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Wnt and FGF signals interact to coordinate growth with cell fate specification during limb development

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A fundamental question in developmental biology is how does an undifferentiated field of cells acquire spatial pattern and undergo coordinated differentiation? The development of the vertebrate limb is an important paradigm for understanding these processes. The skeletal and connective tissues of the developing limb all derive from a population of multipotent progenitor cells located in its distal tip. During limb outgrowth, these progenitors segregate into a chondrogenic lineage, located in the center of the limb bud, and soft connective tissue lineages located in its periphery. We report that the interplay of two families of signaling proteins, fibroblast growth factors (FGFs) and Wnts, coordinate the growth of the multipotent progenitor cells with their simultaneous segregation into these lineages. FGF and Wnt signals act together to synergistically promote proliferation while maintaining the cells in an undifferentiated, multipotent state, but act separately to determine cell lineage specification. Withdrawal of both signals results in cell cycle withdrawal and chondrogenic differentiation. Continued exposure to Wnt, however, maintains proliferation and re-specifies the cells towards the soft connective tissue lineages. We have identified target genes that are synergistically regulated by Wnts and FGFs, and show how these factors actively suppress differentiation and promote growth. Finally, we show how the spatial restriction of Wnt and FGF signals to the limb ectoderm, and to a specialized region of it, the apical ectodermal ridge, controls the distribution of cell behaviors within the growing limb, and guides the proper spatial organization of the differentiating tissues.

KEY WORDS: FGF signaling, Wnt signaling, Cell fate specification, Cell proliferation, Limb development, Multipotency

INTRODUCTION

Growth, patterning and cellular differentiation are inextricably linked during development. In this paper, we investigate how growth of the vertebrate limb bud is coordinated with the segregation of undifferentiated progenitor cells into specific lineages. The limb emerges from the flank of the embryo as a bud of rapidly proliferating mesenchymal cells covered by surface ectoderm. Proximodistal growth is mediated by the apical ectodermal ridge (AER), an ectodermal thickening at the distal aspect of the limb (Saunders, 1948) (for reviews see Capdevila and Izpisua Belmonte, 2001; Niswander, 2003; Tickle, 2003). The AER maintains a population of multipotent progenitors in the most distal 200-300 μm of the limb (here called the subridge region), and descendents from these cells form the limb connective tissues, including cartilage, perichondrium, tendons, muscle connective tissues and dermis (Pearse et al., 2007; Searls, 1965; Stark and Searls, 1973). Soon after establishment of the limb bud, cartilage precursors accumulate in the center and form a chondrogenic core, whereas other connective tissue types become specified in the periphery surrounding this core (Fell and Canti, 1934; Searls, 1965; Thorogood and Hinchliffe, 1975). Myogenic cells, originating from the somite (Chevallier et al., 1977; Christ et al., 1977), migrate into the limb bud and differentiate into a pattern dictated by the connective tissue (Chevallier and Kieny, 1982; Chevallier et al., 1977; Chiquet et al., 1981; Kardon, 1998; Kardon et al., 2003; Kieny and Chevallier, 1979).

What controls the establishment of this basic pattern, a skeletal core surrounded by connective tissue and muscle? In vitro, the subridge cells undergo chondrogenic differentiation and form little of the other connective tissues (Ahrens et al., 1977; Cottrill et al., 1987; Cottrill et al., 1990; Swalla et al., 1983). In vivo, the ectoderm inhibits chondrogenesis in the periphery and limits it to the core (Kosher, 1979; Searls and Janners, 1969; Solursh et al., 1981). This suggests that the AER maintains the subridge cells in an undifferentiated state, whereas the ectoderm directs their differentiation into chondrogenic and non-chondrogenic lineages (Kosher, 1979; Solursh, 1984; Wolpert, 1990). If this is true, by what signals do AER and surface ectoderm regulate differentiation and cell fate specification? And how is this coordinated with the simultaneous growth of the organ?

The AER and surface ectoderm express multiple members of the Wnt family of signaling molecules, including Wnt3 and Wnt6 (Barrow et al., 2003; Geetha-Loganathan et al., 2005; Parr et al., 1993; Roelink and Nusse, 1991) (for reviews, see Clevers, 2006; Logan and Nusse, 2004). Genetic activation of the Wnt pathway inhibits chondrogenesis of limb mesenchymal cells (Hartmann and Tabin, 2001; Rudnicki and Brown, 1997), and Wnts are therefore candidate signals for the chondroinhibitory effect of ectoderm. In addition to Wnts, the AER secretes fibroblast growth factors (FGFs), and these are essential for continued outgrowth of the limb bud (Fallon et al., 1994; Niswander et al., 1993; Sun et al., 2002). Proliferation is, however, not associated with the AER (Fernández-Terán et al., 2006; Janners and Searls, 1970; Köhler et al., 2005), and appears unaffected by its removal (Janners and Searls, 1971). Instead, the AER and the FGFs it produces are required for cell survival (Dudley et al., 2002; Sun et al., 2002), and the tissues and molecules that control cell proliferation during limb development have yet to be elucidated.

In this paper, we identify the effects of Wnt and FGF signals on proliferation, cell fate specification and differentiation using in vitro and in vivo approaches. We find that the combination of Wnt and FGF signals has effects different than either signal alone: they synergistically drive proliferation and maintain limb mesenchymal cells in an undifferentiated state that retains the ability to undergo

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chondrogenesis. We identify target genes that mediate these effects, and present a molecular mechanism showing how Wnts and FGFs regulate growth of the limb and simultaneously guide the segregation of multipotent progenitor cells into specific tissue lineages.

MATERIALS AND METHODS

Purified proteins and beads

Mouse Wnt3a protein was purified without a heparin-purification step (Willert et al., 2003), and concentrated to 250 ng/μl in PBS with 1% CHAPS using Microcon concentrators (Millipore). Heparin acrylic beads (Sigma) were resuspended in three volumes of Wnt3a protein and used within 2 days. Fz8CRD-IgG fusion protein was produced as described previously (Hsieh et al., 1999); mouse Fgf8b protein was purchased from R&D Systems.

Cell and organ culture

Micromass cultures were established from the distal half of limb buds (DeLise et al., 2000) and cultured in DMEM (mouse) or DMEM/F12 (chick) plus 10% fetal bovine serum (FBS). For ectoderm/mesenchyme co-culture, E11.5 mouse or stage 22-24 chick limb buds were dissected in 10% FBS, incubated in 1% trypsin at 4°C for 30 minutes, and the ectoderm peeled off using tungsten needles. The mesenchyme was triturated, resuspended at 2×10^7 cells/ml, and 10 μl micromass cultures established. After 6 hours, pieces of non-AER ectoderm were placed on top, together with Fz8CRD or IgG control protein (5 μg/ml final concentration). BrdU labeling was carried out after another 12 hours for 4 hours using 20 μg/ml BrdU (Sigma), X-gal staining after 20 hours or Alcian Blue staining after 4 days (DeLise et al., 2000). Limb buds were cultured on Millicell-CM filters (Millipore) in DMEM/0.5% fetal bovine serum, in the presence of Wnt3a (250 ng/ml) or Fgf8b (150 ng/ml), as indicated. Limb bud size was determined by imaging the limb buds from above, and counting the number of pixels in each limb using Photoshop (Adobe). BrdU labeling was carried out 16 hours after bead implantation for 4 hours using 20 μg/ml BrdU (Sigma). For secondary micromasses, stage 22-23 chick limb mesenchyme cells were plated in presence of 250 ng/ml Wnt3a and/or 150 ng/ml Fgf8b at a density of 2×10^6 cells/cm^2. Medium was replaced daily. After 4 days, cells were trypsinized and 10 μl micromass cultures established.

Gene expression profiling

E11.5 mouse whole limb bud or stage 22-23 chick limb mesenchyme cells were plated in six-well plates at 12×10^6 cells/well (mouse) or 96-well plates at 7.5×10^5 cells/well (chick). Mouse cells were stimulated with Wnt3a protein at 200 ng/ml or vehicle for 2 hours. Poly A$^+$ RNA was isolated using RNeasy and Oligotex kits (Qiagen). Mouse 42k cDNA microarray (Stanford Functional Genomics Facility) hybridization was performed in quadruple as described (Eisen et al., 1999). Data are available at the Stanford Microarray Database (http://smd.stanford.edu) and at GEO with accession number GSE12441. Wnt3a-regulated genes were identified using SAM (Tusher et al., 2001). Chick cells were cultured overnight, then stimulated with 250 ng/ml Wnt3a and/or 150 ng/ml Fgf8b. Total RNA was isolated and reverse transcribed using RNeasy (Qiagen) and Thermoscript II (Invitrogen) kits and random hexamer primers. Real-time PCR was performed using a Roche Lightcycler and FastStart DNA Master^{plus} SYBR Green I reagents (Roche). Primer sequences are available upon request.

Mutant animals

Prx1::Nmyc transgenic mice were generated by zygote microinjection of a DNA construct containing the Prx1 XB2.4 promoter/enhancer (Martin and Olson, 2000) driving a rabbit *β-globin* intron, an N-terminally FLAG-tagged mouse *Nmyc*-coding sequence, and a rabbit *β-globin* polyA signal. *Nmyc*$^{-/-}$ mutant mice (*Mycn*$^{-/-}$ – Mouse Genome Informatics) are described elsewhere (Knoepfler et al., 2002).

Detection of BrdU, gene expression and reporter activity

Embryos were labeled for 1 hour by intraperitoneal injection of the mother with 50 mg/kg BrdU (Sigma). Tissues were fixed with Bouin's fluid (Sigma) for Sdc1 staining, 4% paraformaldehyde for Col1 in situ hybridization, and Histochoice MB (Amresco) for other immunohistochemistry and in situ hybridization. Monoclonal α-BrdU (Sigma, 1:1,000), α-myosin heavy chain MF20 and α-procollagen 1 M-38 (Developmental Studies Hybridoma Bank, 1:100), rat monoclonal α-Syndecan-1 (BD Pharmingen, 25 μg/ml) and rabbit α-Sox9 (Santa Cruz Biotechnology, 1:100) antibodies were detected using Vectastain elite ABC kit and DAB (Vector Labs), or via fluorescence using biotinylated secondary antibodies and Cy3- or Alexa488-streptavidin. For MF20/M38 double staining, cryosections were stained with MF20 and detected using Alexa488-streptavidin, blocked with avidin and biotin, stained with M-38 and detected using Cy3-streptavidin. For in situ hybridization, DIG-labeled probes were detected with NBT (Roche) for Col1 (Metsaranta et al., 1991), or TSA amplified and detected with DAB using a GenPoint kit (DAKO, Nmyc). BrdU density around beads was determined by counting BrdU-positive nuclei in 50×50 μm squares around the beads in every third 7 μm section. At least 20 squares per bead were counted. *lacZ* was detected in whole mounts (E9.5) or cryosections (E10.5 and E11.5) by X-gal staining, and sections counterstained with nuclear Fast Red (Vector Labs).

Adenovirus infection and proliferation assays

Limb bud cells plated in 96-well plates at 5×10^4 cells/well were infected with 5×10^5 pfu/well of adenovirus (Kuhnert et al., 2004) for 12 hours. The cells were cultured an additional 12 hours followed by labeling with 10 μM BrdU for 4 hours. Cells were then trypsinized, split over 2 plates and assayed for BrdU (Cell proliferation ELISA, Roche) and total DNA content (Cyquant cell proliferation assay, Invitrogen). BrdU values were normalized using the DNA values. Unpaired two-tailed *t*-test or one-way ANOVA, as indicated, was performed to determine significance. Results shown are the mean±s.e.m.

RESULTS

Limb ectoderm inhibits chondrogenesis and promotes proliferation of limb mesenchyme via Wnt signals

Limb ectoderm inhibits chondrogenesis (Kosher, 1979; Solursh et al., 1981), and we tested whether this was mediated by Wnt signals. We visualized Wnt signaling using cells derived from Axin2$^{lacZ/+}$ mutant mice, in which a *lacZ* reporter gene has been inserted into the Wnt target gene Axin2 (Aulehla et al., 2003; Jho et al., 2002; Lustig et al., 2002). Thus, X-gal staining indicates Wnt responsiveness. Wild-type limb ectoderm cultured on top of Axin2$^{lacZ/+}$ limb mesenchyme induced the Wnt reporter (Fig. 1A), and inhibited chondrogenesis in the mesenchymal cells around it (Fig. 1B). In the presence of the Wnt antagonist Fz8CRD (Hsieh et al., 1999), Wnt reporter activity was markedly reduced (Fig. 1C) and chondrogenesis occurred (Fig. 1D). These data indicate that limb mesenchyme responds to a Wnt signal from the ectoderm and that this signal inhibits chondrogenesis.

Several studies have shown that genetic activation of the Wnt pathway inhibits chondrogenesis (Hartmann and Tabin, 2000; Rudnicki and Brown, 1997). We tested here whether purified Wnt3a protein was able to do this. In culture, limb mesenchyme responded to Wnt3a protein by induction of the reporter (Fig. 1E,F) and chondrogenesis in these cells was inhibited (Fig. 1G,H). To confirm that Wnt signals were able to inhibit chondrogenesis in vivo as well, we implanted Wnt3a beads into developing limb buds. Wnt3a beads induced reporter activity (Fig. 1I,J) and the protein alone was sufficient to block chondrogenic differentiation in cells around the Wnt source (Fig. 1K,L). Combined, these results demonstrate that Wnts are necessary and sufficient for the chondroinhibitory effect of limb ectoderm.

Cell proliferation in the limb bud is associated with the presence of nearby ectoderm (Fernández-Terán et al., 2006; Janners and Searls, 1970; Köhler et al., 2005), and we next tested whether limb ectoderm had proliferation-inducing activity. Indeed, ectoderm cultured on top of limb mesenchyme induced proliferation as

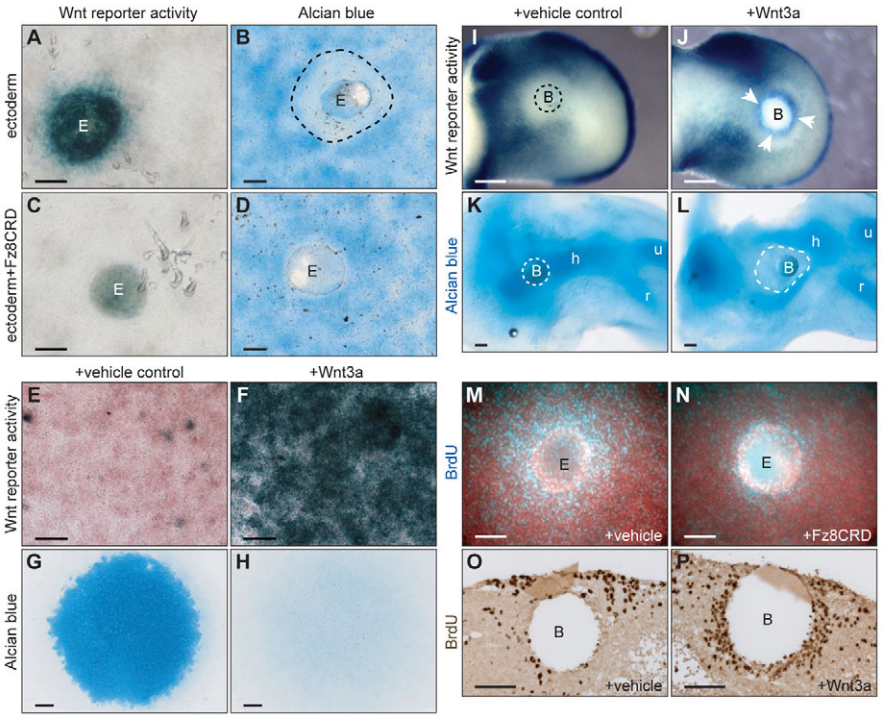


Fig. 1. Limb ectoderm inhibits chondrogenesis and promotes proliferation via Wnt signals. (**A**) Chick limb ectoderm cultured on top of E11.5 *Axin2*$^{lacZ/+}$ limb mesoderm cells induces the reporter in the mesoderm (*n*=10). (**B**) Chick limb ectoderm cultured on top of chick limb mesoderm inhibits chondrogenesis (stained with Alcian Blue) in a ~150 μm wide zone around the explant (*n*=30). (**C**) The Wnt antagonist Fz8CRD inhibits induction of the *Axin2*$^{lacZ/+}$ reporter by the ectoderm (*n*=10). The ectoderm in A and C is derived from stage 24 chick limb buds and does not carry the reporter. (**D**) Fz8CRD protein abrogates the chondro-inhibitory effect of the ectoderm (45 out of 48). (**E**,**F**) E11.5 *Axin2*$^{lacZ/+}$ limb bud cells induce the *lacZ* reporter (blue) in response to Wnt3a protein (100 ng/ml for 19 hours; F). (**G**,**H**) Chick stage 22 limb mesenchyme cells undergo chondrogenesis (stained with Alcian Blue) in micromass cultures (G), which is inhibited by 100 ng/ml Wnt3a protein (H, *n*>200). (**I**,**J**) Activation of the reporter by Wnt3a beads (arrowheads, J) but not vehicle beads (I) implanted in E11.5 *Axin2*$^{lacZ/+}$ limb buds (*n*=4). (**K**,**L**) Vehicle beads implanted in stage 22 wing buds become embedded in the cartilage of the humerus (K), whereas chondrogenesis (stained with Alcian Blue) is inhibited around Wnt3a beads (L, *n*=8). (**M**,**N**) Chick limb ectoderm cultured on top of chick limb mesoderm induces BrdU incorporation (blue) in the surrounding cells (M, *n*=17), which is inhibited by Fz8CRD (N, *n*=18; nuclei labeled in red). (**O**,**P**) Sections through limb buds cultured with implanted vehicle (O) or Wnt3a (P) beads. Wnt3a induces BrdU incorporation around the bead (*n*=11). Scale bars: 100 μm in A-D,M-P; 500 μm in E-H; 200 μm in I-L. h, humerus; r, radius; u, ulna; B, bead; E, ectoderm.

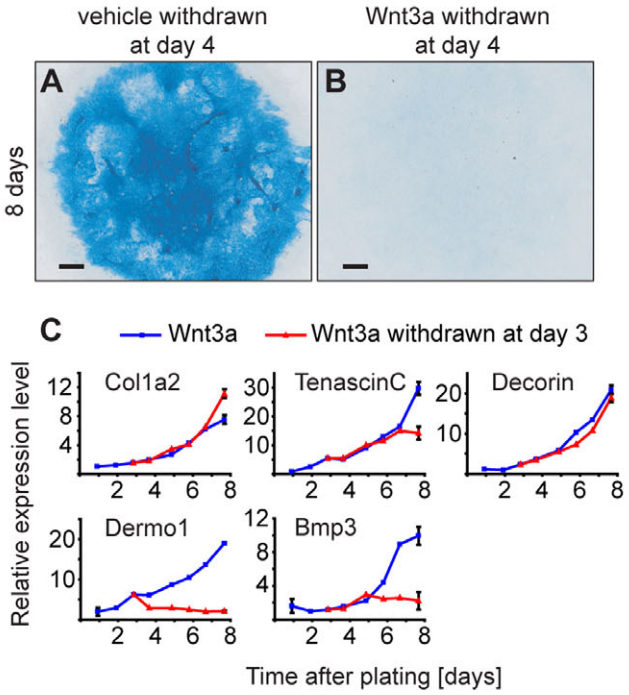
measured by BrdU incorporation (Fig. 1M). Moreover, this effect depends on Wnt signals as it was abolished by the Wnt antagonist Fz8CRD (Fig. 1N). To test whether Wnt signals were sufficient to promote proliferation in vivo, we implanted Wnt3a beads into limb buds and assayed for proliferation using BrdU labeling. Whereas vehicle beads had no effect (Fig. 1O), a strong increase in BrdU labeling was observed around Wnt3a beads (Fig. 1P). Combined, our data show that the limb ectoderm, by secreting Wnts, not only inhibits chondrogenic differentiation but also promotes proliferation in the underlying mesenchyme.

Wnt signals re-specify limb progenitors from cartilage towards soft connective tissue fates

We next addressed whether Wnt3a maintained limb mesenchyme in an undifferentiated state or allowed differentiation into other tissues. We first tested whether Wnt3a maintained the chondrogenic potency of the cells, which would indicate that they remained undifferentiated. Although the cells remained chondrogenic following short exposures to Wnt3a, they lost their chondrogenic potency following prolonged (>42 hours) exposure (see Fig. S1A in the supplementary material; Fig. 2A,B), suggesting that they differentiated into other tissue types. During limb development, the connective tissues that envelop the chondrogenic core form in the vicinity of the Wnt-producing ectoderm. The ectodermal Wnt signal might therefore change cell type specification from chondrogenic towards soft connective tissues. To test this, we cultured cells in the presence of Wnt3a and monitored the expression of a panel of differentiated tissue markers (Table 1). Over the course of 8 days, the cells upregulated expression of collagen 1, tenascin C, decorin, Dermo1 and Bmp3 (Fig. 2C), whereas expression of scleraxis or the (pre)osteoblast marker osteopontin was not detected (Table 1). This combination of

Table 1. Connective tissue markers and their expression in response to Wnt3a in limb mesenchyme cells

Marker	Tissue lineage marked	Expressed in response to continuous Wnt3a exposure	Expressed in response to transient Wnt3a exposure	References
Collagen 1	All soft connective tissues and osteoblasts	+	+	(Kieny and Mauger, 1984; Shellswell et al., 1980; von der Mark et al., 1976)
Tenascin C	Tendon, muscle connective tissue and perichondrium	+	+	(Chiquet and Fambrough, 1984)
Decorin	Tendon, muscle connective tissue, perichondrium and dermis	+	+	(Lennon et al., 1991; Olguin and Brandan, 2001; Wilda et al., 2000)
Scleraxis	Tendon	–	–	(Schweitzer et al., 2001)
Osteopontin	Pre-osteoblasts and osteoblasts	–	–	(Mark et al., 1987)
Dermo1/Twist2	Perichondrium and dermis	+	–	(Li et al., 1995)
Bmp3	Perichondrium	+	–	(Colnot and Helms, 2001)



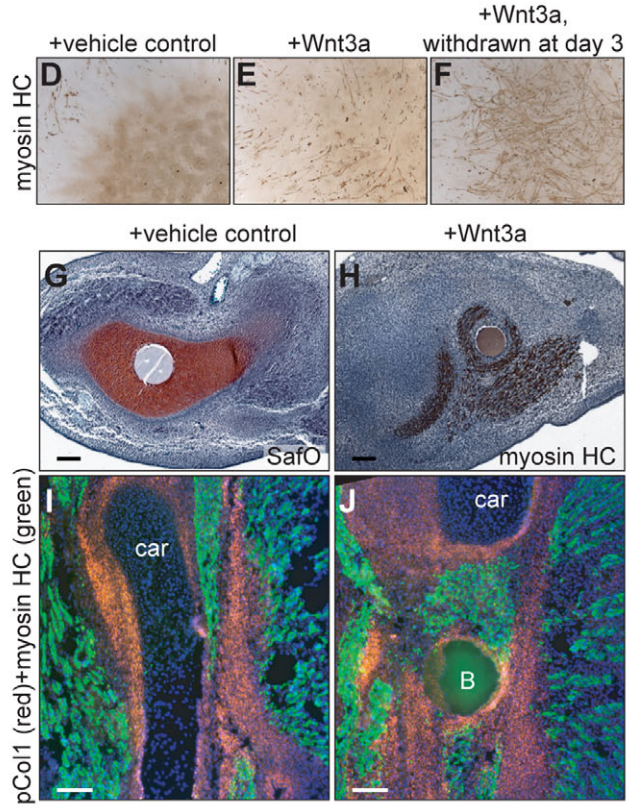


Fig. 2. Wnt3a protein re-specifies chondrogenic cells towards soft connective tissues. (**A**,**B**) Micromass cultures from chick limb mesenchyme cells treated with vehicle (A) or Wnt3a (B) for 4 days, cultured another 4 days in absence of Wnt3a. Wnt3a-treated cells failed to undergo chondrogenesis (Alcian Blue; *n*>50). (**C**) Collagen 1, tenascin C, decorin, Dermo1 and Bmp3 expression levels in high density cultures grown in continuous presence of Wnt3a (blue), or during the first 3 days of culture, after which the Wnt3a was replaced by vehicle (red). Time points 1 and 8 days were sampled twice (mean±s.e.m.). (**D**) Small numbers of myotubules, immunostained for myosin heavy chain (brown), accumulate in the periphery of 6-day-old mouse limb mesenchyme micromass cultures (*n*=6). (**E**) Continuous treatment with Wnt3a (250 μg/ml) slightly expands the myotubule number (*n*=6). (**F**) When Wnt3a is removed after 3 days of culture, large numbers of myotubules spread over the tissue layer (*n*=6). (**G**) Section through a chick wing 3 days after implantation of a vehicle bead at embryonic stage 22, showing the bead embedded in cartilage (*n*=14, Safranin O). (**H**) Wnt3a beads are never embedded in cartilage (*n*=14) but surrounded by ectopic muscle fibers, visualized by myosin heavy chain immunostaining (brown, *n*=7). (**I**,**J**) Section through a control chick wing (I) and a wing with implanted Wnt3a bead (J), immunostained for pro-collagen 1 (red) and myosin heavy chain (green), nuclei stained blue (DAPI) (*n*=4). Both sections are at a similar location and plane. Scale bars: 500 μm in A,B; 100 μm in G-J. B, bead; car, cartilage.

markers suggested differentiation towards soft connective tissue, specifically perichondrium or perhaps dermis. These tissues are indeed Wnt responsive in vivo, as demonstrated by Axin2$^{lacZ/+}$ expression in perichondrium and dermis of an E13.5 limb bud (see Fig. S1B in the supplementary material).

Transient exposure to Wnt3a changed the type of connective tissue that was formed: following withdrawal of Wnt3a after 3 days of culture, collagen 1, decorin and, to a lesser extent, tenascin C continued to be up regulated; scleraxis and osteopontin remained undetectable, again suggesting differentiation towards soft connective tissue (Fig. 2C). But as Dermo1 and Bmp3 were not induced (Fig. 2C), the combination of markers suggested differentiation towards muscle connective tissue. This is consistent with a previous report showing that in vivo activation of the Wnt signal transducer β-catenin induces formation of ectopic muscle connective tissue (Kardon et al., 2003). In vivo, muscle connective tissue controls muscle differentiation (Chevallier and Kieny, 1982; Chiquet et al., 1981; Kardon, 1998; Kieny and Chevallier, 1979), and we tested whether this occurred in vitro as well. We therefore established micromass cultures from whole limb bud mesenchyme, which includes myoblasts, and cultured the cells for 6 days. Indeed, transient exposure to Wnt3a strongly promoted the formation of myotubules in micromass cultures (Fig. 2D,F). Continuous exposure to Wnt3a protein, which does not promote formation of muscle connective tissue, led to a small increase in the number of myotubules (Fig. 2D,E), and it is possible that Wnt signals also promote the proliferation of muscle progenitors (Anakwe et al., 2003; Geetha-Loganathan et al., 2005). So far, our in vitro data suggest that Wnt signals promote the formation of specific types of connective tissue, which in turn influences myogenesis.

To confirm that Wnt3a re-specifies limb progenitors away from a chondrogenic and towards a soft connective tissue fate in vivo, we implanted Wnt3a beads into stage 22 chick wing buds. Vehicle beads became incorporated into cartilage, and no disruption to tissue patterning or cell differentiation was observed (Fig. 2G and data not shown). By contrast, Wnt3a beads were never in contact with cartilage (Fig. 2H, and see Fig. 1L) but disrupted the pattern of cartilage differentiation and that of muscle and connective tissue (Fig. 2H). Ectopic bundles of muscle fibers were aligned around the Wnt3a beads, and in all cases there was a layer of non-muscle tissue between the ectopic muscle and the beads (Fig. 2H). Combined staining for muscle fibers and pro-collagen 1-positive

connective tissue demonstrated that this was ectopic connective tissue (Fig. 2I,J). Although we have no data regarding the duration for which the beads provide active Wnt3a protein, the stimulus can only be transient and would therefore promote the formation of muscle connective tissue. In combination, our in vitro and in vivo data suggest that Wnt signals re-specify limb progenitors from a chondrogenic towards a soft connective tissue fate. The duration of Wnt exposure influences which type of connective tissue forms, which in turn controls the pattern of myogenesis.

Wnt and FGF signals combine to maintain limb progenitor cells in an undifferentiated state that retains the ability to undergo chondrogenesis

Our data show that Wnt signals control the segregation of multipotent progenitor cells into chondrogenic and connective tissue lineages. The multipotent progenitors themselves originate from the subridge region (Pearse et al., 2007), but what prevents their differentiation in this region? Cells in the subridge are exposed to FGFs from the AER, in addition to Wnts from the ectoderm and AER. We therefore tested whether FGF signals, alone or in combination with Wnt signals, inhibited differentiation. Fgf8 protein delayed, but did not prevent, chondrogenesis in micromass cultures (Fig. 3A,C), whereas the combination of Fgf8 with Wnt3a inhibited chondrogenesis altogether (Fig. 3B). But in contrast to the effect of Wnt3a alone, the combination of Fgf8 and Wnt3a maintained the undifferentiated state of the cells: following withdrawal of both factors, they retained their ability to differentiate into cartilage (Fig. 3D).

We then cultured limb mesenchyme cells for 4 days at high density in the presence of Wnt3a alone, or in combination with Fgf8, and established secondary micromass cultures. As expected, these micromasses were non-chondrogenic when derived from cells expanded in presence of Wnt3a alone, as they have switched to soft connective tissue fates (Fig. 3E). By contrast, when derived from cells expanded in presence of Wnt3a and Fgf8, the micromasses differentiated into cartilage, similar to freshly isolated limb mesenchyme (Fig. 3F). Moreover, Wnt3a was still able to inhibit this chondrogenesis (Fig. 3G), indicating that the secondary micromasses remained responsive to developmental signals and retained their multipotency.

Wnt and FGF signals combine to synergistically promote proliferation

We next tested whether FGF signals, alone or in combination with Wnt signals, contribute to the proliferation of limb progenitors. Whereas Wnt3a promoted growth in micromass cultures, Fgf8 protein

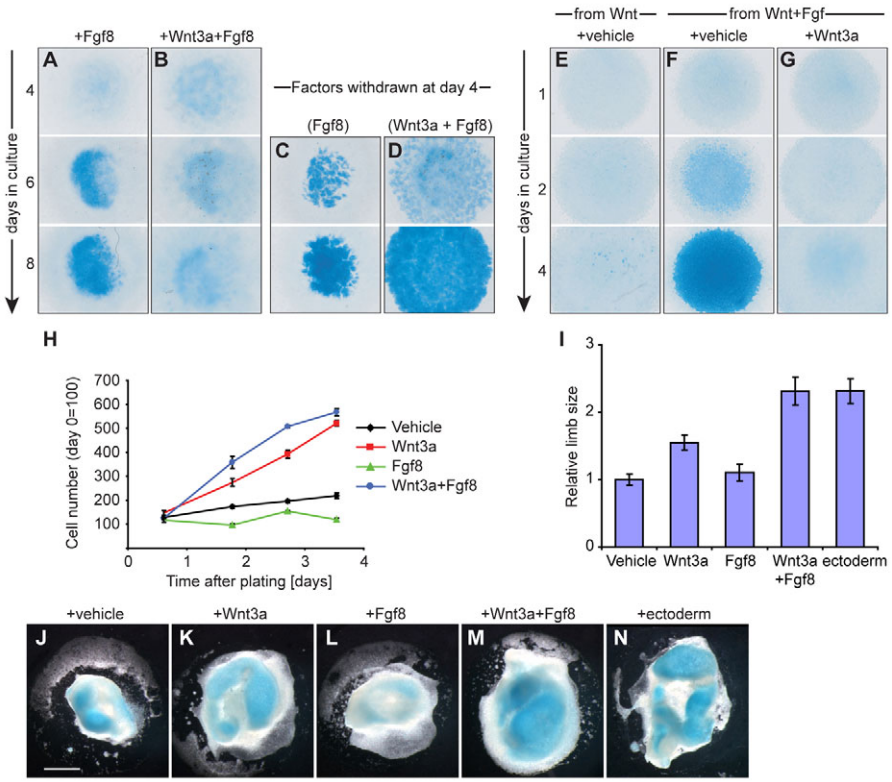


Fig. 3. Wnt and FGF proteins act in synergy to promote proliferation and maintain the undifferentiated state. (A-G) Alcian Blue staining. (**A**) Fgf8 protein delays chondrogenesis in micromass cultures of chick stage 22-23 limb mesenchyme (n=12). (**B**) Wnt3a combined with Fgf8 blocks chondrogenesis (n=12). (**C**) Removal of Fgf8 at day 4 of culture has little effect on chondrogenesis (n=12). (**D**) Cells treated with Wnt3a and Fgf8 resume chondrogenesis upon removal of the factors at day 4 (n=12). (**E-G**) Limb mesenchyme cells were expanded for 4 days in presence of Wnt3a alone (E) or in combination with Fgf8 (F,G), trypsinized and replated as micromass cultures. Cells expanded in Wnt3a alone lost their chondrogenic potential (E). Cells expanded in Wnt3a and Fgf8 retained their chondrogenic potential (F), whereas Wnt3a was still able to inhibit their chondrogenesis (G) (n=4). (**H**) Wnt3a promotes proliferation of limb mesenchyme in micromass cultures, which is enhanced by Fgf8. Fgf8 alone does not enhance proliferation (n=4). (**I**) Size of limbs cultured 4 days with intact ectoderm, or without ectoderm in presence of the indicated factors (n=8, mean±s.e.m.). (**J-N**) Representative examples of Alcian Blue-stained limb buds cultured without ectoderm in presence of vehicle (J), Wnt3a (K), Fgf8 (L), Wnt3a and Fgf8 (M), or with ectoderm left intact (N). Scale bar: 500 μm in J-N.

alone was ineffective (Fig. 3H). However, Fgf8 enhanced the proliferative effect of Wnt3a (Fig. 3H). We observed the same phenomenon in cultures of whole limb buds from which ectoderm and AER had been removed: Fgf8 had little effect on growth, whereas the combination of Fgf8 and Wnt3a strongly promoted growth, to a level on par with that of limb buds cultured with intact ectoderm and AER (Fig. 3I-N). Alcian Blue staining revealed that the extra tissue was largely of a chondrogenic nature (Fig. 3J-N), confirming that Wnt3a and Fgf8 promoted growth of progenitors with a chondrogenic potential. Combined, our data show that the combination of Wnt and FGF signals strongly promotes growth of limb progenitor cells, while maintaining their undifferentiated multipotent state.

Synergistic and antagonistic regulation of target genes by Wnt and FGF

To determine which genes mediate the effects of Wnt3a and Fgf8 in limb progenitor cells, we performed microarray analysis on E11.5 limb bud cells treated with Wnt3a. The candidate target genes were then tested by real-time PCR analysis of chicken stage 22-23 limb mesenchyme treated with Wnt3a. From these two experiments, we identified a set of genes whose regulation by Wnt3a was conserved between mouse and chick. In addition, we studied the response of these genes to Fgf8, and to the combination of Wnt3a and Fgf8. Surprisingly, more than half of the Wnt3a targets were also regulated by Fgf8, in some cases synergistically and in other cases, in an antagonistic fashion.

The target genes fell into five categories (Fig. 4): four genes were induced by Wnt3a only (*Apcdd1*/*Drapc1*, *Msx1*, *Sostdc1*/*WISE*/ectodin and *Axin2*); one gene was induced by Fgf8 only [*Dusp6*/*Mkp3*, a known FGF target included for comparison (Eblaghie et al., 2003; Kawakami et al., 2003; Pascoal et al., 2007)]; two genes were induced synergistically by Wnt3a and Fgf8 (*Nmyc* and syndecan1/*Sdc1*); one gene was induced by Wnt3a but this induction was antagonized by Fgf8 (*Nbl1*/*DAN*); and one gene was repressed synergistically by Wnt3a and Fgf8 (*Sox9*).

We next examined whether the expression domains of these target genes were consistent with their regulation by Wnts and FGFs, and with the temporal and spatial distribution of cell behaviors (i.e. proliferation and cell fate specification) within the limb bud. In both E10.5 and E11.5 limb buds, the Axin2 reporter was expressed in ~100 μm layer of mesenchyme underneath the ectoderm in all regions of the limb bud (Fig. 5A-C), consistent with the expression of several Wnts in limb ectoderm (Barrow et al., 2003; Geetha-Loganathan et al., 2005; Parr et al., 1993; Roelink and Nusse, 1991). *Apcdd1*, another gene induced by Wnt alone (Fig. 4), is similarly expressed (Jukkola et al., 2004).

Our data show that Wnt stimulates proliferation (Fig. 1O,P) and BrdU labeling indeed occurred predominantly in the Wnt-responsive region of the limb (Fig. 5D-F). Wnt3a and Fgf8 synergize in promoting proliferation in culture (Fig. 3H-N) and we found that proliferation is highest in the subridge region where Wnt and FGF signals overlap (Fig. 5D-F) (Pascoal et al., 2007). A similar pattern of expression was displayed by *Nmyc* and *Sdc1* (Fig. 5J-M) (Sawai et al., 1993; Solursh et al., 1990), in accordance with their synergistic induction by Wnt and FGF signals (Fig. 4).

Wnt signals inhibit cartilage differentiation (Fig. 1G,H,K,L). Consistent with this, expression of the chondrogenic marker *Sox9* (Bi et al., 1999) was limited to the center of the limb bud, and absent from the region of Wnt signaling and high cell proliferation (Fig. 5G-I). *Nbl1* is induced by Wnt3a, and this induction is antagonized by Fgf8 in vitro (Fig. 4); in vivo, *Nbl1* is indeed expressed in peripheral mesenchyme and excluded from the subridge region (Pearce et al., 1999). Although we classified *Axin2* as a Wnt-only target, its induction by Wnt3a is to some extent antagonized by Fgf8 (Fig. 4). In contrast to *Nbl1*, *Axin2* is expressed underneath the AER, although slightly weaker ventrally, suggesting that this antagonism is not strong enough to overcome the inducing signal (Fig. 5A-C). As Axin2 is an inhibitor of Wnt signaling, FGF might stimulate the response to Wnt signaling by repressing *Axin2*.

Thus, we have identified target genes that are indicative of the presence, the absence, or the overlap of Wnt and FGF signals. Moreover, the Wnt and FGF-mediated cell behaviors (e.g. proliferation, differentiation, multipotency) predicted from our in vitro analyses occur within the expression domains of these genes in the limb bud. We observed these relationships between cell behaviors and gene expression domains throughout the E10.5 limb, but only in the distal half of the E11.5 forelimb (Fig. 5B,E,H,K). This suggests that at E11.5, subsequent patterning mechanisms come into operation in the proximal limb to refine the patterns set up earlier by Wnts and FGFs.

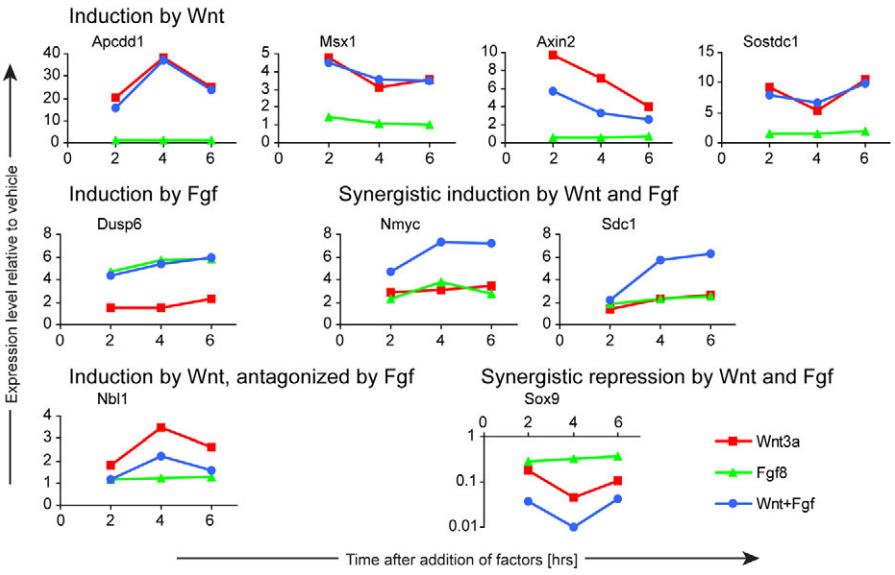


Fig. 4. Regulation of target genes by Wnt3a and Fgf8 in chick limb mesenchyme. Cells were cultured at high density in the presence of Wnt3a, Fgf8, or both, and samples taken 2, 4 or 6 hours after addition of the factors. Gene expression levels were plotted relative to vehicle controls. Note synergistic regulation of *Nmyc*, *Sdc1* and *Sox9* by the combination of Wnt3a and Fgf8 (blue line), and the antagonistic effect of Fgf8 on the induction of *Nbl1* by Wnt3a.

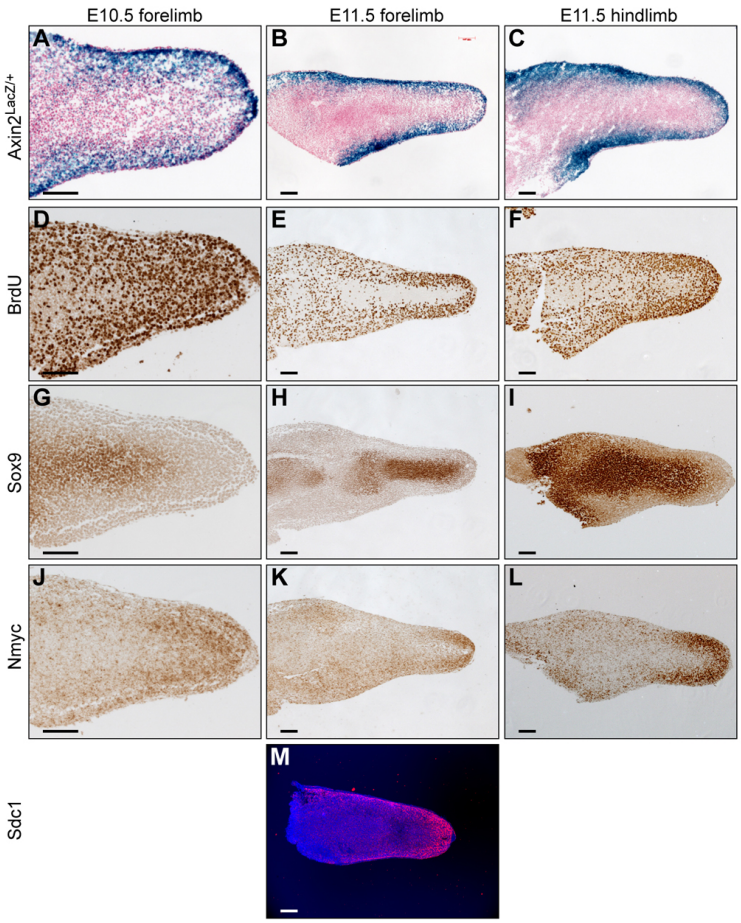


Fig. 5. Expression patterns of Wnt and FGF target genes correlate with cell behaviors in mouse limb buds. (**A-C**) Expression of *Axin2*$^{lacZ/+}$ reporter, (**D-F**) BrdU labeling, (**G-I**) Sox9 immunostaining, (**J-L**) *Nmyc* in situ hybridization, (**M**) Sdc1 fluorescent immunostaining. Sections through E10.5 forelimb buds (A,D,G,J), through the level of the central metacarpal in E11.5 forelimb buds (B,E,H,K,M), through E11.5 hind limb buds (C,F,I,L). Note co-localization of proliferation (BrdU labeling, D-F) with the *Axin2*$^{lacZ/+}$ reporter (A-C) and *Nmyc* expression (J-L), whereas chondrogenic differentiation (Sox9 expression, G-I) is mutually exclusive with proliferation and reporter expression. Dorsal is upwards, distal is rightwards. Scale bars: 100 μm.

Wnt promotes proliferation via *Nmyc* and inhibits chondrogenic differentiation via repression of *Sox9*

One of the target genes we found, *Nmyc*, is a member of the *myc* family of oncogenes that mediate cell cycle entry in response to proliferative signals (Trumpp et al., 2001). Loss of *Nmyc* reduces proliferation and impairs limb outgrowth starting at day E10.5 (Charron et al., 1992; Ota et al., 2007; Sawai et al., 1993; Stanton et al., 1992). In situ hybridization confirmed that *Nmyc* expression colocalized to the zone of proliferating cells in the limb (Fig. 5D-F,J-L) and in the absence of *Nmyc*, cell division in this zone was dramatically reduced (Fig. 6A,B). Nmyc also stimulates cell proliferation, as shown by viral overexpression of the gene in limb mesenchyme and comparing the incorporation of BrdU relative to a *lacZ* viral control (Fig. 6C, one-way ANOVA, P=0.0141).

To determine whether *Nmyc* was required for Wnt-induced proliferation, we impaired Nmyc function by overexpressing a dominant-negative form of the gene, *NmycΔMB2* (MacGregor et al., 1996; McMahon et al., 2000), in E11.5 limb bud cells. Wnt3a increased BrdU incorporation in control infected cells by 46% (±13%, P=0.0090), which was significantly reduced in *NmycΔMB2*-overexpressing cells (P=0.1809) (Fig. 6C). This reduction is on par with the reduction in cell proliferation achieved using the cell-autonomous negative regulator of the Wnt pathway, Axin (Zeng et al., 1997) (P=0.0820) (Fig. 6C). The remaining proliferation is probably from cells that resisted infection (~25% of the cells, not shown).

Following a second strategy to demonstrate that Wnt-mediated cell proliferation depends upon *Nmyc*, we implanted Wnt3a beads into *Nmyc*$^{-/-}$ limb buds and found that the extensive cell proliferation previously observed was abrogated (Fig. 6D-F, compare with Fig. 1O,P). Together, these data show that proximity to a Wnt source maintains cells in a proliferative state and that this is achieved via transcriptional activation of Nmyc.

Proliferation and differentiation are often mutually exclusive cell states. Are they achieved through independent regulation, or does one state actively curtail the other? We addressed this question using E11.5 *Nmyc*$^{-/-}$ limb buds, in which Wnt3a beads could no longer induce cell proliferation. Despite this, Wnt3a still repressed *Sox9* (Fig. 6G,H) and blocked chondrogenic differentiation (Fig. 6I,J). Moreover, Wnt3a beads also induced the formation of ectopic Col1-positive connective tissue in absence of *Nmyc* (Fig. 6K,L). Thus, the Wnt3a source switched limb mesenchyme cells from a chondrogenic towards a soft connective tissue fate, independently from its mitogenic effect. This reinforces our hypothesis that Wnt signals re-specify cell fate, as opposed to selectively expanding connective tissue precursors.

As Sox9 is essential for chondrogenesis (Akiyama et al., 2002; Bi et al., 1999), its repression by Wnt signaling (Fig. 4) explains how Wnt signals inhibit chondrogenesis. This is supported by the observation that deletion of the Wnt signal transducer β-catenin leads to expansion of *Sox9* expression in limb mesenchyme (Hill et al., 2005). Thus, Wnt controls proliferation and chondrogenic differentiation through the independent transcriptional regulation of Nmyc and Sox9.

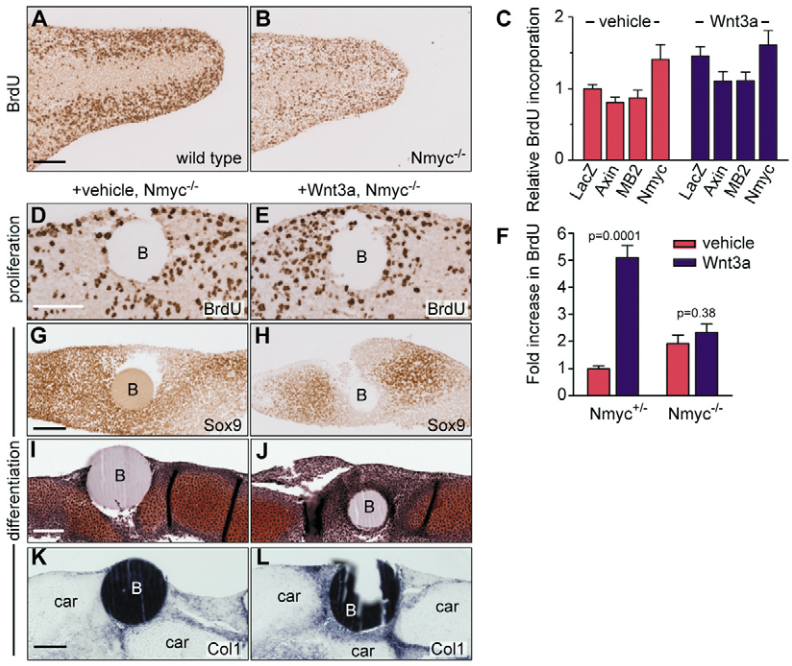


Fig. 6. Wnt promotes proliferation of limb mesenchyme via *Nmyc* and inhibits chondrogenesis independently via *Sox9*. (**A**,**B**) BrdU staining on longitudinal sections through the midregion of E11.5 forelimb bud of wild-type (A) and *Nmyc*$^{-/-}$ littermate (B). (**C**) Proliferation in E11.5 limb bud cells infected with adenovirus expressing the indicated genes (*n*=6). (**D**,**E**) Vehicle (D) or Wnt3a (E) beads implanted in E11.5 *Nmyc*$^{-/-}$ limb buds do not affect BrdU labeling (*n*=8). (**F**) BrdU density around vehicle or Wnt3a beads implanted in E11.5 Nmyc$^{+/-}$ and Nmyc$^{-/-}$ limbs (*n*=4). Wnt3a promotes proliferation 5-fold in *Nmyc*$^{+/-}$ limb buds (*P*=0.0001, *n*=4), but fails to promote proliferation in *Nmyc*$^{-/-}$ limb buds (*P*=0.38, *n*=4). (**G**,**H**) Wnt3a beads repress *Sox9* in *Nmyc*$^{-/-}$ limb buds (H); vehicle beads (G) have no effect (*n*=4). (**I**,**J**) Wnt3a inhibits cartilage formation around the bead in *Nmyc*$^{-/-}$ limb buds (J); vehicle beads have no effect (I). Cartilage stained red with Safranin O (*n*=4). (**K**,**L**) Wnt3a beads (L), but not vehicle beads (K), induce collagen 1 in *Nmyc*$^{-/-}$ limb buds (*n*=4). Dorsal is upwards, distal rightwards (A,B). Scale bars: 100 μm. B, bead; car, cartilage.

Expansion determines differentiation

The finding that the limb ectoderm inhibits chondrogenic differentiation led to various models wherein the size and location of the chondrogenic core is determined by the size of the limb bud and the range of the inhibitory signal (Kosher, 1979; Solursh, 1984; Wolpert, 1990). Several predictions can be made based on such models: (1) chondrogenic cells will only appear where the distance to the ectoderm is larger than the range of the inhibitory signal; and (2) increasing or reducing the growth of the limb bud, without manipulating the range of the inhibitory signal, will increase or reduce the size of the chondrogenic core, whereas the thickness of the prospective soft connective tissue layer will remain unchanged. As we have identified Wnt proteins as the ectodermal signal and Nmyc as a critical growth mediator, we are able to test these predictions.

At E9.5, limb buds have a radius of about 100 μm, which is approximately the range of the Wnt signal (Fig. 5A-C). Indeed, reporter activity indicated that all cells were responding to a Wnt signal (Fig. 7A), and absence of *Sox9* expression indicated that no chondrogenic cells were present (Fig. 7B). As the limb bud expanded to ~200 μm, the center of the developmental field escaped

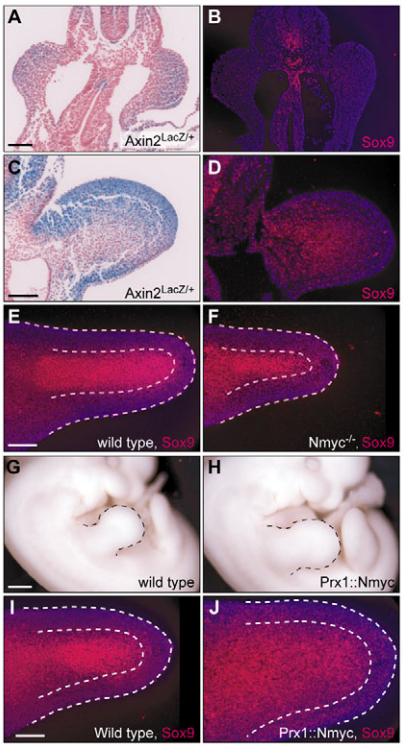


Fig. 7. Wnt couples expansion to differentiation. (**A**) *Axin2*^{lacZ} expression (blue) is visible throughout the forelimb buds of E9.5 embryos (22 somites). (**B**) *Sox9* expression (red) is absent in a nearby section through the same embryo as in A. Expression of *Sox9* can be seen in other areas of the embryo, such as the neural tube. (**C**,**D**) At late E9.5 (27 somites), *Axin2*^{lacZ} is no longer active in the centre of the forelimb (C), where *Sox9* is now expressed (D). (**E**,**F**) *Sox9* immunostaining on longitudinal sections through the midregion of stage-matched wild-type (E) and *Nmyc*$^{-/-}$ (F) E11.5 forelimbs. (**G**,**H**) Stage-matched E11.5 wild type (G) and Prx1::*Nmyc* embryo (H, *n*=5). (**I**,**J**) *Sox9* immunostaining on longitudinal sections through the midregion of the forelimbs of the embryos shown in G,H. Dorsal is upwards and distal is rightwards (C-F,I,J). Nuclei are labeled in blue (B,D,E,F,I,J). Scale bars: 100 μm in A-F,I,J; 500 μm in G,H.

the range of the Wnt signal (Fig. 7C), and we now observed a chondrogenic population expressing *Sox9* in this location (Fig. 7D). Thus, initiation of chondrogenesis is regulated by the size of the limb bud.

If the size of the non-chondrogenic zone is determined by the range of the Wnt signal, then it should retain its dimensions regardless of the size of the developmental field. *Nmyc*$^{-/-}$ embryos develop smaller limb buds (Charron et al., 1992; Sawai et al., 1993; Stanton et al., 1992), and, as predicted, we observed that the non-chondrogenic, proliferative zone remained similar in both its size (100 μm) and its location compared with the wild-type limb bud (Fig. 6A,B). But because the overall size of the *Nmyc*$^{-/-}$ limb bud was reduced, proportionally more cells were under the influence of the ectodermal Wnt signal and consequently the *Sox9* domain was reduced (Fig. 7E,F).

By manipulating Nmyc levels, we were also able to expand limb bud size: we promoted mesenchymal expansion by over-expressing *Nmyc* under control of the *Prx1* promoter (Martin and Olson, 2000). Prx1::Nmyc embryos had larger limb buds, confirming the proliferative function of *Nmyc* (Fig. 7G,H). As before, the non-chondrogenic zone was unaffected, but the region of *Sox9* was considerably expanded (Fig. 7I,J). Thus, the size of the non-chondrogenic, proliferative zone is independent of the size of the limb because it is controlled by the range of the ectodermal Wnt signal. By contrast, any variation in growth at this stage directly alters the size and location of the chondrogenic population. Combined, these results support a model in which the size and location of the chondrogenic core is determined by the size of the limb bud and the range of the ectodermal Wnt signal. Moreover, they show that growth is a crucial component of cell fate determination.

DISCUSSION

In this study, we investigated how growth of an embryonic organ is coordinated with the simultaneous segregation of cells into specific lineages. We show that, during vertebrate limb development, many aspects of this process are under control of two families of signaling proteins, Wnts and FGFs. The apical ectodermal ridge (AER) is a source of FGF signals, whereas the limb ectoderm produces multiple Wnts, including Wnt3 and Wnt6, which may perform the functions outlined in this paper. One of our key findings is that the combination of Wnt and FGF signals synergistically promotes proliferation while maintaining the cells in an undifferentiated, multipotent state that is pre-specified towards the chondrogenic lineage. Thus, withdrawal of the signals results in cell cycle withdrawal and chondrogenic differentiation, whereas continued exposure to ectodermal Wnt blocks chondrogenesis and re-specifies the cells towards the other connective tissue lineages.

A template for a limb

Our findings support a model that explains how limb growth is coordinated with the establishment of skeletal and soft connective tissues (Fig. 8). In this model, both Wnt and FGF proteins signal throughout the newly established limb bud. They maintain all mesenchymal cells in an undifferentiated, proliferative state (red/blue hatching; marked by Axin2 and Dusp6), leading to rapid outgrowth of the limb bud. Once the cells in the centre of the bud are out of range of the signals, they withdraw from the cell cycle, relieve the repression of Sox9, and undergo chondrogenesis (blue, marked by Sox9). In the periphery meanwhile, cells outside the influence of FGFs from the AER remain within range of Wnts from the ectoderm (red hatching, marked by Nbl1). This Wnt signal maintains proliferation and re-specifies the cells towards soft connective tissue fates. Proliferation therefore occurs throughout the periphery and subridge region (hatched areas, marked by Nmyc and Sdc1), and the limb bud continues to expand in all dimensions. However, as Wnts and FGFs combine to promote proliferation synergistically in the subridge region (red/blue hatched domain), proximodistal growth dominates. As a result of these processes, a proximodistally extended organ forms with a multipotent rapidly growing tip and a chondrogenic core surrounded by soft connective tissues. The connective tissues, in turn, control the pattern of differentiation of the immigrating myoblasts.

How can this model be integrated with the existing insights into limb patterning? Our model functions in absence of dorsoventral and anteroposterior signaling centers. Indeed, limb development can tolerate loss of these patterning systems, and the same basic structure then develops, consisting of a skeletal core surrounded by soft connective tissues and muscle (Chiang et al., 2001; Litingtung et al., 2002; Parr and McMahon, 1995). Our model provides a template upon which the dorsoventral and anteroposterior patterning mechanisms are superimposed, elaborating what would otherwise become a simple fin-like structure.

An area of current debate is the process by which the limb bud is patterned into a proximodistal series of segments. Neither progress zone nor early specification models convincingly describe proximodistal patterning, and the authors of those models have proposed the outline of a replacement (Tabin and Wolpert, 2007). For this, it is first postulated that commitment to differentiation and

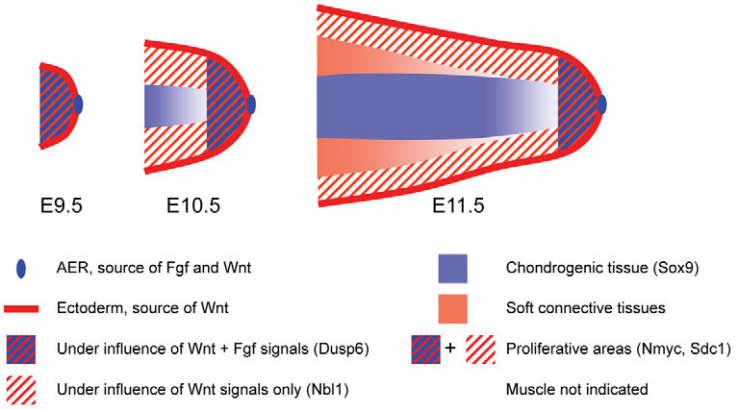


Fig. 8. Wnt and FGF signals interact to coordinate growth and cell fate specification during limb development. In the newly established limb bud (E9.5), both Wnt and FGF proteins signal throughout the limb mesenchyme and maintain all cells in a multipotent, proliferative state (indicated by red/blue hatching, marked by Axin2 and Dusp6). Following limb outgrowth, cells in the center of the limb are no longer within range of the signals. This allows cell cycle withdrawal and expression of Sox9, leading to establishment of the chondrogenic core (indicated in blue, marked by Sox9). In the periphery, meanwhile, cells out of range of FGFs from the AER are still within range of Wnts from the ectoderm (indicated by red hatching, marked by Nbl1), which maintains the proliferative state at a lower level, and respecifies the cells towards soft connective tissue fates. As a result of these processes, a proximodistally extended organ forms with a multipotent, rapidly growing tip and a chondrogenic core surrounded by soft connective tissues.

chondrogenic condensation occurs as cells exit the domain influenced by FGF signals from the AER. Second, it is postulated that the proximodistal specification of a cell is based on the segment-specific genes it expresses at the time it exits this undifferentiated zone (Tabin and Wolpert, 2007). Although the mechanisms that determine segment-specific gene expression are poorly understood, it is hard not to notice the match with our model: we show that the subridge region/undifferentiated zone is maintained by Wnts in addition to FGF signals. The distal truncation following AER removal is explained by the loss of the source of FGF signals coupled with the continued production of Wnts by non-ridge ectoderm: synergistic gene regulation (e.g. *Nmyc*) and polarized outgrowth stops, and the multipotent progenitors start to differentiate. The subridge region becomes like any other peripheral region, where cells are under the influence of Wnt signals, and form connective tissues but no cartilage. Indeed, subridge cells no longer contribute to skeletal structures following AER removal (Dudley et al., 2002). We provide the molecular foundation for further development of a proximodistal patterning model.

Our model describes the formation of a basic, unembellished structure somewhat resembling a paddle, without dorsoventral or anteroposterior pattern. When and where could this basic limb, or Ur-limb, have evolved? An ectopic source of FGF, placed underneath Wnt-expressing ectoderm (Barrow et al., 2003; Parr et al., 1993), induces outgrowth, AER formation and ectopic limb formation (Cohn et al., 1995). The ability of FGF signals to induce ectopic outgrowths is not limited to the paired appendages: in zebrafish, ectopic FGF signals can induce an ectopic median fin (Abe et al., 2007). This fin has a basic tissue arrangement similar to that of a paired appendage, i.e. a skeletal core surrounded by connective tissue and muscle. It is thought that the molecular mechanism of fin and limb development evolved in the midline, before the origin of paired appendages (Freitas et al., 2006). The mechanism we have detailed provides a robust and adaptable molecular framework that might underlie the development and evolution of appendages throughout the vertebrate subphylum.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/19/3247/DC1

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