FGF signaling regulates mesenchymal differentiation and skeletal patterning along the limb bud proximodistal axis

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Fibroblast growth factors (FGFs) are signals from the apical ectodermal ridge (AER) that are essential for limb pattern formation along the proximodistal (PD) axis. However, how patterning along the PD axis is regulated by AER-FGF signals remains controversial. To further explore the molecular mechanism of FGF functions during limb development, we conditionally inactivated fgf receptor 2 (Fgfr2) in the mouse AER to terminate all AER functions; for comparison, we inactivated both Fgfr1 and Fgfr2 in limb mesenchyme to block mesenchymal AER-FGF signaling. We also re-examined published data in which Fgf4 and Fgf8 were inactivated in the AER. We conclude that limb skeletal phenotypes resulting from loss of AER-FGF signals cannot simply be a consequence of excessive mesenchymal cell death, as suggested by previous studies, but also must be a consequence of reduced mesenchymal proliferation and a failure of mesenchymal differentiation, which occur following loss of both Fgf4 and Fgf8. We further conclude that chondrogenic primordia formation, marked by initial Sox9 expression in limb mesenchyme, is an essential component of the PD patterning process and that a key role for AER-FGF signaling is to facilitate SOX9 function and to ensure progressive establishment of chondrogenic primordia along the PD axis.

KEY WORDS: FGF, FGF receptor, Apical ectodermal ridge (AER), Limb bud development, Chondrogenesis

INTRODUCTION

The apical ectodermal ridge (AER) is a specialized ectodermal structure formed at the distal tip of the vertebrate limb bud that is essential for proximodistal (PD) patterning of the limb (Saunders, 1948). Surgical removal of the AER results in skeletal truncation that is limited to more distal segments when the AER is removed at progressively later stages (Rowe and Fallon, 1982; Summerbell, 1974). The AER functions by elaborating signals to the adjacent mesenchyme, and members of the fibroblast growth factor (FGF) family have been identified as key components of AER signaling (Martin, 1998). It has been demonstrated that FGF-soaked beads are sufficient to rescue the skeletal phenotypes that result from AER removal (Fallon et al., 1994; Niswander et al., 1993). Of the four FGFs expressed in the AER, Fgf4 and Fgf8 are necessary for limb skeletal formation and when both genes are disrupted prior to limb bud initiation, the entire limb skeleton fails to form (Boulet et al., 2004; Sun et al., 2002).

FGFs elicit their biological functions by binding and activating a family of four high-affinity receptors (FGFRs), a sub-class of receptor tyrosine kinases (Ornitz and Itoh, 2001; Zhang et al., 2006). Two members of the FGFR family, Fgfr1c and Fgfr2c, are expressed in nascent limb bud mesenchyme (Orr-Urtreger et al., 1991). In vitro studies demonstrate that both Fgfr1 and Fgfr2 are capable of interacting with AER-expressed FGFs (Ornitz et al., 1996; Zhang et al., 2006). After ligand binding, activated FGFRs trigger several downstream intracellular signaling cascades (Eswarakumar et al., 2005). During limb development, the MAP kinase signaling pathway is activated in mesenchymal cells immediately adjacent to the AER, indicating active AER-FGF signaling in distal limb mesenchyme (Corson et al., 2003).

Previous studies indicate that AER-FGF signals are important for maintaining mesenchymal cell survival during limb development (Boulet et al., 2004; Sun et al., 2002). However, although AER-FGF signals are limited to distal mesenchyme, cell death induced by loss of AER-FGFs appears in proximal mesenchyme, far away from the AER. This is inconsistent with current knowledge that FGFs act as paracrine factors due to their interaction with heparan sulfate proteoglycans (HSPGs) in the extracellular matrix (ECM) (Ornitz, 2000). Moreover, when Fgf4 and Fgf8 are disrupted after limb bud initiation, transient AER-FGF signaling allows formation of a severely hypoplastic skeleton (Sun et al., 2002). Notably, despite significant size and number reduction of skeletal elements, all three PD segments are still recognizable. By contrast, AER removal, after limb bud initiation, results in a truncated skeleton owing to loss of all skeletal elements in distal segments. Phenotypic differences between loss of the AER and loss of AER-FGFs suggest that the AER possesses additional functions, which are either not mediated by Fgf4 and Fgf8 or are redundantly mediated by other AER factors such as Fgf9, Fgf17, or other signaling molecules including WNTs and BMPs (Barrow et al., 2003; Wang et al., 2004; Yamaguchi et al., 1999). All of these phenotypes, which undoubtedly have mechanistic implications, are not readily explained by the current model(s) of AER-FGF functions during limb development.

To further understand the functions of AER-FGF signals during limb development, we used a genetic analysis in the mouse embryo to compare phenotypes resulting from loss of the AER with phenotypes resulting from loss of AER-FGF signals. We also compared phenotypes resulting from partial loss of AER-FGF signaling with phenotypes resulting from complete loss of both Fgf4 and Fgf8 in the AER. We conclude that AER-FGF signals regulate multiple molecular and cellular processes during limb development and that the skeletal agenesis after complete loss of both Fgf4 and Fgf8 is due to combined mesenchymal defects, including increased cell death, decreased proliferation and failure of chondrogenic differentiation. We present a model to explain how AER-controlled
proliferation and FGF-regulated mesenchymal differentiation organize the formation of the skeletal primordia along the PD axis during normal limb development.

**MATERIALS AND METHODS**

**Mice**

Sources: Fgfr1<sup>lox/lox</sup> and Fgfr2<sup>1/2<sup> (Trokovici et al., 2003); Fgfr2<sup>lox/lox</sup> and Fgfr2<sup>1/2</sup> (Yu et al., 2003); Msx2-Cre (Sun et al., 2000); Prx1-Cre (Logan et al., 2002). Fgfr2<sup>Msx2-Cre</sup> mice were generated by mating Fgfr2<sup>1/2<sup>/M</sup>; Msx2<sup>Cre/+</sup> mice with Fgfr2<sup>Msx2-Cre</sup> mice. Fgfr2<sup>Msx2-Cre</sup>, Fgfr2<sup>Prx1-Cre</sup>, and Fgfr1<sup>Prx1-Cre</sup> mice were generated by mating Fgfr1<sup>Prx1-Cre</sup>; Fgfr2<sup>1/2<sup>/M</sup>; Prx1<sup>Cre/+</sup> compound heterozygous mice with Fgfr1<sup>Prx1-Cre</sup>; Fgfr2<sup>Msx2-Cre</sup> double homozygous mice. In all matings, Fgfr1<sup>Prx1-Cre</sup> and Fgfr2<sup>1/2<sup> alleles were incorporated to increase the efficiency of Cre-mediated recombination. Mouse embryonic skeletons were prepared as described previously (Yu et al., 2003).

**Staining, immunohistochemistry and in situ hybridization**

Embryos or tissues were fixed in 4% paraformaldehyde in PBS and embedded in paraffin. Sections were stained with Hematoxylin and Eosin (H&E). For β-galactosidase histochemistry, embryos were fixed in 0.2% glutaraldehyde in PBS for 60 minutes, washed in PBS and stained in β-gal staining buffer [5 mM K<sub>3</sub>Fe(CN)₆, 5 mM K<sub>3</sub>Fe(CN)₆ • 3H₂O, 1 mM MgCl₂, 0.01% sodium desoxycholate, 0.009% NP40, 0.002% X-Gal] for 8 hours at 4°C. After post-fixing in 4% paraformaldehyde, embryos were embedded in paraffin and sections counterstained with nuclear fast red.

Immunohistochemistry was carried out using LAB-SA Detection System (Zymed) according to the manufacturer’s instructions. Primary antibodies are anti-cleaved caspase 3 (Cell Signaling) and anti-phosphohistone H3 (pHH3) (Sigma). TUNEL assay was carried out using the In Situ Cell Death Detection Kit (Roche) according to the manufacturer’s instructions. Distal mesenchymal proliferation was analyzed on transverse sections of limb buds and pH3-labeled mesenchymal cells were counted in a 1.5 µm² area immediately adjacent (within 100 µm) to the AER. Fgfr8 whole-mount-in situ hybridization was performed as described previously (Sun et al., 2000).

**RESULTS**

**AER ablation by conditional removal of Fgfr2 in limb ectoderm**

To understand functional differences between the AER and AER-FGFs, we ablated the AER in mouse limb buds, which allowed us to directly compare phenotypes with those resulting from inactivation of Fgf4 and Fgf8 in the AER. Because Fgfr2<sup>b</sup> is essential for the survival of AER epithelial cells (Revest et al., 2001), conditional inactivation of Fgfr2, using the limb ectoderm-specific Msx2-Cre transgene (Sun et al., 2000), could be used to terminate AER functions during limb development. Msx2-Cre/+; Fgfr2<sup>Msx2-Cre</sup> mice appeared normal except for the absence of hindlimbs and severely truncated forelimbs (Fig. 1A-F), consistent with expression of Msx2-Cre before hindlimb bud initiation and after forelimb bud initiation (Lewandoski et al., 2000). Fgfr2<sup>Msx2-Cre</sup> forelimbs formed a normal stylopod (humerus) and zeugopod (radius and ulna), but completely lacked an autopod (Fig. 1D,E). The mutant forelimb skeleton was very similar to the truncated chicken wing skeleton that results from AER removal at stage 23 (Summerbell, 1974), indicating that genetic methods can mimic the effects of surgical means to completely ablate the AER during limb development.

In Fgfr2<sup>Msx2-Cre</sup> mice, forelimb buds were initiated normally, but showed reduced distal outgrowth after embryonic day 10.5 (E10.5). By E11.5, mutant forelimb buds were only comparable in size to control limb buds at E10.5 and showed distal morphological deformities (Fig. 1R, dotted line). By E12.5, mutant forelimbs appeared truncated, lacking any autopod primordial structures (Fig. 1C,F). By contrast, mutant hindlimbs only developed a small bulge protruding from the body wall and never demonstrated further outgrowth (Fig. 1F, arrow and dotted line).

Examination of histological sections indicated that a stratified AER was never formed in Fgfr2<sup>Msx2-Cre</sup> hindlimb buds (Fig. 1G-J). Consistent with agenesis of the AER in mutant hindlimbs, Fgfr8 was
not detected at any stage examined (Fig. 1O,P and data not shown). In mutant forelimbs, transient epithelial thickening was observed in the prospective AER region at E10, which rapidly regressed to a single layer of epithelial cells by E10.5 (Fig. 1K-N). Fgf8 expression was detected in the AER at E10.5, but was prematurely lost by E11.5 (Fig. 1O-R).

In a previous study, conditional disruption of β-catenin (Ctnnb1 – Mouse Genome Informatics) in the AER with Msx2-Cre (β-catenin<sub>Msx2-Cre</sub>) resulted in hindlimb skeletal agenesis that was indistinguishable from the hindlimb phenotypes of Fgfr2<sub>Msx2-Cre</sub> mice. However, β-catenin<sub>Msx2-Cre</sub> mice showed significantly more severe forelimb phenotypes than Fgfr2<sub>Msx2-Cre</sub> mice, with skeletal truncation occurring at the elbow joint level rather than at the wrist level (Barrow et al., 2003). As the truncation level correlates with the timing of AER removal in chick experiments, these results indicate that AER functions are lost at a later stage in Fgfr2<sub>Msx2-Cre</sub> forelimbs than in β-catenin<sub>Msx2-Cre</sub> forelimbs. Since the same Msx2-Cre transgene was used in both studies, it suggests that AER ablation in the forelimbs is not determined by the time of gene targeting but more likely by the time of protein inactivation.

### The AER is essential for proliferation of adjacent limb mesenchyme

Previous studies indicated that AER extirpation results in reduced proliferation and/or increased cell death in distal mesenchyme (Dudley et al., 2002; Niswander and Martin, 1993; Rowe et al., 1982; Sun et al., 2002). Mesenchymal proliferation and cell death were examined in Fgfr2<sub>Msx2-Cre</sub> limb buds using anti-phosphohistone H3 (pHH3) and anti-caspase 3 immunohistochemistry (IHC), respectively. Consistent with the complete absence of AER functions, mutant hindlimb buds showed decreased mesenchymal proliferation beginning at the limb bud initiation stage (E10) (Fig. 2A,B; Table 1). By E10.5, mesenchymal proliferation was further decreased in mutant limb mesenchyme, and few pHH3-positive cells were found throughout the whole hindlimb field (Fig. 2E,F; Table 1). In contrast to the reduced proliferation observed at early stages, mesenchymal cell death did not appear in the hindlimb buds until E10.5, when massive cell death was detected throughout the entire limb mesenchyme, adjacent to the prospective AER (Fig. 2C,D,G,H; Table 1). Mutant forelimbs showed normal mesenchymal proliferation at E10 (Fig. 2I,J; Table 1). However, by E10.5, reduced proliferation was evident in mutant limb mesenchyme and few pHH3-positive cells were found throughout the whole hindlimb field (Fig. 2E,F; Table 1). At E11.5, apoptotic cells were no longer detected in distal mesenchyme (Fig. 2S,T). This is consistent with the reduced or absent cell death observed after AER extirpation at later stages of chick limb development (Dudley et al., 2002; Rowe et al., 1982), further indicating that loss of AER functions occurs at a later developmental stage in Fgfr2<sub>Msx2-Cre</sub> forelimbs.

Comparison of Msx2-Cre, Fgf4/8 double conditional knockout (Fgf4/8<sub>Msx2-Cre</sub>) mice (Sun et al., 2002), which do not show reduced distal mesenchymal proliferation at early stages, with Fgfr2<sub>Msx2-Cre</sub> mice, revealed that either the AER contributes to mesenchymal
proliferation independently of FGF4 and FGF8, or that mesenchymal proliferation is redundantly regulated by these FGFs and other AER signals. As pointed out in previous studies (Sun et al., 2002), it is puzzling that in Fgf4/8MSX2-Cre hindlimbs, the cell-death zone is located in proximal mesenchyme, whereas AER removal leads to cell death in distal mesenchyme. However, what was not rigorously considered during such a comparison was whether loss of all AER functions versus loss of AER-FGFs happens at the same developmental stages. As demonstrated in previous studies, removal of the AER at different times can lead to different mesenchymal defects and skeletal phenotypes (Dudley et al., 2002). Thus, to understand functional differences between the AER and AER-FGFRs, it is necessary to choose and compare phenotypes that occur at the same stages of development. Hindlimb phenotypes of Fgfr2MSX2-Cre mice and Fgf4/8MSX2-Cre mice allow such a comparison. Fgfr8, which is expressed before limb bud initiation, mediates AER-like functions before formation of a morphologically identifiable AER. In Fgf4/8MSX2-Cre hindlimbs, Fgfr8 was never expressed, similar to the situation for Fgf4/8MSX2-Cre hindlimbs, but, additionally, the AER was never formed. By contrast, a morphologically normal AER is present in Fgf4/8MSX2-Cre hindlimbs at early stages (Sun et al., 2002). Thus, it is conceivable that before or during limb bud initiation, all AER functions are lost in Fgf4/8MSX2-Cre hindlimbs, whereas only FGF signals are lost in Fgf4/8MSX2-Cre hindlimbs. However, despite the loss of all AER-derived signals, mesenchymal cell death in Fgf4/8MSX2-Cre hindlimbs occurs at the same time as that of Fgf4/8MSX2-Cre hindlimbs (35 somites, E10.5), strongly suggesting that the survival function of the AER is solely mediated by FGF signals. Phenotypic comparison of Fgf4/8MSX2-Cre and Fgf4/8MSX2-Cre hindlimbs also suggests that the proximal location of the cell-death domain, after loss of AER-FGF signals, is likely to be due to continued proliferation of distal mesenchyme, which is not affected by loss of AER-FGF signals at early stages (Sun et al., 2002). As apoptosis is delayed, continued proliferation gradually separates dying mesenchymal cells from the AER. Without distal mesenchymal proliferation, the same cell-death domain will remain adjacent to the ectoderm/AER, as shown in Fgf4/8MSX2-Cre hindlimbs (Fig. 2H).

**FGFR1 and FGFR2 mediate AER-FGF signaling during early limb development**

To further understand the mechanism of AER-FGF functions, we disrupted FGF signal transduction in limb mesenchyme by targeting mesenchymal FGFRs. To overcome potential functional redundancy between Fgfr1c and Fgfr2c, we inactivated both Fgfr1 and Fgfr2 in limb mesenchyme with a Prx1-Cre transgene (Prx1 is also known as Prx1 – Mouse Genome Informatics) (Logan et al., 2002). Prx1-Cre is expressed in both forelimb and hindlimb mesenchyme, but is activated in the prospective forelimb region prior to limb bud initiation and targets forelimb mesenchyme exclusively at initiation stages of limb bud development (Fig. 3A). Double conditional knockout mice with the genotype Prx1-Cre/+; Fgfr1flox/Δ; Fgfr2flox/Δ (Fgfr1/2Prx1-Cre) were born alive along with compound single conditional knockout littermates, Fgfr1Prx1-Cre (Prx1-Cre/+; Fgfr1flox/Δ; Fgfr2flox/Δ+) and Fgfr2Prx1-Cre (Prx1-Cre/+; Fgfr1flox/Δ; Fgfr2flox/Δ+). Fgfr1Prx1-Cre mice showed mild limb-skeletal defects (Fig. 3B,C and see Fig. S1 in the supplementary material) and Fgfr2Prx1-Cre skeletons were indistinguishable from wild-type skeletons at post-natal day 0 (P0) (data not shown), consistent with previous findings (Eswarakumar et al., 2002; Li et al., 2005; Verheyden et al., 2005; Yu et al., 2003). In contrast to the single-gene knockouts, Fgfr1/2Prx1-Cre mice showed severe skeletal hypoplasia in both forelimbs and hindlimbs (Fig. 3B,D and see Fig. S1 in the supplementary material). All three segments of the forelimb skeleton were severely affected and bony fusion was found at the elbow joint and between skeletal elements in the autopod. These results suggest that the functions of Fgfr1 and Fgfr2 are partially redundant during limb skeletal development and that Fgfr1 has unique functions in distal limb skeletal formation.

When examined at initiation stages (E9.5), Fgfr1/2Prx1-Cre forelimb buds appeared normal. However, by E10, Fgfr1/2Prx1-Cre forelimb buds were significantly smaller (72%, P<0.03) than control limb buds along the PD axis (Fig. 3E,H,L; Table 2), a phenotype not found in either Fgfr1Prx1-Cre or Fgfr2Prx1-Cre littermates (Fig. 3E,G,I; Table 2). Smaller limb bud size in Fgfr1/2Prx1-Cre embryos could result from decreased mesenchymal cell proliferation or increased cell death. However, proliferation in the distal mesenchyme of Fgfr1/2Prx1-Cre forelimb buds was not affected (Fig. 3M-P, similar to the observations in Fgf4/8MSX2-Cre limb buds (Sun et al., 2002). Increased mesenchymal cell death was not detected in mutant forelimb mesenchyme at E9.5 (25 somites; data not shown); however, at E10 (30-32 somites), increased levels of cell death in Fgfr1/2Prx1-Cre forelimb mesenchyme were clearly present, as shown by caspase 3 IHC (Fig. 3Q,R) and TUNEL labeling (see Fig. S2 in the supplementary material). Apoptotic cells were found in both proximal and central
regions of the limb bud, but were excluded from the most-distal limb mesenchyme. At E10.5, no abnormal cell death was observed in Fgfr1/2Prx1-Cre forelimbs (Fig. 3S,T). As reported previously, after loss of AER-FGFs, forelimbs show similar mesenchymal defects, including increased proximal cell death and reduced limb bud size, that occur at similar stages of development (E10) (Boulet et al., 2004; Sun et al., 2002), demonstrating that AER-FGF signals are mediated by both FGFR1 and FGFR2 during early mesenchymal development.

When compared with RAR-Cre (RAR is also known as Rarb – Mouse Genome Informatics), Fgf4/8 double conditional knockout (Fgf4/8Rar-Cre) forelimbs (Boulet et al., 2004), in which complete loss of FGF4 and FGF8 activity results in skeletal agenesis, Fgfr1/2Prx1-Cre forelimbs were hypoplastic but formed a normally segmented skeleton. Notably, the Fgfr1/2Prx1-Cre skeletal phenotype was similar to that of Fgf4/8Msx2-Cre embryos, in which AER-FGF signals are transiently present at early stages. This suggested that in Fgfr1/2Prx1-Cre forelimbs, early mesenchymal cells still receive FGF signals, albeit at a significantly reduced level. Consistent with this, it has been reported that complete mesenchymal deletion mediated by Prx1-Cre may occur by as late as E12.5 (Hill et al., 2006; Kmita et al., 2005; Lewis et al., 2001), suggesting incomplete mesenchymal inactivation of FGRFs by Prx1-Cre during early limb development. However, the hypomorphic nature of the Fgfr1/2Prx1-Cre mutation allows transient FGF signaling, which can then be contrasted with both pre-limb bud initiation and post-limb bud initiation deletions of Fgf4 and Fgf8.

**DISCUSSION**

Mesenchymal cell death is not sufficient to cause skeletal agenesis

Previous studies concluded that AER-FGF signals regulate limb skeletal formation by maintaining mesenchymal cell survival and, in turn, maintaining the progenitor cell population that will be needed for PD segment formation. After loss of AER-FGF signals, mesenchymal cell death reduces progenitor cell numbers. It has been proposed that skeletal agenesis occurs when progenitor cell numbers fall below some threshold (Boulet et al., 2004; Sun et al., 2002). However, when compared with Fgf4/8Rar-Cre forelimbs, which completely lack a limb skeleton, both Fgf4/8Msx2-Cre and Fgfr1/2Prx1-Cre forelimbs show similar patterns of mesenchymal cell death but they do form a skeleton (Fig. 4B,D,E). It is worth noting that although proximally located cell death domains overlap with the prospective stylopod region, the humerus is the least affected.

<table>
<thead>
<tr>
<th>Somite</th>
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<th>Fgfr1Prx1-Cre P value</th>
<th>Fgfr1/2Prx1-Cre P value</th>
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<tr>
<td>28</td>
<td>4, 2, 4</td>
<td>72* &lt;0.03</td>
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<tr>
<td>30-31</td>
<td>2, 2, 2</td>
<td>84 NS</td>
<td>74* &lt;0.02</td>
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<tr>
<td>32-33</td>
<td>4, 6, 4</td>
<td>97 NS</td>
<td>72* &lt;0.0001</td>
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n, Number of limb buds measured (control, Fgfr1Prx1-Cre, Fgfr1/2Prx1-Cre); ~, not examined.

*Proximodistal limb length as percentage of wild-type control.

†Compared to wild-type control and Fgfr1Prx1-Cre limb buds.
skeletal element in Fgf4/8Msx2-Cre forelimbs. These observations suggest that increased mesenchymal cell death is not sufficient to cause skeletal agenesis.

The notion that there is a threshold number of mesenchymal cells to form limb skeletal segments was originally proposed to explain limb PD patterning defects that result from X-irradiation-induced mesenchymal cell death (Wolpert et al., 1979). However, skeletal defects following X-irradiation are very different from those following loss of both Fgf4 and Fgf8. After X-irradiation, proximal skeletal elements are either not formed or reduced in size, whereas skeletal phenotypes in the autopod are much less severe. It is worth noting that in the X-irradiation model, similar levels of cell death occur in proximal and distal mesenchyme. By contrast, after loss of both Fgf4 and Fgf8, even though...
mesenchymal cell death is limited to proximal mesenchyme, distal skeletal elements are either not formed or are severely hypoplastic. As AER functions are not affected by X-irradiation (Wolpert et al., 1979), such phenotypic differences strongly suggest that loss of AER-FGFs must cause additional mesenchymal defects that result in severe distal skeletal phenotypes.

**Continual proliferation of distal mesenchyme is essential for distal skeletal formation**

Previous studies concluded that skeletal defects, after loss of both Fgf4 and Fgf8, are not due to reduced mesenchymal proliferation, as distal mesenchymal proliferation was not affected by loss of AER-FGF signals when examined before E10.5 (Sun et al., 2002). Although AER-FGF signals are dispensable for mesenchymal proliferation, they are essential to maintain mesenchymal gene expression. One such gene is Fgf10, which is abundantly expressed in distal mesenchyme throughout early limb development (Ohuchi et al., 1997). After loss of both Fgf4 and Fgf8, Fgf10 expression is greatly reduced in distal mesenchyme and is barely detectable at later stages (Boulet et al., 2004; Sun et al., 2002). During limb development, Fgf10 functions to maintain FGFR2b signaling in the AER and the integrity of the AER (Min et al., 1998; Ohuchi et al., 2000; Sekine et al., 1999). This is supported by the observation that after loss of both Fgf4 and Fgf8, there is a higher than normal level of cell death in the AER between E10.5 and E11.5, when Fgf10 expression is no longer maintained (Boulet et al., 2004; Sun et al., 2002).

As illustrated by Fgfr2<sup>Msx2-Cre</sup> forelimb defects (Fig. 4F), the integrity of the AER is important for maintaining distal mesenchymal proliferation. Therefore, we suggest that after loss of both Fgf4 and Fgf8, distal mesenchymal proliferation is eventually reduced following downregulation of Fgf10 and premature degeneration of the AER. Thus, in the absence of both Fgf4 and Fgf8, increased mesenchymal cell death results in an initial reduction in limb bud size. The extreme reduction in limb size that occurs by E11.5 is likely to be due to the combined effects of reduced distal mesenchymal proliferation and sustained cell death that result in the complete cessation of limb bud outgrowth. Continual proliferation of distal mesenchyme is important for distal skeletal element formation. When the AER is excised from chick limb buds at late stages, distal skeletal truncation (loss of autopod elements) correlates with reduced distal mesenchymal proliferation, as mesenchymal cell death no longer occurs after AER removal at late stages of development (Dudley et al., 2002). Fgfr2<sup>Msx2-Cre</sup> forelimb phenotypes further indicate that autopod skeleton formation requires proliferation of distal mesenchymal cells to form a primordium prior to overt morphological changes. The resurgence of autopod skeletal formation after X-irradiation is also likely to be due to restoration of distal mesenchymal proliferation by the intact AER after X-irradiation. Thus, we suggest that distal skeletal defects after loss of both Fgf4 and Fgf8 are more likely to be due to reduced mesenchymal proliferation than to increased mesenchymal cell death.

**A role for FGF signaling during limb mesenchymal differentiation**

As reported previously, disruption of Fgf8 prior to limb bud initiation results in a limb skeleton that completely lacks the stylopod (Fig. 4C). Formation of the zeugopod and autopod are less affected, presumably owing to increased Fgf4 expression that maintains some AER-FGF functions (Lewandoski et al., 2000; Moon and Capecchi, 2000). During limb bud development, Fgf8 expression precedes that of other AER-FGFs, resulting in a window of time in which only FGF8 is present (from E9.0 to ~E10 in mouse forelimbs). After that, AER-FGF signals comprise FGF8 and other FGFs (FGF4, 9 and 17) (Sun et al., 2000; Sun et al., 2002). In the absence of Fgf8, even though Fgf4 is precociously expressed (Lewandoski et al., 2000), there is still a period of time (from E9.0 to ~E9.75 in mouse forelimbs) when mesenchymal cells fail to receive FGF signals from the AER. This gap in FGF signaling results in stylopod agenesis. Moreover, as shown by Fgf4/8<sup>Msx2-Cre</sup> forelimb phenotypes (Fig. 4D), when mesenchymal cells receive FGF signals during this period of time, stylopod formation proceeds relatively normally and is not affected by extensive proximal cell death or dramatic limb bud size reduction. Thus, stylopod agenesis after loss of Fgf8 cannot simply be due to reduced mesenchymal cell number, as suggested in previous studies (Lewandoski et al., 2000; Sun et al., 2002).

Previous studies also indicate that loss of AER-FGF signals do not prevent mesenchymal cells from expressing Sox9, the earliest marker for chondrogenic differentiation (Akiyama et al., 2002; Wright et al., 1995), but do prevent formation of mesenchymal condensations (Boulet et al., 2004; Sun et al., 2002). Normally, Sox9 functions to commit undifferentiated mesenchymal cells to osteochondroprogenitors, a cell type that will give rise to both chondrocytes and osteoblasts and to promote mesenchymal condensation formation (Akiyama et al., 2002). During forelimb development, Sox9 expression begins at E10 in a subpopulation of mesenchymal cells (Akiyama et al., 2005). It is worth noting that at E9.5, prior to Sox9 expression, AER-FGF-induced MAP kinase activity is already present in the entire forelimb mesenchyme (Corson et al., 2003). Thus, we hypothesize that AER-FGF signals are required for Sox9-mediated mesenchymal condensation formation. In support of this hypothesis, previous studies have shown that FGF signaling through the MAP kinase signaling pathway can function synergistically with SOX9 to upregulate Sox9 expression and that the level of SOX9 protein is critical for the formation of a mesenchymal condensation (Bi et al., 2001; Murakami et al., 2000). Thus, stylopod agenesis after loss of Fgf8 is not due to reduced mesenchymal cell numbers, but rather to the failure of Sox9-expressing cells to undergo further chondrogenic differentiation.

**AER-FGF functions during PD pattern formation**

During limb skeletal development, prior to initiation of a mesenchymal condensation, some mesenchymal cells undergo initial stages of chondrogenic differentiation, characterized by expression of Sox9, but without apparent changes in cell morphology (chondrogenic primordia). Initial Sox9 expression is localized to central regions of the limb bud, surrounded by undifferentiated mesenchymal cells. Later, the Sox9 expression domain expands following limb outgrowth, but is still excluded from distal mesenchyme beneath the AER (Akiyama et al., 2005). Owing to the lack of definitive markers for each PD segment at early stages, it is difficult to determine whether the initial Sox9 expression domain contains progenitors for all three PD segments, or only for the most-proximal segment.

Recent lineage-tracing studies with Shh-Cre and the ROSA26 (R26R) β-galactosidase reporter suggest that the initial Sox9 expression domain might not contain progenitors for all three PD segments. Along the PD axis, progeny of Shh-Cre-expressing cells are found in the autopod and part of the zeugopod, but never in the stylopod (Harfe et al., 2004). The origin of Shh-Cre-expressing cells is limited to the zone of polarizing activity (ZPA) in the posterior
edge of limb mesenchyme, and does not appear to overlap with the central Sox9 expression domain. This suggests that skeletal lineages in the zeugopod and autopod are not derived from the initial Sox9 expression domain, but rather are formed at later stages after their progenitors move out of the ZPA. Thus, Sox9 expression is sequentially regulated along the PD axis, which is consistent with the conclusion that chondrogenic differentiation along the PD axis follows a proximal-to-distal sequence (Summerbell et al., 1973).

We suggest that the initial Sox9 domain demarcates a chondrogenic primordium for the stylopod, in which Sox9-expressing cells only contribute to very proximal skeletal elements. With continual transition of distal undifferentiated cells into Sox9-expressing cells, chondrogenic primordia for the zeugopod and autopod become sequentially established (Fig. 4A). During this sequential differentiation process, AER-controlled proliferation provides a source of undifferentiated mesenchymal cells that continually receive AER-FGF signals and progressively differentiate into more-distal skeletal elements. Such a mechanism ensures continual commitment of mesenchymal cells to the osteochondrogenic primordia that are essential for mesenchymal condensation formation along the PD axis. This could explain the different skeletal phenotypes in Fgfr2Msx2-Cre and Fgf4/8Msx2-Cre as well as Fgfr2M2msx2-Cre forelimbs, in which AER-FGFs or all AER functions are inactivated at different times during development (Fig. 4C,D,F).

In addition to temporal differences in Sox9 expression, different PD segments might also regulate Sox9 expression through distinct molecular mechanisms. This is supported by recent studies of hindlimb phenotypes (lack of the stylopod and zeugopod but normal autopod) in Gli3 and Plzf (also known as Zhub16 – Mouse Genome Informatics) double-knockout mice (Barna et al., 2005). In the absence of both Gli3 and Plzf, Sox9 is not expressed in early limb mesenchyme, which, as we suggest, results in failure of proximal chondrogenic primordia formation and subsequent failure of proximal skeletal element formation. However, owing to normal mesenchymal proliferation and normal Sox9 expression in distal mesenchymal cells at later stages, autopod chondrogenic primordia and distal skeletal development proceeds normally in mutant hindlimb buds.

In a recent review, after examination of segment-specific marker gene expression, Tabin and Wolpert present ideas that distal proliferation and proximal differentiation are tightly coupled at early stages and that segmental primordia are sequentially established along the PD axis (Tabin and Wolpert, 2007). It is worth noting that establishment of chondrogenic primordia, marked by Sox9 expression, occurs in a similar timeframe to this PD segmentation process that is defined by segment-specific gene expression. Since, in the absence of Sox9, segment-specific genes themselves are insufficient to induce the formation of a mesenchymal condensation (Akiyama et al., 2002), these results suggest that PD skeletal pattern formation requires coordination of chondrogenic- and segment-specific differentiation.

In summary, AER-FGF signals directly or indirectly regulate mesenchymal survival, proliferation and differentiation during limb development. The complete skeletal agenesis after loss of both Fgf4 and Fgf8 or all AER functions before limb bud initiation (Fig. 4E,G) is likely to be due to the loss of all of these functions. Finally, in contrast to previous conclusions that AER-FGF signals regulate PD skeletal pattern formation by controlling mesenchymal cell number, our analysis indicates that the AER and AER-FGFs regulate PD skeletal pattern formation through coordinating distal mesenchymal proliferation and chondrogenic differentiation.

We thank C. Smith and G. Schmid for mouse husbandry and C. Tabin and R. Kopan for helpful discussions. This work was funded by NIH grant HD049808 and NIH training grant T32AR007033.

Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/3/483/DC1

References
FGF regulation of limb bud development


