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Comprehensive expression analysis of all Wnt genes and their major secreted antagonists during mouse limb development and cartilage differentiation

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ABSTRACT

Wnt signalling plays important roles in patterning and outgrowth of the vertebrate limb. Different mutations in Wnt genes, their antagonists or (co-)receptors result in patterning and outgrowth defects as well as chondrocyte and bone phenotypes in mouse and human. Understanding Wnt activity during mouse limb development and chondrogenesis requires a temporal and spatial overview of Wnt signalling key factor expression. Here we present a comparative expression analysis of all 19 *Wnt* genes and their major secreted antagonists of the *Dickkopf* (*Dkk*), *Wisp* and the *secreted frizzled related protein* (*Sfrp*) families during mouse limb development. Our study reveals new domains of expression for *Wnt2*, *Wnt2b*, *Wnt5b*, *Wnt6*, *Wnt7b*, *Wnt9a*, *Wnt10a*, *Wnt10b*, *Wnt11* and *Wnt16*, in the limb. We also identified novel expression domains for the Wnt antagonists *Sfrp1*, *Sfrp3*, *Sfrp5*, *Wisp1* as well as *Dkk2* and *Dkk3*. We provide a full expression pattern for *Wif1* in limb development, for which no limb expression had been documented so far.

1. Results and discussion

Wnt signalling cascades are implicated in several processes at many stages of vertebrate limb development. They are involved in proximal-distal outgrowth and dorso-ventral patterning as well as later during chondrogenesis, osteogenesis, muscle development or joint initiation and maintenance (reviewed in Church and Francis-West, 2002). Mutations in human Wnt genes Wnt3 and Wnt7a result in severe skeletal malformations (Niemann et al., 2004; Woods et al., 2006) and several mouse lines with misregulated Wnt signalling exhibiting various limb phenotypes have been described until now (reviewed in Logan and Nusse, 2004). Different inhibitors of the Wnt signalling cascade add a second level of regulation. Whereas members of the family of secreted frizzled related proteins (Sfrp) directly bind to Wnt ligands and thus preventing receptor binding (reviewed in Bovolenta et al., 2008; Kawano and Kypta, 2003), antagonists of the Dickkopf family (Dkk) act via interaction with Wnt coreceptors named low density lipoprotein receptor-related proteins (LRPs) (reviewed in Niehrs, 2006). A balanced combination of ligand expression and antagonistic regulation ensures accurate Wnt signalling and therefore proper limb outgrowth, patterning and cartilage differentiation.

In order to comprehensively understand Wnt signalling during limb development it is therefore desirable to provide a complete overview of temporal and spatial expression patterns of all Wnt ligands and their secreted antagonists. So far analysis of Wnt and Wnt antagonist expression has been focussed on single aspects of limb development with the exception of a recent study by Summerhurst et al. (2008), which described expression of all *Wnt* and *Frizzled* genes at a single embryonic stage (E11.5). Thus, we re-analysed all mouse *Wnt* genes and genes for secreted Wnt antagonist of the Sfrp, Dkk and CCN/Wisp families by whole-mount in situ hybridization (ISH) from embryonic day E9.5 to E13.5 and additionally by section ISH at E13.5 and E15.5. This covers limb development from the early limb bud stage to the formation and differentiation of different cell types and tissues in the limb such as cartilage and bone. All sections of E15.5 limbs show the distal zeugopode (radius and ulna). For orientation, hybridisation of cartilage and bone marker genes *Collagen type 1 alpha 1 (Col1a1), Collagen type 2 alpha 1 (Col2a1) and Collagen type 10 alpha 1 (Col10a1)* on E15.5 sections are shown in Supplementary Fig. 1. In the body text and figures of this paper only expression patterns are shown and discussed that were newly identified in this screen and have not been described in the mouse previously. Figures containing

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all probes and embryonic stages analysed are provided as Supplementary information.

1.1. Novel expression domains of Wnt genes in mouse limb development

All 19 mouse *Wnt* genes were analysed by whole-mount and section ISH. We were not able to detect expression of *Wnt1*, *Wnt3a*, *Wnt8a*, *Wnt8b* in the limb, however detection of *Wnt1* signal in dorsal neural tube (Wilkinson et al., 1987), *Wnt3a* in the presomitic mesoderm (Aulehla et al., 2003), Wnt8a in the ear (Summerhurst et al., 2008) and *Wnt8b* in the forebrain (Lako et al., 1998) (Supplementary Figs. 2 and 4 and data not shown) ensure fidelity of the probes. For *Wnt3*, *Wnt5a*, *Wnt7a* and *Wnt9b* no novel expression patterns could be detected. For these Wnt molecules we verified previously described mRNA localizations and completed the time course of expression patterns (Supplementary Figs. 2–4). For *Wnt2*, *Wnt2b*, Wnt4, *Wnt5b*, *Wnt6*, *Wnt7b*, *Wnt9a*, *Wnt10a*, *Wnt10b*, *Wnt11* and *Wnt16*, we identified new expression domains or describe expression that had been partially observed in other species, but not in the mouse before.

Wnt2 was not expressed during early limb development. Summerhurst et al (2008) have noted a weak, restricted expression in the proximal limb mesenchyme at E11.5, which we did not identify using WM-ISH, potentially owing to the higher sensitivity of the OPT method. At E15.5 however, we found expression of *Wnt2* specifically in the distal epimysium of zeugopode muscles (Fig. 1A).

Wnt2b expression has not been shown in the limb so far. Expression in cartilage was weakly detected in post-natal growth plates by RT-PCR (Andrade et al., 2007). *Wnt2b* showed a specific transient expression in proximal interdigital mesenchyme at E13.5 (Fig. 1E and Supplementary Fig. 2). No expression of *Wnt2b* could be detected before or after this stage in the limb (Supplementary Fig. 2).

Wnt3 was expressed in limb ectoderm as reported before (Barrow et al., 2003). At E13.5 and 15.5 we found that expression in the skin was confined to the basal cell layer (Fig. 1I and J).

Wnt4 was expressed strongly in the ectoderm at E9.5 and E10.5 but decreased distally at E11.5 (Fig. 1B and Supplementary Fig. 2, arrows). We were not able to detect mesenchymal expression as described by Summerhurst et al. (2008) at E11.5. Insufficient penetration of the probe into the tissue could be an explanation, but since we detected Wnt4 expression in the neural tube (Supplementary Fig. 2) this appears unlikely. Thus, the higher sensitivity of the OPT technique used by Summerhurst et al. might account for the difference. At E13.5 expression was observed in the hand plate interdigital region. Vibratome sections revealed expression in ectoderm and peripheral interdigital mesoderm (Fig. 1c,c' arrowheads) and also in cells surrounding ventral blood vessels (Fig. 1c arrows). Section ISH at E13.5 showed expression in synovial joints (Supplementary Fig. 2, arrows) as demonstrated before in chick and mouse (Barrow et al., 2003; Guo et al., 2004; Hartmann and Tabin, 2000). At E15.5 section ISH showed expression of *Wnt4* mainly in basal cells of the skin (Fig. 1D).

The expression of *Wnt5a* in the limb is well described in the literature, our ISH results confirmed previous observations in mouse and chick (Hartmann and Tabin, 2000; Yamaguchi et al., 1999; Yang et al., 2003) (Supplementary Fig. 3).

Wnt5b mRNA was detected in superficial limb mesenchyme from E9.5 to E13.5 (Fig. 1F and Supplementary Fig. 3), but we did not detect expression in ventral-proximal mesenchyme as described previously at E11.5 (Summerhurst et al., 2008). Section ISH at E13.5 revealed expression of *Wnt5b* in distal mesenchyme surrounding the cartilaginous condensations and in the perichondrium of the metacarpals. (Fig. 1G). *Wnt5b* was also expressed in perichondrium, prehypertrophic and hypertrophic chondrocytes at stage E15.5 (Fig. 1H), so far only shown for the chicken ortholog (Church et al., 2002; Hartmann and Tabin, 2000).

Wnt6 was detected in the limb ectoderm in all stages analysed. From E9.5 to E11.5 an intense signal in the apical ectodermal ridge (AER) was noticed. (Fig. 1K and Supplementary Fig. 3, arrows). In the hand plate at E13.5, *Wnt6* was expressed in the ectoderm, expression was more intense in areas covering interdigital mesenchyme than in areas dorsal and ventral to developing digits (Fig. 1L). Section ISH at E13.5 and E15.5 showed restriction of *Wnt6* expression to the basal cell layer of the skin (Fig. 1M and N).

Wnt7a showed the well-described early limb bud expression in dorsal ectoderm (Parr and McMahon, 1995) (Supplementary Fig. 3, arrows). Ectodermal expression appeared to cease at E12.5 and was not detectable at E13.5 by whole-mount or section ISH. However, section ISH at E15.5 showed reappearing expression of *Wnt7a* in the ectoderm predominantly in basal cells (Fig. 1O and Supplementary Fig. 3).

Wnt7b, in contrast to its well-described close relative *Wnt7a*, shows an expression in both dorsal and ventral ectoderm at early stages E10.5–E11.5, however, we noted that at E11.5 expression of Wnt7b was weaker in dorsal than in ventral ectoderm (Fig. 1P and Supplementary Fig. 4, arrow). This is in accordance with the finding of Summerhurst et al. (2008) of predominant ventral expression at E11.5. In later stages, *Wnt7b* becomes restricted to basal cells of the epidermis (Fig. 1Q and R) and shows weak expression in the perichondrium flanking the prehypertrophic chondrocytes (Supplementary Fig. 4, arrowhead) as described before (Hu et al., 2005).

Wnt9a mRNA was detected in tissue flanking hand plate condensations dorsally and ventrally at E12.5 and E13.5 (Fig. 1S and Supplementary Fig. 4, arrows) as well as in muscle at E15.5 (Fig. 1T), in addition to the known expression in the joints in chick and mouse (Guo et al., 2004; Hartmann and Tabin, 2001) (Fig. 1T and Supplementary Fig. 4, arrows).

Wnt9b showed ectodermal expression as demonstrated by whole-mount ISH and section ISH (Supplementary Fig. 4). At E15.5 *Wnt9b* appeared to be expressed in the ectoderm in a patchy pattern (Supplementary Fig. 4, arrows). Ectodermal expression of *Wnt9b* in the mouse has been reported before (Lan et al., 2006) during craniofacial development.

Wnt10a was expressed weakly in limb ectoderm from E9.5 to E13.5 and prominently in the AER until E11.5 but not thereafter in accordance with expression previously reported in chick and for E11.5 in the mouse (Narita et al., 2005; Summerhurst et al., 2008) (Fig. 1U and V and Supplementary Fig. 5, arrows). At E15.5 expression was strong in the epidermis, where *Wnt10a* transcripts were predominantly found in the basal cell layer. (Fig. 1W).

Wnt10b was expressed throughout the limb ectoderm in all stages analysed. Expression in the AER at E11.5 has been described by Summerhurst et al. (2008), interestingly we found AER expression specifically only at E11.5, not at earlier or later stages (Fig. 1X and Supplementary Fig. 5). At E13.5 and E15.5 *Wnt10b* was expressed in a patchy pattern in the epidermis (Supplementary Fig. 5, arrows). It was reported before, that *Wnt10b* is expressed in hair placodes (Reddy et al., 2001).

Wnt11 was expressed in the mesenchyme of developing limb buds at E9.5 to E11.5 in a pattern overlapping with recently published results at E11.5 by Summerhurst et al. (2008). In general we detected a weaker and more superficial pattern than reported by Summerhurst et al, again indicating a difference in sensitivity between the techniques used. We detected expression of *Wnt11* in the AER strongly at stage E9.5 and faintly at E10.5 and E11.5. (Supplementary Fig. 5, arrows). In the autopod at E12.5, *Wnt11* showed a mesenchymal expression flanking to the condensations that expanded further distally at the dorsal side at stage E13.5 (Fig. 1Z and Supplementary Fig. 5). Section ISH at E13.5 and E15.5 revealed expression of *Wnt11* in mesenchyme and dermal