IM) adjacent to the presumptive forelimb area. FGF10 (in blue) is expressed in the lateral plate mesoderm (LPM), and in the segmental plate (SP). In the caudal portion of the LPM, FGF10 is coexpressed with Wnt8c (in red). Somites are indicated with their corresponding segment numbers. (B) During limb induction (HH stage 14), both FGF8 (in grey) and Wnt2b (in red) are expressed in the IM. Wnt2b is also expressed in the LPM of the presumptive forelimb area and signals through β -catenin to regulate FGF10 specifically in the prospective forelimb. FGF10 expression remains in the caudal LPM in a diffuse pattern. (C) At HH stage 16, expression of Wnt2b and FGF10 is confined to the forelimb bud field in the LPM. Additionally, expression of FGF10 is now confined to the presumptive hindlimb bud field. It may be Wnt8c in the caudal LPM contributes to this restriction of FGF10. In the LPM of the presumptive limb areas, FGF10 signals to the surface ectoderm (SE) to induce Wnt3a (green) resulting in activation of FGF8. For reason of simplicity, only the expression patterns in the relevant regions are shown, while the expression in somites and other areas is neglected.

in the early LPM or IM before limb bud formation is required for limb initiation. Nevertheless, canonical Wnt signaling was shown to sustain high levels of *FGF10* expression during limb initiation also in the mouse, even though *Tbx5* alone is able to activate *FGF10* expression.²⁵ Another member of the Wnt family, Wnt10a, has been found to be expressed in the chick limb ectoderm and to induce FGF8 via the canonical Wnt pathway.²⁹

Wnt Signaling During Limb Bud Outgrowth

The AER is induced at the junction between the dorsal and ventral ectoderm at an early stage of limb bud formation, and produces growth factors such as FGF8 and FGF4 to promote proximo-distal (P/D) limb outgrowth. Wnt3a has been proposed to

act downstream of FGF10 and to induce AER formation and *FGF8* expression through the canonical Wnt pathway.³⁰ Inactivation of mesenchymal β -catenin leads to degeneration of the AER and limb truncation.³¹ Thus, in the chick, the β -catenin/LEF pathway is thought to play a major role in both in the initiation of limb bud outgrowth and in the establishment of AER. Surprisingly, *Wnt3a* is not expressed in murine limb ectoderm, and limb development is normal in *Wnt3a* mutant mice.^{32,33} However, mice lacking TCF-1 and LEF-1 develop significantly smaller limb buds and lack a functional AER. This indicates that limb outgrowth is initiated but stalls due to the absence of a functional AER.³⁴ Wnt3, which is signaling via the β -catenin pathway, has been found to be involved in the formation and maintenance of the AER in mouse, similar to Wnt3a in the chick.³⁵

It is not only the presence of a functional AER, but also continued proliferation of the limb bud mesenchyme that is necessary for limb outgrowth. In the limb, loss of Wnt5a activity results in reduced proliferation of the progressive zone underneath the AER, a progressive shortening of the appendicular skeleton in a proximal-to-distal fashion and a total loss of the most distal structures such as the digits.³⁶ Wnt5a is expressed in the distal mesenchyme underneath the AER in both chick and mouse.^{37,38} It is not known through which pathway Wnt5a acts in this context. In other systems, Wnt5a was shown to act via either the canonical³⁹ or non-canonical pathway^{22,40} depending on which type of Frizzled receptor is present.

Wnt Signaling in Limb Patterning

AER formation is intimately linked to dorso-ventral (D/V) patterning of the limb ectoderm. Because canonical Wnt signaling is required upstream of *Engrailed 1* (*En1*) in the ventral ectoderm for determining ventral limb identity,^{34,35} the AER formation defects in the limb-specific mutants of *Wnt3* or β -catenin might be a result of a failure to pattern the ventral ectoderm properly. This hypothesis is consistent with the observation that formation of the mature AER involves the convergence of broadly distributed ventral precursor cells towards the D/V boundary of the limb field; expression of AER marker genes such as *FGF8* is initially observed in a broad domain of thickened ventral ectoderm and progressively becomes restricted to the mature AER at the distal margin in the mouse.⁴¹⁻⁴⁴ Another ectoderm-derived Wnt ligand that is thought to play a role in AER formation is Wnt7a. Unlike *Wnt3*, *Wnt7a* is expressed specifically in the dorsal ectoderm, where it activates *Lmx1b* in the dorsal limb mesenchyme, which encodes a LIM-homeodomain transcription factor that determines the dorsal mesodermal cell fate in the limb.^{37,38,45-47} Misexpression of *Lmx1* dorsalises the ventral distal limb bud showing that it is a key downstream mediator of Wnt7a signaling.^{47,48} Strikingly, loss of Wnt7a function in the mouse resulted in embryos with ventralized paws having sole pads on both surfaces, showing that Wnt7a is necessary for specifying dorsal cell fates in the distal limb.⁴⁶ To restrict the activity of Wnt7a to dorsal ectoderm requires the activity of *Engrailed-1* in the ventral

ectoderm.^{42,49} Ectopic expression of *En-1* throughout the limb ectoderm in chick, including the dorsal ectoderm, results in suppression of *Wnt7a* in the dorsal ectoderm,⁴⁹ whereas in *En-1* deficient mice, *Wnt7a* expands into the ventral ectoderm of the limb bud, leading to double-dorsal paws.⁴²

Next to its decisive role in P/D limb outgrowth and patterning, the AER is also important for antero-posterior (A/P) limb patterning, as it indirectly maintains *Shh* expression in the Zone of Polarizing Activity (ZPA) through a positive feedback loop between *Shh* in the posterior limb mesenchyme and *FGF4* expressed in the AER.⁵⁰⁻⁵³ Canonical Wnt signaling is required for AER maintenance. First, the AER forms but is not maintained, when β -catenin is ablated slightly later in limb development.³⁵ Second, AER activity and structure change in response to alterations of canonical Wnt signaling. *Dkk1* encodes a secreted antagonist of the canonical Wnt pathway and is, in the developing limb bud, expressed in the AER.⁵⁴⁻⁵⁶ In the *Dkk1*^{-/-} mouse embryo, *FGF8* expression in the AER is stronger, and is expanded both dorsally and ventrally in the limb bud as a result of upregulated canonical Wnt signaling. An expanded AER is also observed in *En-1*-deficient mice, indicating an interaction of *Dkk*, *En-1* and *Wnt7a* during mouse limb development.⁵⁷ This is in line with data on *Lrp6*, which is a Wnt coreceptor required for the canonical Wnt signaling pathway.⁵⁸⁻⁶⁰ In *Lrp6*^{-/-} embryo, *FGF8* expression is significantly reduced, indicating that the AER degenerates prematurely as a result of weakened canonical Wnt signaling.^{59,61} These results demonstrate that after AER formation, proper modulation of canonical Wnt signaling is necessary to maintain normal function and structure of the AER. Interestingly, Thalidomide-induced limb deformities in human patients have now been proposed to be at least in part due to defects in the interaction of *Dkk1*, Wnt and BMP signaling.⁶²

Apart from its role in DV patterning and AER formation, *Wnt7a* is also involved in AP patterning, as *Wnt7a* deficient mice suffer from loss of posterior skeletal elements. This suggests that Wnt7a signaling may be necessary for normal function of the ZPA. Consistent with this, the expression of *Shh*, which mediates ZPA activity, is progressively lost in *Wnt7a* deficient limbs.⁴⁶ After removal of dorsal limb ectoderm, both *Shh* expression and posterior skeletal development can be rescued by applying *Wnt7a* in combination with *FGF4*, arguing that maintenance of *Shh* expression is under the combinatorial influence of Wnt and FGF signaling. However, the activity of *Wnt7a* in regulating *Shh* expression is independent of its dorsalizing activity, as *Shh* expression is normal in *Lmx1*^{-/-} limbs.⁴⁵ Since the expression of *Axin2* and *Nkd*, both being transcriptional targets and antagonists of canonical Wnt signaling, is located dorsally in the limb mesoderm,⁶³⁻⁶⁵ it will be interesting to investigate whether *Axin2* and *Nkd* expression depends on *Wnt7a* or β -catenin signaling and whether any of them is involved in D/V patterning, *Shh* expression, or the establishment of the difference between dorsal and ventral ectoderm in response to Wnt3 signaling.

Wnt Signaling in Limb Morphogenesis

Next to their functions in overall limb patterning, Wnts control the morphogenesis of specific tissues in the limb such as musculature, synovial joints, cartilage, and bone.

Muscle differentiation. Precursors of limb musculature are formed by deepithelization of the lateral dermomyotome and subsequent

Fig 3. Expression of Wnt genes (4, 5a, 5b, 11, 14) in the mesenchyme surrounding the developing cartilage elements of HH-stage 26 chick forelimb. Limbs shown in dorsal view (Loganathan et al., 2005). (A) Expression of Wnt 4 in the central elbow region and in the joint interzones of the wrist. (B) Wnt 5a expression is seen at the distal tip of the limb bud and in the entire AER, with lower expression levels proximally. (C) Expression of Wnt 5b in the dorsal and ventral mesenchyme of the limb. (D) Wnt 11 transcripts in the dorsal and ventral mesenchyme of the limb. (E) Wnt 14 expression as a transverse stripe in the mesenchyme of the presumptive joint region.

migration of somitic mesenchymal myogenic precursor into the limb bud.^{3,4} Wnts play a number of roles during muscle development including the initiation of myogenesis. The myogenic precursor cells are specified by Wnts secreted from the neural tube and the overlying surface ectoderm through induction of *Pax3*,⁶⁶ likely via the canonical pathway. Once at their homing sites within the limb bud, the precursor cells switch on the expression of the myogenic determination factors *MyoD* and *Myf5*. There is compelling evidence indicating the requirement of canonical Wnt signaling in the induction of myogenic differentiation. Blocking canonical Wnt signaling leads to reduction in myocyte numbers in the chick limb bud.⁶⁷ Further, the expression of the Wnt antagonist *Sfrp2* in uncommitted myogenic precursors is downregulated as the myogenic cells differentiate.⁶⁸⁻⁷⁰ Recently it was shown that *Wnt6* from the limb ectoderm promotes limb myogenesis via *Pax3* and *Myf5*, whereas the other putative signaling pathway involving *MyoD* expression is negatively regulated by *Wnt6* signaling,⁷¹ and that *Wnt6* can compensate for the ectoderm during the formation of limb muscle cells.⁷² In addition, Wnt signaling modulates other aspects of myogenic development such as migration and cell morphology. A recent study has identified a Wnt-dependent TCF4 binding site in the enhancer region of *Msx1*, which represses differentiation in migratory myoblasts.⁷³ In the limb bud, N-cadherin, which is essential for myogenic migration and during chondrogenesis, is maintained by *Wnt7a*.⁷⁴ Myoblasts terminally differentiate into slow or fast muscle fibres that have different metabolic activities and contain different isoforms of myofibrillar contractile proteins, such as myosin heavy chain (MHC). The specific combination of slow and fast muscle fibres in each muscle determines its function. Recent cell labelling experiments in chick limb buds indicate that the specification of slow and fast myotubes involves both extrinsic and intrinsic mechanisms.⁷⁵ Wnt family members such as *Wnt5a* and *Wnt11* may be involved in instructing the fate determination of slow and fast muscle fibres, likely through noncanonical pathways.⁶⁷ The ability of Wnts to induce stem cells to differentiate into a specific muscle cell type has tremendous impact on tissue engineering and repairing. Adult muscle stem cells are

induced to differentiate into myoblasts by canonical Wnt signaling, showing that the generation and regeneration of skeletal muscles are governed by a common mechanism.⁷⁶ A better understanding of how Wnt signaling regulates muscle development will be very beneficial for therapy of neuromuscular diseases in the future.

Skeletal development. Wnt signaling controls several key developmental processes during skeletal morphogenesis in the limb. It has been shown that canonical Wnt signaling acts to suppress chondrocyte differentiation from condensed mesenchymal progenitors.^{74,77,78} Several candidate Wnt genes (*4, 5a, 5b, 11, 14*) are expressed in the mesenchyme surrounding the developing cartilage elements (Fig. 3) along with their receptors *Fz4* and *Fz6*, and the Wnt antagonists *Dkk2* and *Dkk3*.^{77,79–85} Wnt1, 7a and 14 have been shown to inhibit chondrogenesis in micromass culture. Thus, the presence of Wnt antagonists, particularly *Sfrp3* which is expressed in the central mesenchymal condensation, may allow chondrogenesis to proceed.^{77,78,86} However, in vitro studies indicate that misexpression of the Wnt antagonist *Frzb-1* inhibits chondrogenesis, whereas misexpression of constitutively active LEF-1 or Wnt8 promotes chondrocyte differentiation.⁸⁷ The inhibition by Wnt7a has been correlated with the maintenance of N-cadherin expression and hence cell adhesion which halts chondrogenesis just after the initiation stage.⁷⁴ This process seems to be mediated by *Frz7* and the MAPK-pathway.^{88,89} Misexpression of Wnt5a or dominant negative forms of its receptors *Fz1* and *Fz7* results in the formation of shorter skeletal elements due to a delay in chondrocyte differentiation. Conversely, Wnt5a was shown to promote chondrocyte differentiation in the distal limb bud by inhibiting canonical Wnt activity.⁹⁰

Other Wnts such as Wnt14 have been implicated in initiating synovial joint-forming interzone cells prior to cartilage segmentation.^{77,81} Ectopic expression of Wnt14 in the chick limb bud induces morphological and molecular changes in chondrocytes characteristic of early stages of joint formation, such as the expression of *Gdf5*, and Wnt14 can induce dedifferentiation of chondrocytes in micromass cultures, a process that occurs during normal joint development.⁷⁷ In addition, several other Wnts such as Wnt4 and Wnt5a are also expressed in the future joint region.^{77,91}

It is interesting to note that Wnt5a and Wnt4 have opposing effects on chondrocyte maturation. Misexpression of Wnt5a causes a delayed appearance of hypertrophic chondrocyte, Wnt4 misexpression results in an acceleration of chondrocyte maturation.⁸¹ Wnt9a deficient mice show a relatively mild phenotype in joint formation, such as synovial chondroid metaplasia in some joints, while mice mutant for Wnt4 have no anomaly in joints. However, mice which are double mutant for Wnt4 and Wnt9a show severe phenotypes, suggesting that Wnt4 and Wnt9a play redundant roles in the maintenance of joint integrity.⁹² Overexpression of Wnt9a or β -catenin in early differentiating chondrocytes causes ectopic joint formation and conditional inactivation of β -catenin by crossbreeding with *Col2a1-Cre* mice results in the disappearance of *Gdf5* expression and a reduction of joint formation.⁹³ Conditional deletion of β -catenin in mouse limbs also results in fused joints.⁹⁴ These findings suggest that Wnt/ β -catenin signaling is necessary and sufficient for joint development initiation. Defective Wnt signaling has been described to be manifest in many skeletal diseases. For instance, the Wnt pathway has been implicated in degenerative joint diseases such as rheumatoid arthritis.⁹⁵ Mutations in the *WISP3* gene, which encodes for

Wnt1-induced signaling protein subfamily of the connective tissue growth factor (CTGF) family, are associated with the autosomal recessive skeletal disorder progressive pseudorheumatoid dysplasia (PPD).⁹⁶ Mutation of LRP6, which are expressed in osteoclasts, results in osteoporosis-pseudoglioma syndrome.⁹⁷ This syndrome is characterized by a low bone mass and increased vulnerability to fractures and deformation. Wnt signaling has also been implicated in osteoarthritis where *Sfrp4* expression has been shown to correlate with apoptotic chondrocytes within degenerating articular cartilage.⁹⁸

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