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Wnt signalling during limb development

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ABSTRACT Wnts control a number of processes during limb development - from initiating outgrowth and controlling patterning, to regulating cell differentiation in a number of tissues. Interactions of Wnt signalling pathway components with those of other signalling pathways have revealed new mechanisms of modulating Wnt signalling, which may explain how different responses to Wnt signalling are elicited in different cells. Given the number of Wnts that are expressed in the limb and their ability to induce differential responses, the challenge will be to dissect precisely how Wnt signalling is regulated and how it controls limb development at a cellular level, together with the other signalling pathways, to produce the functional limb capable of co-ordinated precise movements.

KEY WORDS: *Wnt, limb, development, chondrogenesis, myogenesis*

The *Wnt* Gene Family

The *Wnt* family of secreted glycosylated factors consists of 22 members in vertebrates which have a range of functions during development from patterning individual structures to fine tuning at a cellular level controlling cell differentiation, proliferation and survival. The founding members of this family are the *Drosophila* segment polarity gene *Wingless* (*Wg*), required for wing development, together with Wnt1 (originally named int-1) in the mouse. The latter was identified due to integration of the mouse mammary tumour virus into the Wnt1 locus, which resulted in epithelial hyperplasia and increased susceptibility to mammary carcinomas (Nusse and Varmus, 1982; Nusse *et al.*, 1984; Tsukamoto *et al.*, 1988). Initially, based on their ability to induce a secondary axis in *Xenopus* embryos and to transform the mammary epithelial cell line C57MG, the family was divided into two subgroups. The Wnt1 class, which comprises Wnt1, -3a and -8, can induce a secondary axis in *Xenopus*, whilst the Wnt5a class, consisting of Wnt4, -5a and –11, cannot. Similarly, Wnt1, -3a and -7a have transforming activity whilst Wnt4 and -5a do not (www.stanford.edu/~rnusse/wntwindow.html).

The Wnt family signals through the frizzled receptors which are seven pass transmembrane receptors, like the smoothened receptors involved in transduction of the hedgehog signalling pathway. The frizzled protein family consists of 10 genes characterised by an N-terminal cysteine-rich domain responsible for ligand binding. The family can be divided simplistically on the basis of the intracellular motif S/T-X-V proposed to be involved in binding PDZ domains such as that found in the Wnt signalling component dishevelled. The S/T-X-V motif is not present in Fz3, -6 and -9 but is found in the others (Cadigan and Nusse, 1997). The frizzled receptors can function together with the LRP co-receptors, which are single transmembrane proteins containing LDL receptor repeats, two frizzled motifs and four EGF type repeats in the extracellular domain (reviewed by Pandur and Kühl, 2001; also see Roszmusz *et al.*, 2001). The LRPs, which include the vertebrate genes LRP4, -5 and -6 and the *Drosophila* gene *arrow*, form a complex with frizzled in a Wnt-dependent manner and signal in the canonical pathway (Tamai *et al.*, 2000; Wehrli *et al.*, 2000; reviewed by Pandur and Kühl, 2001).

Heparan sulphate proteoglycans (HSPGs) are also important for signalling by Wnt family members (reviewed by Perrimon and Bernfield, 2000). For example, the *Drosophila* HSPGs division abnormally delayed (dally*)* and dally-like are essential for Wingless signalling and are suggested to act either as co-receptors stabilising the Wingless/frizzled complex or by restricting the extracellular diffusion of the ligand (Tsuda *et al.*, 1999; Baeg *et al.*, 2001). Also important in HSPG regulation of Wnt signalling are the enzymes required for biosynthesis and sulphation of heparan sulphate.

Abbreviations used in this paper: AER, apical ectodermal ridge; BMP, bone morphogenetic protein; CamKII, calcium/calmodulin-dependent kinase II; CTGF, connective tissue growth factor; Dkk, dickkopf; Dsh, dishevelled; EGF, epidermal growth factor; Fgf, fibroblast growth factor; GSK3β, glycogen synthase kinase 3β; HSPG, heparan sulphate proteoglycan; Ihh, Indian hedgehog; JNK, c-Jun N-terminal kinase; LDL, low density lipoprotein; LRP, LDL-related protein; MyHC, myosin heavy chain; PKC, protein kinase C; PTHrP, parathyroid hormone-related peptide; RA, rheumatoid arthritis; RTK, receptor tyrosine kinase; Sfrp, secreted frizzled-related protein; WIF, Wnt inhibitory factor; WISP, Wnt induced secreted protein.

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Indeed, mutations in the *Drosophila* genes *sugarless* (also called *kiwi* and *suppenkasper*) and *sulphateless*, which encode homologues of UDP-glucose dehydrogenase and *N*-deacetylase/*N*-sulphotransferase respectively, have revealed the necessity of these glycosaminoglycan biosynthetic enzymes in Wingless signalling (Binari *et al.*, 1997; Häcker *et al.*, 1997; Haerry *et al.*, 1997; Lin and Perrimon, 1999). These proteins are needed for heparan sulphate modification of the co-receptors dally and dally-like. Similarly, the avian extracellular sulphatase QSulf1 can regulate heparan-dependent Wnt signalling and is required in the Wnt regulation of *MyoD* expression in myogenic C2C12 cells (Dhoot *et al.*, 2001).

In the classical Wnt signalling pathway, Wnts signal via β-catenin (see Fig. 1). Briefly, Wnt signalling represses the axin/glycogen synthase kinase-3β (GSK3β) complex, which normally stimulates the degradation of β-catenin via the ubiquitin pathway (reviewed by Kikuchi 2000). Therefore, in Wnt-activated cells, cytoplasmic β–catenin accumulates and is translocated to the nucleus where, in conjunction with T cell-specific factor/lymphoid enhancer binding factor 1 (Tcf/Lef1) transcription factors, it activates the transcription of Wnt target genes. A second pathway activated in response to Wnt signalling signals via the small GTPases Rho and Cdc42 to c-Jun N-terminal kinase (JNK) (Fig. 1; Boutros *et al.*, 1998; Li *et al.*, 1999; reviewed by McEwen and Peifer, 2000). This pathway is utilised in *Drosophila* planar cell polarity and by Wnt11 in convergent extension movements during gastrulation. Both of these pathways utilise dishevelled (Dsh): the β–catenin pathway is dependent on all three domains present in Dsh (DIX, PDZ and DEP) whilst the JNK pathway only requires the DEP domain. Thirdly, some Wnts, such as Wnt5a, can stimulate the release of intracellular Ca^{2+}, activating protein kinase C (PKC) and Ca^{2+}/calmodulin-dependent kinase II (CamKII) (see Fig. 1; Sheldahl *et al.*, 1999; reviewed by Kühl *et al.*, 2000). The pathway that is utilised appears to depend on the receptor profile and intracellular signalling molecules and is not determined by the specificity of the ligand. For example, Wnt1, which has classically been shown to signal through the β-catenin pathway, has also been implicated in the PKC pathway and more recently has been shown to signal through a novel GTPase in the JNK pathway (Tao *et al.*, 2001; Ziemer *et al.*, 2001). Similarly, Wnt5a and Wnt3a, which have distinct effects in *Xenopus* embryos, can both induce β-catenin accumulation in cardiac myocytes (Toyofuku *et al.*, 2000; Yamanaka *et al.*, 2002). Differentiation between the β-catenin and JNK pathway is controlled by the Dsh-associated kinase PAR-1, which promotes signalling via β-catenin (Sun *et al.*, 2001).

The Wnt Antagonists

As with other growth factors such as members of the TGF-β and fibroblast growth factor (Fgf) families, Wnt signalling can be antagonised by secreted factors. These antagonists include the **s**ecreted **f**rizzled **r**elated **p**roteins (Sfrps), cerberus, dickkopfs (Dkks) and **W**nt **i**nducible **f**actor (WIF-1). However, unlike the other antagonists, *cerberus* has not been reported to be expressed in the developing limb. The Sfrp family consists of at least five members and contains the frizzled related N-terminal domain, which can bind to Wnts, but lacks the intracellular sequence found in the frizzled receptors. They antagonise Wnt function by binding to the Wnt

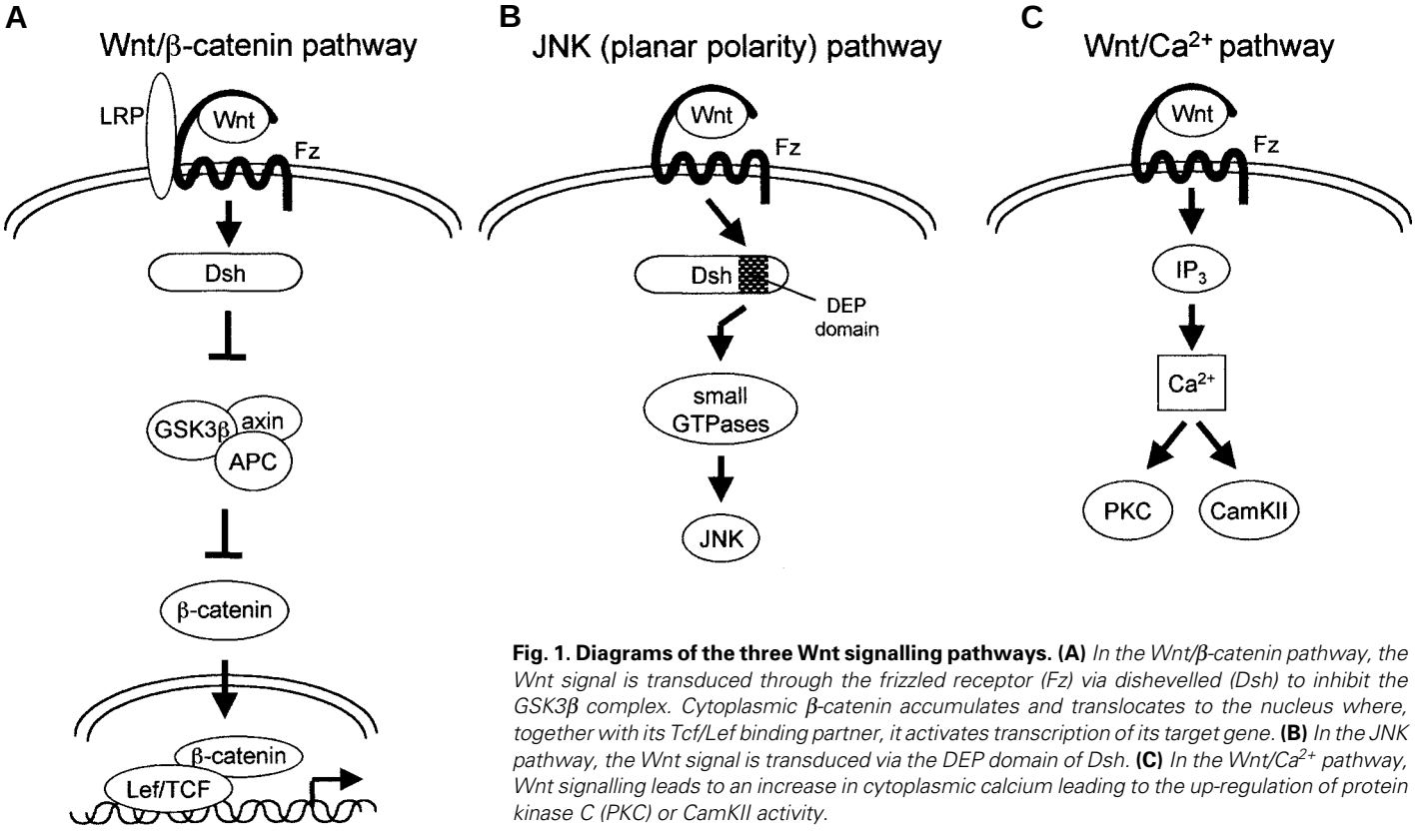

Fig. 1. Diagrams of the three Wnt signalling pathways. (A) *In the Wnt/β-catenin pathway, the Wnt signal is transduced through the frizzled receptor (Fz) via dishevelled (Dsh) to inhibit the GSK3β complex. Cytoplasmic β-catenin accumulates and translocates to the nucleus where, together with its Tcf/Lef binding partner, it activates transcription of its target gene.* **(B)** *In the JNK pathway, the Wnt signal is transduced via the DEP domain of Dsh.* **(C)** *In the Wnt/Ca^{2+} pathway, Wnt signalling leads to an increase in cytoplasmic calcium leading to the up-regulation of protein kinase C (PKC) or CamKII activity.*

molecule and preventing receptor activation. However, they have also been proposed to act as agonists, thus facilitating Wnt function – one possible mechanism may be to increase the solubility of the Wnt molecule to aid its diffusion (Lin *et al.*, 1997). Alternatively, as Sfrps are membrane bound, like the HSPG core protein dally, they may help to localise Wnts to the cell surface, effectively increasing the concentration of Wnt ligand for the receptor. WIF-1 has an extracellular "WIF" domain, five EGF-like repeats and a small hydrophilic C-terminal domain (Hsieh *et al.*, 1999). The WIF-1 domain alone can block Wnt activity suggesting that it binds Wnts. Interestingly, this domain shares homology with the extracellular sequence present in the orphan tyrosine kinase Ryk receptors but whether there is any functional significance of this is at present unknown (Patthy, 2000). However, Ryk, like Wnts, has been suggested to have transforming activity whilst the Ryk mouse knockout bears resemblances to the Wnt5a mouse null mutant (Katso *et al.*, 1999; Yamaguchi *et al.*, 1999; Halford *et al.*, 2000). In both mutants, there is overall growth retardation characterised by a shorter snout and limbs. In contrast to the other antagonists, Dkk1 and -4 bind directly to the Wnt co-receptor LRP6 and block Wnt signalling through the β-catenin pathway (Mao *et al.*, 2001; Zorn, 2001). Surprisingly however, the related molecules Dkk2 and -3 do not antagonise Wnt signalling and the former actually promotes Wnt signalling at least in *Xenopus* (Krupnik *et al.*, 1999; Wu *et al.*, 2000).

Limb Outgrowth and Patterning

Members of the Wnt family are differentially expressed either within the ectoderm or mesenchyme where they play a number of roles (see Fig. 2). Analogous to their role in *Drosophila* and emphasising the importance of this gene family, members of the Wnt family are expressed in two key signalling centres in the limb – the apical ectodermal ridge (AER), which controls outgrowth, and the dorsal ectoderm, which controls dorso-ventral patterning. *Wnt8c* and *Wnt2b* (also known as *Wnt13*), which are transiently expressed in the lateral plate mesoderm, initiate outgrowth of the leg and wing respectively (Kawakami *et al.*, 2001). Thus, ectopic overexpression of these Wnts in the interflank region of a stage 13/14 chick embryo, prior to limb outgrowth, induces ectopic *Fgf10* expression and limb formation via the β-catenin pathway. Fgf10 subsequently induces *Wnt3a* expression in the AER, which in turn switches on the expression of *Fgf8*, again via the β-catenin pathway, and hence promotes AER formation (Kengaku *et al.*, 1998; Kawakami *et al.*, 2001). The Wnt antagonists *Sfrp3* and *Dkk1* are expressed in the lateral plate mesoderm where they may limit the limb- and AER-inducing activity of Wnt2b, -8c and –3a, either temporally or spatially (Ladher *et al.*, 2000a; Mukhopadhyay *et al.*, 2001). Misexpression of Dkk1 in the chick results in the loss of or irregularities in the AER whilst loss of Dkk1 in the mouse broadens the AER along the dorso-ventral axis (Mukhopadhyay *et al.*, 2001). The defects seen in the Dkk studies may reflect the role of Wnt signalling in both limb initiation and subsequent limb outgrowth.

In contrast to the chick, Wnt3a in the mouse is not expressed in the AER. However, consistent with the essential role of Wnts in limb outgrowth, gene-inactivation of both *Lef1* and *Tcf1,* which are expressed in the early developing limb, prevents limb outgrowth (Galceran *et al.*, 1999). In these mice, the AER does not form as shown by the lack of *Fgf8* expression and the absence or down-

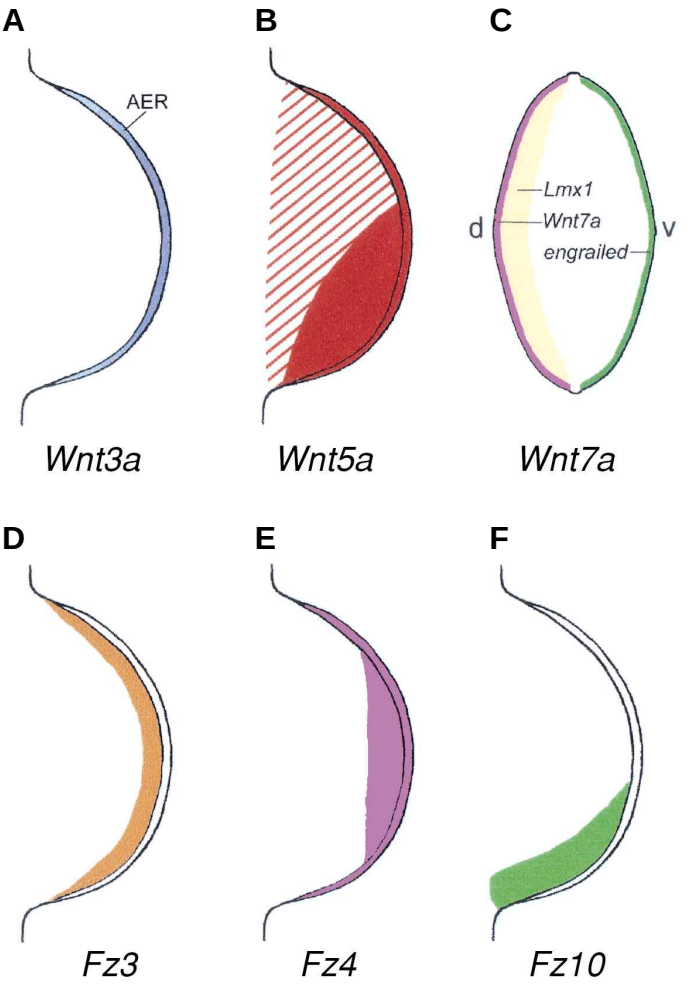

Fig. 2. The expression of *Wnts* and *frizzled* receptors in stage 20 chick limb buds. *(A,B,D,E, F) Sketches of dorsal views showing the expression of* **(A)** Wnt3a*,* **(B)** Wnt5a*,* **(D)** Fz3*,* **(E)** Fz4 *and* **(F)** Fz10. **(C)** *is a sketch of a transverse section showing the expression of* Wnt7a *(purple) in the dorsal (d) ectoderm, with underlying mesenchymal expression of* Lmx1 *(yellow) and* engrailed *in ventral (v) ectoderm (green).*

regulation of the distal mesenchymal markers *Msx1* and *Wnt5a*. In addition, dorso-ventral patterning is disrupted: expression of the ventral ectodermal marker *engrailed1* is absent whilst expression of *Lmx1b*, normally restricted to the dorsal mesenchyme, is found ventrally indicating that a double dorsal limb has formed. This defect possibly indicates a very early role of Lef1/Tcf1 during the establishment of the dorso-ventral axis. In the chick, Wnt3a has been shown to induce mesenchymal expression of *Lef1* (Kengaku *et al.*, 1998). However, as with other progress zone markers such as *Fgf10* and *Msx1*, it is possible that Wnt3a acts via Fgf8 in the ectoderm and that *Lef1* is not directly induced by Wnt3a (Kengaku *et al.*, 1998). Indeed Fgf8 can substitute for the AER and induce/maintain *Lef1* expression in the limb mesenchyme (Grotewold and Rüther, 2002).

In the chick, *Wnt3a* expression persists in the AER throughout limb development where it maintains *Fgf8* expression and AER function (Kengaku *et al.*, 1998). The regulation of *Fgf8* expression in the ectoderm suggests that Wnt signalling within the AER can act

cell-autonomously. Consistent with this is the expression of *β-catenin*, *Tcf1*, *Lef1* and the receptor *Fz4* in the AER (Oosterwegel *et al*., 1993; Kengaku *et al*., 1998; Lu *et al*., 1997; Nohno *et al*., 1999). Another possible role for cell-autonomous Wnt function within the AER would be to regulate the expression of gap junctions, which are needed to maintain the integrity of the AER (Becker *et al*., 1999; Makarenkova and Patel, 1999). Wnt signalling via the β-catenin pathway has been shown to transcriptionally activate Cx43, a component of gap junctions, in P19 cells and cardiomyocytes and to increase gap junction communication in *Xenopus* (Olson *et al*., 1991; van der Heyden *et al*., 1998; Ai *et al*., 2000). Misexpression of Wnt1 in the mesenchyme has also been associated with a change in Cx43 expression – Cx43 is down-regulated in the Wnt1-expressing cells, but is up-regulated in the surrounding mesenchyme (Meyer *et al*., 1997). Whether these are direct effects or due to changes in patterning/cell differentiation is unclear, although it is possible that Wnts, together with Fgf signalling from the AER, also regulate gap junctional expression in the limb mesenchyme (Makarenkova *et al*., 1997). Several other Wnts, such as *Wnt5a* and *–12,* together with the Wnt antagonists *Sfrp1* and *Dkk1,* are also expressed in the AER, although their functions are unknown (Christiansen *et al*., 1995; Esteve *et al*., 2000; Mukhopadhyay *et al*., 2001; Grotewold and Rüther, 2002).

In addition to controlling *Fgf8* expression, in the chick Wnt3a and –7a signal in conjunction with Fgfs to induce/maintain the expression of *Csa1*, a gene mutated in the human Townes-Brocks syndrome which is characterised by pre-axial polydactyly (Farrell and Münsterberg, 2000). Neither Wnt nor Fgf signalling alone can induce/maintain *Csa1* expression in the progress zone. This shows that in addition to acting alone, Wnts may also act in synergy with other factors, again highlighting the complexity of Wnt signalling in limb bud development. This synergy is also seen during otic and neural induction in chicks, and in antero-posterior patterning of the neuroectoderm in *Xenopus*, suggesting that it may be a fundamental process of many aspects of development, which to date, has not been investigated (McGrew *et al.,* 1997; Ladher *et al*., 2000b; Wilson *et al*., 2001). This in part may be due to the ability of Fgfs to substitute almost wholeheartedly for the AER making investigation into the role of other AER factors almost redundant.

Another Wnt required for limb outgrowth is Wnt5a. Gene inactivation of *Wnt5a* in mice results in a shortening of the limb reflecting an overall retardation in development (Yamaguchi *et al*., 1999). All skeletal structures are affected but in general the severity increases in a proximal to distal direction. Thus, the distal phalanges are missing whilst the proximal elements are shortened. However, patterning of the elements is normal. This truncation is also observed in other regions of the body – the rostro-caudal axis, the jaws and the genitalia - suggesting that a common mechanism mediated by Wnt5a controls the development of all these structures. This hypothesis, at least for the genitals and limbs, has previously been put forward on the basis of gene-inactivation of members of the *Hox* gene complex which affects both structures (Kondo *et al*., 1997). In both the paraxial mesoderm and the limb bud mesenchyme, loss of Wnt5a results in a decrease in proliferation (Yamaguchi *et al*., 1999). Whether a similar proximo-distal progression of defects occurs in the face is unclear but in light of the parallels that are often drawn between limb and face development, it will be of interest to compare. Analysis of the regulation of *Wnt5a* expression has shown that, as with many progress zone markers, Wnt5a is regulated by Fgf signalling from the AER (Kawakami *et al*., 1999).

In contrast, Wnt7a controls dorso-ventral patterning. In the absence of *Wnt7a* (i.e. following gene-inactivation of *Wnt7a* in mice, and the naturally occurring *Wnt7a* mutant, *postaxial hemimelia,* which is due to a splicing defect in *Wnt7a*) the sesamoid bones and foot pads are duplicated, being found both ventrally and dorsally, and the dorsal tendons assume a ventral pattern (Parr and McMahon, 1995; Parr *et al*., 1998). However, there is not a complete transformation of a dorsal to ventral fate as the dorsal ectodermal derivatives, the nails and hair, are still present, although they are abnormal (Parr and McMahon, 1995). Wnt7a maintains *Shh* expression which is necessary for antero-posterior patterning (Parr and McMahon, 1995; Yang and Niswander, 1995). Thus, in the *Wnt7a* mutants, the posterior digits are lost consistent with the loss of *Shh* expression (Parr and McMahon, 1995; Parr *et al*., 1998). Wnt7a signalling may depend on LRP6 as loss of LRP6 function in the mouse also results in dorso-ventral patterning and anterior-posterior patterning defects similar to that seen in the *Wnt7a* mouse mutant (Pinson *et al*., 2000). In addition, in *LRP6* mutants, the AER is not maintained possibly reflecting a role in Wnt3a signalling. As Wnt7a is initially expressed throughout the dorsal limb ectoderm, only later becoming confined to the distal dorsal ectoderm, it is unclear why the proximal structures are unaffected. There may be redundancy with other Wnts substituting for Wnt7a function or this may reflect the differences in patterning mechanisms between the autopod and the zeugopod/stylopod.

Wnt7a regulates the expression of the LIM-homeobox-containing gene *Lmx1* in the chick and its homologue *Lmx1b* in the mouse. Indeed, the onset of *Lmx1/1b* expression is coincident with *Wnt7a* expression (Riddle *et al*., 1995; Cygan *et al*., 1997). In the chick and mouse limb bud, *Lmx1/1b* is expressed in the dorsal subectodermal mesenchyme over a distance of 9-12 cell layers (Riddle *et al*., 1995; Vogel *et al*., 1995; Cygan *et al*., 1997). This hints, but does not definitively prove, how far ectodermal Wnts diffuse or signal through the mesenchyme and is similar to that seen in *Drosophila*. Misexpression of Lmx1 dorsalises the ventral distal limb bud showing that it is a key downstream mediator of Wnt7a signalling (Riddle *et al*., 1995; Vogel *et al*., 1995). In *Wnt7a* mutants, *Lmx1b* expression in the limb bud is initiated but is down-regulated distally by E11.5 showing that Wnt7a is not required for the early and proximal expression of *Lmx1b*, and consistent with the absence of proximal defects in the $Wnt7a^{-/-}$ limb bud (Cygan *et al*., 1997).

Gene inactivation of *Lmx1b* in the mouse again shows that Lmx1b mediates some of the effects of Wnt7a signalling on dorsal patterning (Chen *et al*., 1998). As with *Wnt7a* mutants, the distal ventral tendons and muscles, footpads and sesamoid bones are duplicated dorsally whilst the distal ulna and the hair follicles are absent. In contrast to *Wnt7a* mutants, some proximal structures are also affected with the pelvis, clavicle and scapula being slightly abnormal. Also, *Shh* expression is not down-regulated showing that Wnt7a regulates *Shh* expression via an Lmx1b-independent pathway (Chen *et al*., 1998). Likewise, loss of function of Lmx1 in the chick can affect dorsal derivatives (Rodriguez-Esteban *et al*., 1998). Analysis of molecular markers also shows that *Shh* expression is unaffected together with the

expression of *Fgf8*, *Fgf10*, *Wnt3a*, *Wnt7a* and *Lmx1*. The normal expression of *Wnt7a* and *Lmx1* suggests that their expression is not autoregulated. In humans, mutation of *LMX1B* results in the autosomal dominant nail-patella syndrome characterised by dorsal limb defects (Dreyer *et al.*, 1998). The knee caps are hypoplastic or absent, the elbows are abnormal, and the fingernails may be brittle or absent.

Wnt7a appears to signal through Fz10, which is expressed in the dorsal-posterior-distal mesenchyme underlying *Wnt7a* expression in the ectoderm and colocalising with *Shh* (Kawakami *et al.*, 2000). Assays in *Xenopus* have shown that Fz10 and Wnt7a synergise when co-injected showing that Wnt7a may signal through this receptor (Kawakami *et al.*, 2000). In the limb bud *Fz10* expression can be induced by Shh and Wnt7a providing a positive feedback loop to maintain the expression of these genes in the distal mesenchyme (Kawakami *et al.*, 2000). *Wnt7a* expression is regulated negatively by engrailed1, a homeobox-containing gene which is essential for formation of the ventral structures. In the absence of engrailed1, *Wnt7a* is ectopically expressed in the ventral ectoderm resulting in the formation of bi-dorsal distal limbs (Loomis *et al.*, 1996; Cygan *et al.*, 1997). Conversely, misexpression of engrailed1 in the chick results in a down-regulation of *Wnt7a* in the dorsal ectoderm (Logan *et al.*, 1997).

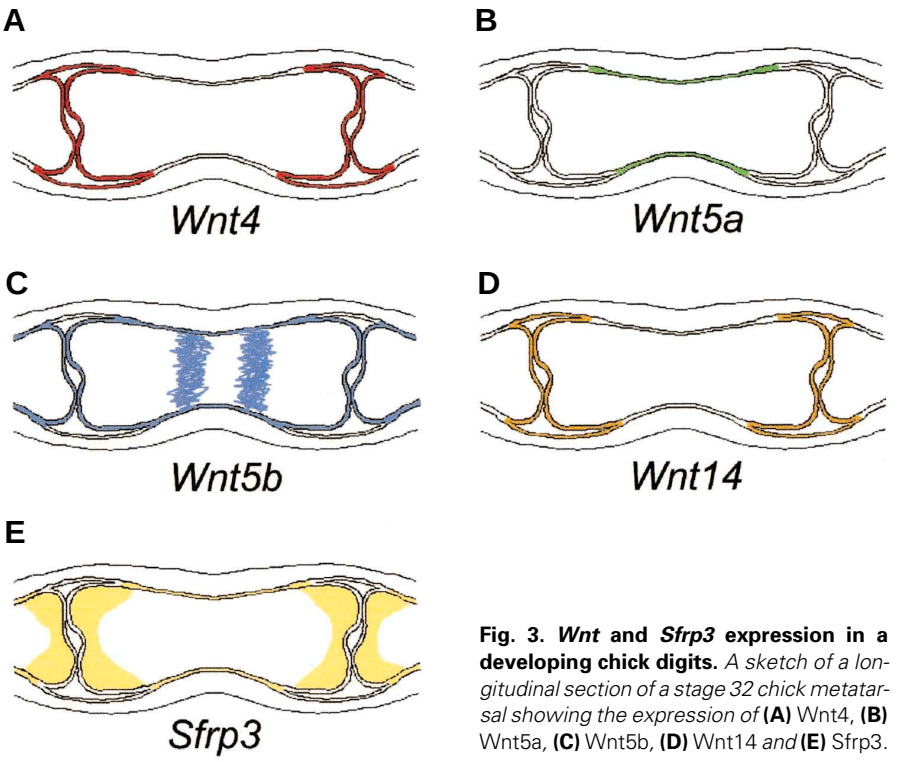

Fig. 3. *Wnt* and *Sfrp3* expression in a developing chick digits. *A sketch of a longitudinal section of a stage 32 chick metatarsal showing the expression of* **(A)** Wnt4, **(B)** Wnt5a, **(C)** Wnt5b, **(D)** Wnt14 *and* **(E)** Sfrp3.

Finally, Wnt signalling has been implicated in the sculpting of the limb bud, removing "excess" tissue by programmed cell death. Indeed, the ability of BMP4 to induce cell death in the developing limb appears to be mediated by Dkk1 (Grotewold and Rüther, 2002). Loss of function of Dkk1 in mice results in the down-regulation of *Msx1*, a component of the cell death pathway, in the anterior and posterior necrotic zones and the interdigital mesenchyme, whilst gain of Dkk1 function in chicks causes excessive cell death via activation of the c-jun pathway (Mukhopadhyay *et al.*, 2001; Grotewold and Rüther, 2002). The decrease in cell death in the mouse mutants contributes to the polydactyly and fusion of digits that occurs in Dkk1 mouse mutants (Mukhopadhyay *et al.*, 2001). In addition, *Fz2*, *Fz3* and *Fz4,* and *Dkk-2,and –3*, are expressed in the interdigital mesenchyme suggesting that a fine balance of Wnt signalling controls cell death/survival in this region (Monaghan *et al.*, 1999; Nohno *et al.*, 1999).

Other Wnts that are expressed in the limb bud include *Wnt3*, *-4*, *-6* and *-7b* in the ectoderm (Parr *et al.*, 1993). Their role during patterning and outgrowth of the limb is unknown although Wnt-4, -7a and -7b can induce *neurotrophin-3* expression and hence survival of sensory neurons (Patapoutian *et al.*, 1999).

Muscle Differentiation

Wnts may play a number of roles during muscle development including the initiation of myogenesis. As the limb myogenic precursors migrate into the limb bud, they are uncommitted to myogenic differentiation. Once within the limb bud, they become committed switching on the expression of the myogenic determination factors *MyoD* and *Myf5*. During migration and within the limb bud they come into contact or close proximity with a number of Wnt signals, including Wnt5a and -11 in the mesenchyme and ectodermal Wnts such as Wnt3 and -4. Whether Wnt signalling initiates myogenesis in the limb bud is unclear. However, the ability of Wnts or β-catenin to activate myogenesis in the somite and in P19 cells respectively, together with the expression of the Wnt antagonist *Sfrp2* in uncommitted myogenic precursors, which is down-regulated as the myogenic cells differentiate, suggests that this is possible (Cossu *et al.*, 2000; Ladher *et al.*, 2000a; Petropoulos and Skerjanc, 2002).

In addition, Wnt signalling may modulate other aspects of myogenic development such as migration and cell morphology. In the limb bud, N-cadherin is essential for myogenic migration and, during chondrogenesis, Wnt7a has been shown to maintain the expression of this cadherin (Brand-Saberi *et al.*, 1996; Tufan and Tuan, 2001). Thus, if parallels between muscle and skeletal development can be drawn, this may suggest an additional potential role of Wnts during limb muscle development. Finally, work in our laboratory has shown that Wnt signalling can regulate the terminal stages of muscle differentiation and can alter the number of myoblasts expressing either slow or fast Myosin heavy chains (MyHCs) (Anakwe *et al.*, submitted). Each muscle is composed of a unique combination of slow and fast fibres which will determine how it will function. Slow fibres have oxidative metabolism, contract slowly and in general are required for posture whilst fast fibres contract rapidly with high force, have glycolytic metabolism and are necessary for movement. Therefore, our data suggest that Wnt signals determine the intricate pattern of slow/fast MyHC expression in the musculature.

Skeletal Development

The expression patterns and putative functions of several of the Wnts and components of the signalling pathway have been described during skeletal development. Their particular spatiotemporal expression patterns, together with functional analyses in the chick, suggest that different members of the Wnt family possess distinct roles during chondrogenesis and joint development.

During the early stages of chondrogenesis, *Sfrp3* (*Frzb*) is expressed in the central mesenchymal condensation where it encompasses the domain of *Sox9* expression which marks chondrogenic commitment (Wada *et al*., 1999; Baranski *et al*., 2000; Esteve *et al*., 2000; Ladher *et al*., 2000a). *Wnt5a*, *-5b*, *-11* and *–14,* together with the receptors *Fz4 and –6*, and the Wnt antagonists *Dkk2* and *–3,* are also expressed in the mesenchyme surrounding the developing cartilage elements (Christiansen *et al*., 1995, 1996; Tanda *et al*., 1995; Kawakami *et al*., 1999; Monaghan *et al*., 1999; Nohno *et al*., 1999; Hartmann and Tabin, 2000; 2001). Wnt1, -7a and –14 have been shown to inhibit chondrogenesis in micromass culture and, therefore, the presence of the antagonists, particularly Sfrp3, may allow chondrogenesis to proceed (Rudnicki and Brown, 1997; Stott *et al*., 1999; Hartmann and Tabin, 2001). 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 (Tufan and Tuan, 2001).

As chondrocytes proceed through the differentiation pathway, the expression profile of Wnts, receptors and antagonists alters (see Fig. 3). For instance, *Sfrp3* becomes confined to the epiphyseal (i.e. relatively undifferentiated) chondrocytes (Hoang *et al*., 1996; Wada *et al*., 1999; Baranski *et al*., 2000; Ladher *et al*., 2000a). *Wnt5b* is expressed in a sub-population of the diaphyseal chondrocytes whilst *β-catenin* and *Tcf4* are more widely expressed with transcripts of both overlapping in the articular, prehypertrophic and hypertrophic chondrocytes (Hartmann and Tabin, 2000; Church *et al.,* in press).

Misexpression of Wnt5a results in the formation of shorter skeletal elements due to a delay in chondrocyte differentiation. Thus, there is a reduction in the number of hypertrophic chondrocytes and a smaller *Ihh*-expressing (i.e. prehypertrophic) domain (Kawakami *et al*., 1999; Hartmann and Tabin, 2000). Similarly, misexpression of dominant-negative versions of *Fz1* and *–7,* two of the receptors expressed in developing elements, has a similar effect to Wnt5a in that misexpression results in shorter elements: the hypertrophic domain is absent and the prehypertrophic zone is smaller (Hartmann and Tabin, 2000). In contrast, *in vitro* micromass studies have shown that these two receptors can have different effects when overexpressed: Fz7, but not Fz1, inhibits chondrogenesis (Tufan *et al*., 2002).

Several Wnts and their signalling pathway components are expressed in the perichondrium, a layer of fibroblasts, surrounding the cartilage element. The perichondrium inhibits chondrocyte differentiation by a PTHrP-Ihh signalling loop, contributes to appositional growth and also ultimately will be become the site of bone collar formation. Other molecular players that control perichondrial development and mediate signalling pathway interactions are uncharacterised. However, candidate factors include *Wnt5a, -5b, -11*, *Fz1*, *Fz2*, *β-catenin* and *Dkk1* which are expressed here (Christiansen *et al*., 1995; Tanda *et al*., 1995; Lako *et al*., 1998; Monaghan *et al*., 1999; Hartmann and Tabin, 2000; Church *et al.,* in press).

In contrast to the other Wnts, Wnt4 and -14 have been associated with joint formation. During early joint development, both genes are expressed in the presumptive joint regions (Kawakami *et al*., 1999; Hartmann and Tabin, 2000; 2001). Additionally, *cWnt14* is expressed in the mesenchyme adjacent to the joint regions (Hartmann and Tabin, 2001). As the joint undergoes differentiation, both genes are expressed in the synovial membrane and the connective tissue of the joint capsule (Church *et al.,* in press; Hartmann and Tabin, 2000; 2001). Mouse *Sfrp2* (in contrast to chick *Sfrp2*) is also expressed at sites of joint development (Leimeister *et al*., 1998). Misexpression of Wnt14 in the developing chick limb has shown that Wnt14 can induce the joint phenotype as indicated by the absence of cartilage matrix and loss of chondrogenic markers concomitant with the acquisition of joint markers (Hartmann and Tabin, 2001). The study also implicated Wnt14 as a key factor in defining joint spacing in development: in addition to the induction of ectopic joints, Wnt14 seemed to prevent the formation of endogenous joints in the adjacent region (Hartmann and Tabin, 2001). Furthermore, Wnt14 can induce dedifferentiation of chondrocytes in micromass cultures, a process that occurs during normal joint development (Hartmann and Tabin, 2001). In contrast to Wnt14, overexpression of Wnt4 does not induce joint formation but instead accelerates chondrocyte differentiation and bone collar formation as suggested by an expanded hypertrophic zone and ultimate shortening of the elements, as well as a thicker osteoid layer with increased expression of osteogenic markers and calcification (Hartmann and Tabin, 2000). It has been proposed that Wnt4 signals from the developing joint to control chondrocyte differentiation (Hartmann and Tabin, 2000).

At present it is unclear which pathways are utilised by Wnt signalling during skeletal differentiation. β-catenin can mimic the effect of Wnt1, -7a and –14 in inhibiting chondrogenic differentiation *in vitro* whilst overexpression of β-catenin *in vivo* mimics the effect of Wnt4 to accelerate terminal chondrocyte differentiation (Hartmann and Tabin, 2000). It is interesting to note that Wnt4 and –5a are in the same class of Wnts yet have opposing effects on chondrocyte differentiation (Hartmann and Tabin, 2000). These different effects may result from the activation of different pathways.

Degenerative Skeletal Disorders

Classically, misregulation of the Wnt pathway has been associated with carcinogenesis. However, recent evidence has emerged to show that regulation of Wnt function is essential for both skeletal development and maintenance of the skeleton in the adult. There are currently three skeletal dsyplasias which are linked to the Wnt signalling pathway. These are progressive pseudorheumatoid dysplasia, characterised by the loss of the articular cartilage and joint space starting during early childhood; Robinow syndrome, characterised by a severe skeletal dysplasia with short bones, brachydactyly, spinal defects and facial anomalies; and osteoporosis-pseudoglioma syndrome, which results in decreased bone mass in adults (Hurvitz *et al*., 1999; Afzal *et al*., 2000; Gong *et al*., 2001). In the former, ***W**nt-**I**nduced **S**ecreted **P**rotein 3 (WISP3)* is mutated whilst Robinow syndrome is due to mutations in the tyrosine kinase receptor *ROR2* and osteoporosis-pseudoglioma syndrome is the result of mutations in the Wnt co-receptor *LRP5*. All are thought to be loss-of-function mutations. In contrast, there are predicted gain-of-function mutations in human *ROR2* which result in the dominant

syndrome brachydactyly type B, possibly reflecting a Wnt-mediated patterning defect (Schwabe *et al.*, 2000). In addition, as other players that modulate Wnt signalling emerge, it is possible that other human syndromes may also be linked to deregulation of the Wnt signalling pathways. For example, β–catenin can interact with Sox proteins which antagonise Wnt function (Zorn *et al.*, 1999; Takash *et al.*, 2001). Mutations in human *Sox9* give rise to the syndrome campomelic dysplasia, a bone dysmorphogenesis. Although not tested it is possible that this syndrome is in part due to increased Wnt activity. Similarly, the paired-type homeobox protein Alx4 can bind Lef1 and may modulate the β-catenin/Lef1 signalling pathway (Boras and Hamel, 2002). In humans, haploinsufficiency of *ALX4* results in a bone mineralisation defect in the skull (Wuyts *et al.*, 2000; Mavrogiannis *et al.*, 2001). Likewise, notch signalling, which is important for skeletal development, interacts with components of the Wnt pathway (Axelrod *et al.*, 1996; Ross and Kadesch, 2001). Thus, a number of signalling pathways that control skeletogenesis may converge on or modify the Wnt signalling pathway.

Wisps are members of the CCN (CTGF/Cyr61/Nov) family of growth factors which participate in a multitude of processes, including chondrogenesis (reviewed by Perbal, 2001; Lau and Lam, 1999). The subfamily of Wisps comprises three proteins: Wisp1 and –2 were initially identified as proteins that are up-regulated in Wnt1-transformed mammary epithelial (C57MG) cells and Wisp3 was identified by screening EST databases for homologous genes (Pennica *et al.*, 1998). Further analysis showed that the *Wisp1* promoter contains Tcf/Lef1 binding sites and is directly activated by Wnt1 signalling (Xu *et al.,* 2000). Furthermore, like Wnt1, Wisp1 also has oncogenic activity and, therefore, appears to mediate some of the effects of Wnt1 signalling. Whilst Wisp1 is under the control of Wnt1/β-catenin signalling, connective tissue growth factor (CTGF), the founder member of the family, is under the control of TGF-β (Grotendorst *et al.*, 1996). Therefore, distinct sets of growth factor signals appear to converge on different CCN proteins to mediate their effects. *Wisp3* is expressed in synoviocytes, chondrocytes and bone marrow stem cells undergoing chondrogenesis *in vitro*, and *Wisp2* is expressed by chondrocytes and osteoblasts (Hurvitz *et al.*, 1999; Kumar *et al.*, 1999). These data, together with the *Wisp3* mutation in progressive pseudorheumatoid dysplasia, strongly suggest that Wisps may mediate Wnt regulation of the formation and maintenance of cartilage and joints.

The 120 residue cysteine-rich Frizzled motif is found not just in frizzed receptors and Sfrps, but is also present in type XVIII collagen, carboxypeptidase Z and receptor tyrosine kinases (RTKs), which include the ROR family (Rehn *et al.*, 1998). Thus it is possible that Wnts may bind to these molecules and, in the case of the receptors, activate or block (by steric competition for the "legitimate" ligand) the signalling pathway. *ROR2* is expressed during face and limb skeletal development in the mouse (Matsuda *et al.*, 2001). For example, *ROR2* transcripts are found in the perichondrium of the digits. *ROR1* is expressed in the anterior and posterior necrotic zones of the limbs and in interdigital areas, in a similar region to the Wnt antagonist Dkk1, and thus may control cell death (Matsuda *et al.*, 2001). Like the human Robinow syndrome, mice null for ROR2 have a severe skeletal phenotype which is also characterised by misshapen bones and abnormal growth plate morphology (Afzal *et al.*, 2000; Takeuchi *et al.*, 2000). Thus, ROR2 has essential roles in chondrocyte differentiation and ossification. As yet, the ligands for the RORs are not known but the presence of a frizzled domain suggests that Wnts may modify/induce ROR signalling.

Finally, mutation of LRP5, which is expressed in osteoclasts, results in osteoporosis-pseudoglioma syndrome (Gong *et al.*, 2001). This syndrome is characterised by a low bone mass and increased vulnerability to fractures and deformation. A dominant negative LRP5 blocks the ability of bone morphogenetic proteins (BMPs) to induce alkaline phosphatase activity in C3H10T1/2 and ST2 cell lines showing that Wnt signalling acts downstream of BMPs (Gong *et al.*, 2001).

Rheumatoid arthritis (RA) is a debilitating disease characterised by inflammatory responses resulting in the erosion of the articular cartilage. A number of Wnts and frizzled receptors (namely Wnt1, -5a, -10b, -11 and -13, and Fz2, -5 and -7) are expressed in synovial tissue from patients with RA, with a notable upregulation of Wnt5a and Fz5 expression (Sen *et al.*, 2000). Importantly, overexpression of Wnt5a in normal synovial cells *in vitro* stimulates the production of interleukin-6, -8 and -15, cytokines that can contribute to the eventual destruction of the joint in RA, whilst blocking Wnt5a and Fz5 function abolishes these responses (Sen *et al.*, 2000; 2001). Wnt signalling has also been implicated in osteoarthritis where *Sfrp4* expression has been shown to correlate with apoptotic chondrocytes within the degenerating articular cartilage (James *et al.*, 2000). Another member of the Sfrp family, Sfrp2, sensitises MCF7 cells to apoptotic stimuli suggesting that Sfrp4 may directly contribute to the chondrocyte apoptosis (Melkonyan *et al.*, 1997). These are the first reports implicating Wnt signalling in arthritic disease and are sure to be followed by many important discoveries.

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