Wls-mediated Wnts differentially regulate distal limb patterning and tissue morphogenesis

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ABSTRACT

Wnt proteins are diffusible morphogens that play multiple roles during vertebrate limb development. However, the complexity of Wnt signaling cascades and their overlapping expression prevent us from dissecting their function in limb patterning and tissue morphogenesis. Depletion of the ( ) gene, which is required for the secretion of various Wnts, makes it possible to genetically dissect the overall effect of Wnts in limb development. In this study, the gene was conditionally depleted in limb mesenchyme and ectoderm. The loss of mesenchymal prevented the differentiation of distal mesenchyme and arrested limb outgrowth, most likely by affecting Wnt5a function. Meanwhile, the deletion of ectodermal resulted in agenesis of distal limb tissue and premature regression of the distal mesenchyme. These observations suggested that Wnts from the two germ layers differentially regulate the pool of undifferentiated distal limb mesenchyme cells. Cellular behavior analysis revealed that ectodermal Wnts sustain mesenchymal cell proliferation and survival in a manner distinct from Fgf. Ectodermal Wnts were also shown for the first time to be essential for distal tendon/ligament induction, myoblast migration and dermis formation in the limb. These findings provide a comprehensive view of the role of Wnts in limb patterning and tissue morphogenesis.

Introduction

Vertebrate limb patterning and morphogenesis start from a limb bud consisting of multipotent mesenchyme and the overlaying ectoderm. In the process of limb patterning, mesenchymal cells in the limb bud integrate positional information from the three axes. Fgf signal from the apical ectodermal ridge (AER) induces formation of the proximal–distal (PD) axis (Mariani et al., 2008), while the Shh signal from the zone of polarizing activity (ZPA) establishes anterior–posterior (AP) polarity (Mariani and Martin, 2003). To establish dorsoventral (DV) patterning, limb mesenchyme requires a signal from the dorsal ectoderm and an signal from the ventral ectoderm (Riddle et al., 1995). Among the many signals governing limb patterning, the role of AER-derived Fgf signaling in the regulation of PD patterning is particularly well recognized. AER-Fgf signal-

1995). In the chicken, Wnt2b-Wnt8c/β-catenin signaling in the lateral plate mesoderm is required for expression in the presumptive limb bud mesenchyme, mediating the / feedback loop in limb bud formation and AER maintenance (Kawakami et al., 2001). Genetic studies in mice revealed that ectodermal Wnt3/β-catenin signaling establishes and maintains AER function (Barrow et al., 2003). Mesenchymal β-catenin signaling is also required for AER maintenance and mesenchyme survival (Hill et al., 2006). Meanwhile, Wnt5a also promotes limb outgrowth by establishing planar cell polarity (PCP) along the PD axis (Gao et al., 2011; Yamaguchi et al., 1999).

The Wnt protein family can be classified into canonical and non-canonical signaling pathways. Canonical Wnts, such as Wnt1/Wnt3, signal through the intracellular mediator β-catenin to activate downstream targets (Bejsovec, 2000). Non-canonical Wnts, such as Wnt5a/Wnt11, seem to act through PCP or Ca^{2+} cascades to fulfill their function. In genetic studies, single Wnt protein or β-catenin is usually individually manipulated to elucidate the function of Wnt signaling. However, the overlapping expression pattern of the various Wnts and their functional redundancy obscure the true consequences of removing individual genes. Meanwhile, β-catenin does play a central role in the canonical Wnt pathway, but it also serves as a membrane protein of the cell junction complex (Aberle et al., 1996), thus its genetic modification most likely affects additional functions independent of Wnt signaling.

Wls was first identified in the fruit fly and worm as a cargo protein that functions in Wnt ligand secretion (Port et al., 2008), and its conserved function is confirmed in several model organisms (Banziger et al., 2006; Kim et al., 2009). Blocking this gene by different methods causes various phenotypes corresponding to Wnt signaling failure (Adell et al., 2009; Kim et al., 2009). Recently, was both conventionally and conditionally targeted to study the role of Wnts in mouse embryonic development (Fu et al., 2011, 2009). Thus, conditional inactivation makes it possible to dissect the overall role of Wnts in limb patterning and tissue morphogenesis.

Here, we generated a conditional knockout mouse line carrying an exon3-floxed allele. Removal of by limb–mesenchyme-specific and ectoderm-specific showed that Wls-mediated Wnts regulate early patterning along the three axes of the limb bud and also sustain cell proliferation and survival of distal limb mesenchyme. However, the Wnts from the two germ layers exerted distinct effects on modulating the undifferentiated pool of mesenchymal progenitors. At later developmental stages, ectodermal Wnts, most likely canonical Wnts, were found to coordinate soft tissue specification, including tendon/ligament induction, myoblast migration and dermis formation.

Materials and methods

(C57BL/6) mice were crossed with transgenic mouse lines or . or offspring were backcrossed to mice to generate the limb-specific knockout mice or . The floxed status of the conditional allele was genotyped with the primers: P1: 5 -ATACTTTTTCTGATCTGTTGT-3 and P2: 5 -AAGTTTTAATAGGTCTGTGTT-3 . The presence of was identified by PCR using the primers: F-5 -CAAAAGTTGGAGTCTTCGCT-3 and R-5 -CAGAAG CA TTTTCCAGGT AT-3 . The primers for genotyping were F-5 -ACCTGAAGATGTTCGCGATTATCT-3 and R-5 -ACCGTCAGTACGTGAGA TATCTT-3 .

Mice were maintained in a specific pathogen-free environment,

sites (−1156 to −1147; −742 to −733), which were replaced by ATGATGCACG. C3H10T1/2 cells were cultured in DMEM supplemented with 10% FBS. For each transfection, cells were plated in a 24-well plate and then transfected with 0.8 μg of experimental vector and 10 ng of pRL-TK (Promega, WI, USA), the latter as an internal control. Transfections were carried out using FuGene 6 transfection reagent (Roche, Switzerland). Wnt3a-conditioned medium or L-cell supernatant was added to the cells at the time of transfection. After 48 hr of culture, the cells were harvested and a luciferase assay was performed using a dual luciferase reporter assay system and a GloMax 20/20 Luminometer (Promega, WI, USA).

Results

The results of whole-mount hybridization showed that the gene was extensively expressed in mouse limb tissues from E9.5 to E13.5 (

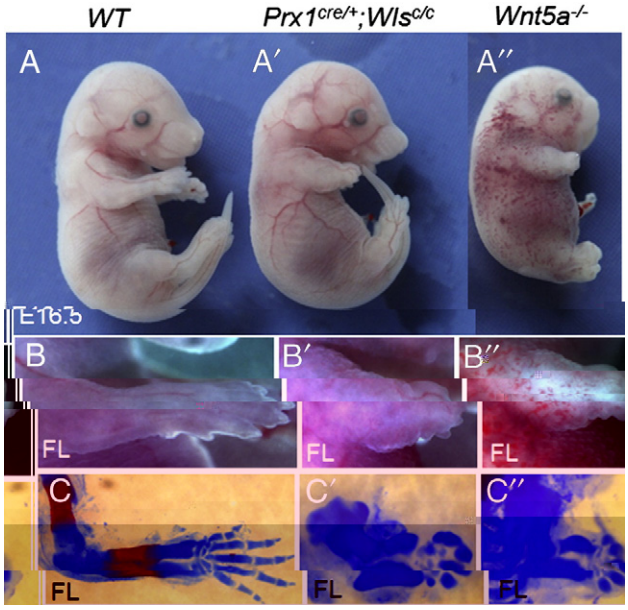

Fig. 2. The similar hypoplastic forelimb skeletons of and embryos at E16.5. (A–A) Similar hypoplastic forelimbs and truncated autopods are detected in and mutant embryos. (B–B) Magnification of the forelimbs in (A–A). (C–C) Skeletal preparation of forelimbs in (B–B). Abbreviation: FL, forelimb.

for undifferentiated distal mesenchymal progenitors. These mesenchymal cells in the ring retained distal identity, as indicated by their expression of , an autopod progenitor marker (Figs. 3D and D). However, they did not differentiate into other tissues, such as muscle, tendon/ligament or dermis, as they did not display changes in the expression of , and , the marker of muscle, tendon progenitors and dermis respectively (Supplemental Fig. S5). The block in cell differentiation may have resulted from the elevated Wnt signaling activity in the distal mesenchyme, reflected by the higher level of β-catenin in the distal tip of mesenchymal null mutants (arrows in Figs. 3E and E). The elevated β-catenin level in the distal mesenchyme was also detected in the embryo (arrow in Fig. 3E). It is in line with previous findings that loss of Wnt5a could lead to increased canonical Wnt activity and inhibited differentiation of distal mesenchyme (ten Berge et al., 2008; Topol et al., 2003). Taken together, these results indicate that the inactivation of limb mesenchymal arrests distal mesenchymal differentiation, most likely due to the blocking of Wnt5a secretion.

To dissect the comprehensive function of Wnts as diffusible morphogens in limb development, was also specifically deleted in limb ectoderm by crossing mice with mice, which express ventral ectoderm and AER-specific (Sun et al., 2002). mutant mice exhibit truncated limbs at the level of autopod in all limbs, but hindlimb also have shortened zeugopod (Figs. 4D–E). Both the fore- and hindlimbs in the mutant were dorsally flexed (Figs. 4B–C). The defects in the hindlimbs are more severe than in the forelimbs (shortened fibula and tibia as arrows in Figs. 4E' and E"), most likely due to earlier and broader Cre activity in the hindlimb (Barrow et al., 2003; Sun et al., 2002). In addition, mice displayed Cre activity in the skull, as indicated by LacZ staining in reporter mice (Fig. 4F). The inhibition of expression in the mutant had deleterious effects on suture fusion and intramembranous ossification in the skull (arrows in Figs. 4G and G), matching phenotypes in mice (Spater et al., 2006).

We first examined expression in the mutant embryo at E12.5. IHC results indicated Wls protein expression was markedly decreased in the mutant forelimb ectoderm and in the underlying mesenchyme (Supplemental Fig. S1D). The decrease in dorsal ectoderm was milder due to weak acitivity. As expected, ectodermal deletion of significantly down-regulated canonical Wnt signaling activity, which was indicated by decreased expression at the distal tip (arrows in Figs. 5A and A). The AER structure, evaluated by the expression of , became thinner and narrower in the embryo than in the control at E10.5 (Figs. 5B

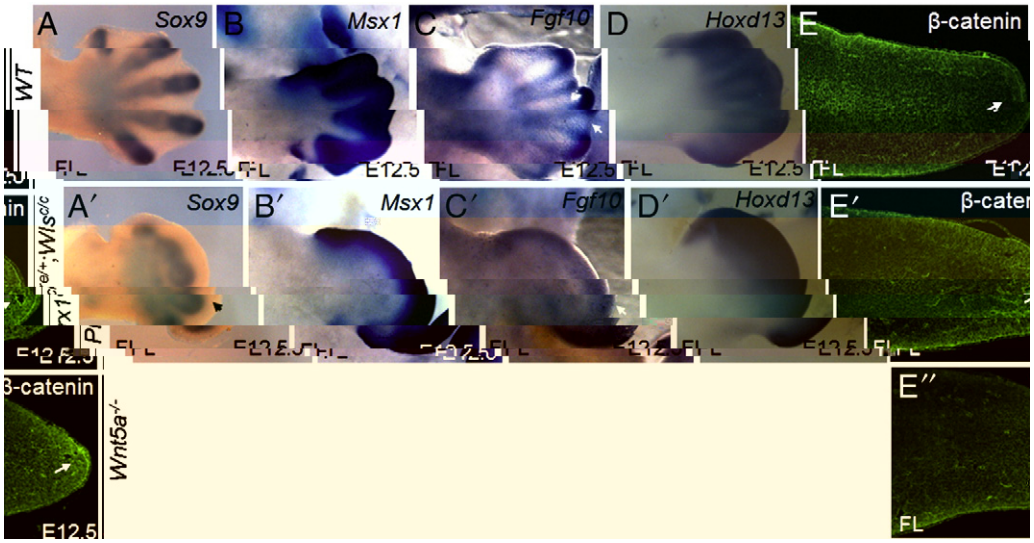

Fig. 3. Distal limb mesenchyme differentiation is arrested in the mutant at E12.5. (A–A) expression is excluded from the distal tip of the mutant forelimb. (B–D) Expression of the progenitor markers (B–B), (C–C ,white arrows) and the autopod marker (D and D) remains at high level in the distal limbs of the mutant. (E–E) An elevated β-catenin level is observed in the distal mesenchyme in the mutant without mesenchymal and (white arrows) compared to wild type. Abbreviation: FL, forelimb.

and B). Correspondingly, expression was weaker in the distal mesenchyme of the mutant due to the impaired AER structure (Figs. 5C and C). The regression of the AER structure may be due to impairment of AER-derived Wnt signaling because Wnt3/β-catenin is necessary for normal AER function and maintenance (Barrow et al., 2003).

The regression of the AER also resulted in the down-regulation of expression in the ZPA (Figs. 5D and D), which is a major regulator of anterior–posterior (AP) polarity (Riddle et al., 1993). The down-regulated Shh activity was further confirmed by a restricted expression domain (data not shown), as is a putative target of hedgehog pathway. Based on previous work, the defect in the Shh expression pattern may be caused by disruption of the Fgf/Gremlin/Shh feedback loop (Verheyden and Sun, 2008). Concerning dorsal–ventral (DV) patterning, we detected expression of was expanded to ventral mesenchyme of the mutant limb at E12.5 (arrow in Fig. 5 E and Supplemental Fig. S6). However, no obvious difference in or expression between the mutant and the wild type limbs was found at E10.5 (data not shown), which are regarded as dorsal and ventral ectodermal signals respectively (Chen and Johnson, 2002). These data suggested that the ventralization of the mutant limb was progressive, being consistent with the phenotypes of the mouse (Barrow et al., 2003). Taken together, the loss of ectodermal directly or indirectly affects limb patterning in three directions.

The abnormal AER structure in the mutant most likely affected the identity of the distal mesenchyme, thus disrupting limb outgrowth and structure. The expression domain was disorganized at E12.5 at the autopod level in the mutant limb (Figs. 5F and F), in agreement with the deformed autopod skeletons. In addition, distal mesenchymal cells prematurely lost their progenitor identity, as demonstrated by significant reduction of and expression (Figs. 5G–H). Expression of another distal mesenchyme marker, , was also greatly reduced (data not shown). Combined with the confined expression at E10.5 (Figs. 5C and C), the diminished expression of these progenitor markers suggests that the undifferentiated distal mesenchyme regresses quickly due to the loss of ectodermal Wnt signaling.

Skeletal agenesis in the and mutants might result from either impaired cell proliferation, impaired cell survival or both during limb development. In mutant limbs at E12.5, mesenchymal cell proliferation was reduced compared with controls (Figs. 6A and A); so did in null mutant (data not shown). However, extensive cell death was not significantly detected in the mutant limb at E12.5 (Supplemental Figs. S3C and C),

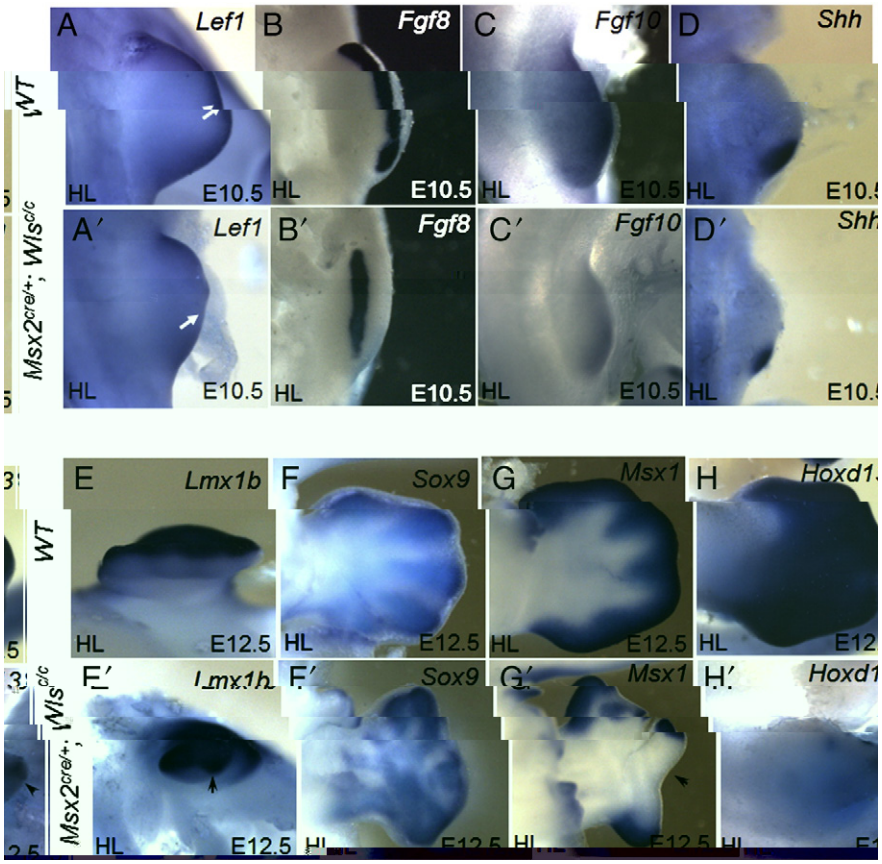

Fig. 5. Disrupted limb patterning with the absence of ectodermal .(A and A) Canonical Wnt signaling activity, indicated by expression, is down-regulated in the distal limb of the mutant (white arrows).(B and B) The AER, labeled by is thinner and narrower in the mutant than in the wild type.(C and C) expression in the distal mesenchyme is compromised with respect to wild type.(D and D) expression is also confined to a much smaller region, thus affecting AP patterning.(E and E) ' is usually expressed in the limb dorsal mesenchyme, but its expression expands ventrally in the mutant limb at E12.5, as indicated by the black arrowhead.(F and F) expression is disorganized in the mutant autopod, which indicates disturbed digit patterning and formation.(G and G) The mutant distal mesenchyme is characterized by remarkably reduced expression, which even disappears in some regions (black arrow).(H and H) In a severely affected mutant, mRNA is almost lost in the distal mesenchyme, only a little expression remains in the most distal region (black arrowhead). Abbreviations: FL, forelimb; HL, hindlimb.

the defective distal limb structures in both the and the mutants, while increased cell death also contributed to the dysgenesis of mutant. Taken together, these data suggest that ectodermal Wnts are indispensable for cell proliferation and survival of the distal mesenchyme, while mesenchymal Wnts are also essential for cell proliferation.

In addition to the defects in limb PD patterning and skeletons of mutant mice, there was also an obvious dysgenesis of limb soft tissues such as muscle, tendon/ligament and dermis, especially in the distal limb. All of these tissues display progressive

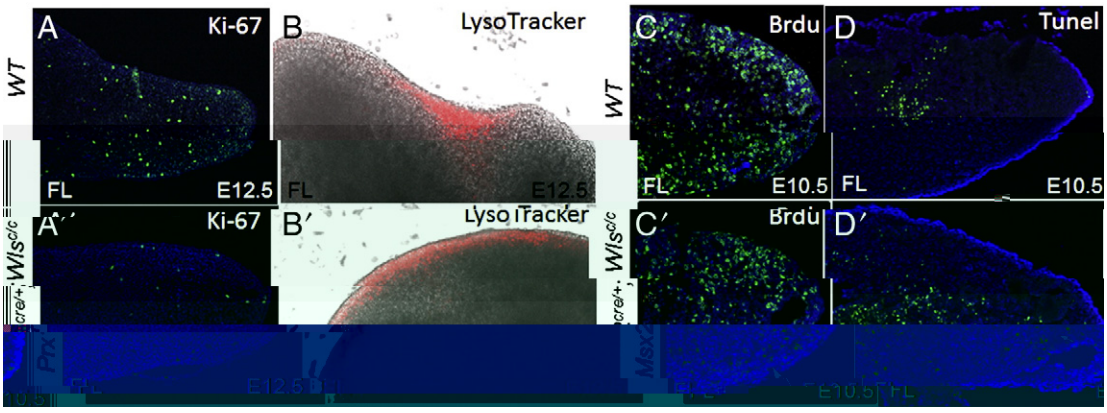

Fig.6. Cell behavior in the distal limbs of and mutant embryos is changed.(A and A) Mesenchymal proliferation is decreased in the forelimb of P at E12.5, as indicated by Ki-67.(B and B) Cells in the undifferentiated mesenchymal ring of

Wls has been reported to mediate the secretion of Wnt3a and Wnt5a in HEK293T cells (Banziger et al., 2006; Bartscherer et al., 2006). In mice, development of the body axis in a null mutant is impaired due to the disruption of Wnt3 generation (Fu et al., 2009). Wls mediates both the production of xWnt4 in and β-catenin-independent functions in Planaria (Adell et al., 2009; Kim et al., 2009). However, there is no strong evidence from mouse genetic analyses that Wls is required for the secretion of non-canonical Wnts. In addition, an mutant in the fruit fly does not display the typical Wnt/PCP defects (Bartscherer et al., 2006). In our experiment, when was depleted in the limb mesenchyme by , limb defects phenocopied those of the mutant (Fig. 2). During the preparation of this paper, another group showed that Wls-mediated Wnt5a secretion regulates angiogenesis (

The removal of ectodermal lead to a distal defect of AER maintenance with premature regression of the distal mesenchyme, as shown by the swift loss of expression of progenitor markers and extensive apoptosis (Figs. 5G–H ; 6D and D). This result is similar to the findings in mice (Barrow et al., 2003) and suggested that the role of ectodermal Wnts is to sustain AER structure and maintain the undifferentiated pool of distal mesenchyme progenitors (Figs. 5 and 6C–D). By contrast, the ablation of mesenchymal prevented distal mesenchyme from differentiating even after AER regression (Fig. 3). Therefore, we speculate that the mesenchymal progenitors are maintained by ectodermal Wnts and repressed by mesenchymal Wnts. They differentially controls the mesenchymal differentiation, thus regulate the pool size of the distal mesenchymal progenitors. This speculation is in agreement with the notion that Wnt5a, the major mesenchymal Wnt, antagonizes and inhibits canonical Wnt signaling in distal limb mesenchyme (Gao et al., 2011; Topol et al., 2003). In other words, we identified mesenchymal Wnt as a negative regulator of distal limb mesenchyme identity, which exerts its function indirectly through antagonizing ectodermal canonical Wnts. It was independent of Fgf signaling because expression was not altered in mesenchymal null mutants (Supplemental Fig. S3).

Wls itself could play a role in mediating the feedback crosstalk between Wnts in the two germ layers. As reported previously, is a direct transcriptional target of the canonical Wnt pathway (Fu et al., 2009). The ectodermal inactivation of decreased the expression itself not only in the ectoderm but also in the underlying mesenchyme (Fig. S1D). The reduction of mesenchymal feedback impaired Wnt secretion and function, contributing to the complex limb deformity in the ectodermal null mutant. However, the ectodermal inactivation of presented much more severe phenotypic consequences than the mesenchymal null mutant. These stronger phenotypes may have resulted from the additional AER defect and impaired Fgf signaling activity in the ectodermal null embryo (Figs. 5B–C).

Based on the phenotypes of the mesenchymal and ectodermal knockouts, Wnt proteins are more likely to regulate distal limb identity. The removal of ectodermal or mesenchymal resulted in defects that were more severe in the distal than in the proximal skeleton. The defects observed in the ectodermal null mutant could be ascribed to the combined effects of increased cell death, decreased cell proliferation and failure of soft tissue specification in the distal mesenchyme (Figs. 6C–D ; 7C and C). The truncated limbs of the mice could also be attributed to decreasedt(-5911695-1.82-

Furthermore, AER-Fgf alone is not necessary to sustain the proliferation of distal mesenchyme (Li et al., 2005). Conversely, both ectodermal and mesenchymal Wnts had positive roles for proliferation of the distal mesenchyme (Figs. 6A and A ; C and C).

Following early limb patterning, limb morphogenesis is mainly referred to as the specification and organization of various limb tissues, such as skeletal elements, muscle, tendon/ligament and dermis. Wnts have been shown to guide the differentiation of various limb tissues. For instance, mesenchyme progenitors preferably differentiate into soft connective tissue under the effect of Wnt signaling (ten Berge et al., 2008); while ectodermal Wnt6 signaling induces myoblast migration and myogenesis through the activation of Pax3/Myf5 transcriptional factors in the chicken (Geetha-Loganathan et al., 2005). Our observations also demonstrate that ectodermal Wnt signaling plays multiple roles in organizing limb morphogenesis, including roles in myoblast migration and differentiation, tendon/ligament induction and dermis specification. The myocytes and muscle mass were remarkably diminished following the loss of ectodermal Wls or mesenchymal β-catenin, as revealed by immunostaining and histochemical analysis (Figs. 7 and 9). Our genetic data confirmed that ectodermal Wnts, possibly Wnt6, induced myoblast migration and myogenesis by a conserved mechanism. In addition, in null mutants of ectodermal and mesenchymal β-catenin, dermis formation was also obviously inhibited (Figs. 7 and 9). It has been reported that canonical β-catenin signaling is absolutely required and sufficient for specification of dermal cells (Atit et al., 2006; Tran et al., 2011). Therefore, it is speculated that the ectodermal Wnts, especial canonical Wnts, instruct limb dermis formation.

Interestingly, tendon/ligament formation in the distal limb was first found to be affected by the removal of ectodermal and mesenchymal β-catenin. Indeed, distal tendon formation was impaired to a much greater extent than proximal tendon formation, as evidenced by expression (Figs. 8D and D). This difference may be due to the different developmental processes of these tendons. Proximal and distal tendons derive from common progenitors of the lateral plate mesoderm. However, proximal tendon formation is tightly coupled with myogenesis, whereas the distal tendon develops independently from a mesenchymal lamina underlying the ectoderm, and its development is coordinated with skeletal formation (Liu et al., 2010). Although ectodermal signal is supposed to induce distal tendon formation, the pathway that plays this inductive role has not been clarified (Schweitzer et al., 2010). Our data strongly suggest ectodermal Wnt/β-catenin signaling directly induces distal tendon formation, as evidenced by the finding that the promoter luciferase construct displayed obvious responsiveness to Wnt3a induction. However, since there are also severe skeletal defects in the autopod, we could not absolutely exclude that the tendon loss is secondary to skeletal deformity. By contrast, mesenchymal Wnts display no significant effect in limb tissue-specific differentiation (Supplemental Fig. S5). In summary, our results demonstrate that ectodermal and mesenchymal Wnts differentially regulate the limb mesenchymal differentiation. Ectodermal Wnt signaling centrally organizes limb morphogenesis, including myogenesis, dermal specification and distal tendon/ligament formation.

Supplementary materials related to this article can be found online at doi:10.1016/j.ydbio.2012.02.019.

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