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Normal limb development in conditional mutants of *Fgf4*

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SUMMARY

Fibroblast growth factors (FGFs) mediate multiple developmental signals in vertebrates. Several of these factors are expressed in limb bud structures that direct patterning of the limb. FGF4 is produced in the apical ectodermal ridge (AER) where it is hypothesized to provide mitogenic and morphogenic signals to the underlying mesenchyme that regulate normal limb development. Mutation of this gene in the germline of mice results in early embryonic lethality, preventing subsequent evaluation of *Fgf4* function in the AER. A conditional mutant of *Fgf4*, based on site-specific Cre/loxP-mediated excision of the gene, allowed us to bypass embryonic lethality and directly test the role of FGF4 during limb development in living murine embryos. This conditional mutation was designed so that concomitant with inactivation of the *Fgf4* gene by excision of all *Fgf4*-coding sequences, a reporter gene was activated in *Fgf4*-expressing cells, allowing assessment of the site-specific recombination reaction. Although a large body of evidence led us to predict that ablation of *Fgf4* gene function in the AER of developing mice would result in abnormal limb outgrowth and patterning, we found that *Fgf4* conditional mutants had normal limbs. Furthermore, expression patterns of *Shh*, *Bmp2*, *Fgf8* and *Fgf10* were normal in the limb buds of the conditional mutants. These findings indicate that the previously proposed FGF4-SHH feedback loop is not essential for coordination of murine limb outgrowth and patterning. We suggest that some of the roles currently attributed to FGF4 during early vertebrate limb development may be performed by other AER factors in vivo.

Key words: Fibroblast growth factor (Fgf), Limb, Mouse

INTRODUCTION

The vertebrate limb provides a powerful system in which to address fundamental questions in development, such as how genes control cell migration, differentiation and the morphology of complex structures. Intercellular signals that pattern the murine limb also function in other regions of the developing embryo and are conserved across species (Cohn and Tickle, 1996; Tabin, 1991). The apical ectodermal ridge (AER) is an epithelial limb bud structure required to support continued outgrowth of the limb bud by maintaining the underlying mesenchyme, the progress zone (PZ), in a highly proliferative, undifferentiated state (Globus and Vethamany-Globus, 1976; Saunders, 1977). Time of residence in the PZ and exposure to AER-derived signals determine cell fate along the proximal-distal limb axis (Summerbell et al., 1973). The AER is also required for initiation and maintenance of the zone of polarizing activity (ZPA), a region of posterior mesenchyme that specifies the anteroposterior axis of the developing limb (Riddle et al., 1993; Todt and Fallon, 1987; Vogel and Tickle, 1993). Similarly, signals from the PZ and ZPA are necessary for survival and function of the AER (Hinchcliffe and Gumpel-Pinot, 1981; Ohuchi et al., 1997; Reiter and Solursh, 1982; Xu et al., 1998)

Members of the fibroblast growth factor gene family (*Fgfs*) encode secreted signaling proteins (Basilico and Moscatelli, 1992). Subsets of these molecules (FGFs) and their receptors are produced in different regions of the developing vertebrate limb, including the AER and PZ. The importance of FGFs in human skeletal development was confirmed when several dominant human skeletal defects were mapped to genes encoding FGF receptors (Muenke and Schell, 1995). Additionally, mice homozygous for null mutations of *Fgf receptor 2* or *Fgf10* fail to form limbs (Ohuchi et al., 1997; Sekine et al., 1999; Xu et al., 1998). FGFs can induce and support the outgrowth of ectopic limbs in the absence of an AER and can substitute for the AER after it is surgically ablated from early limb buds (Crossley et al., 1996; Fallon et al., 1994; Niswander et al., 1993). These growth factors have thus been hypothesized to mediate AER activities in vivo.

Fgf4 is first expressed in the developing murine forelimb bud during embryonic day 10 (E10.0, 30-32 somites, stage 3; Wanek et al., 1989); expression is strongest in the posterior AER at E10.5-11.0 and then wanes to undetectable levels by E12.0 (Drucker and Goldfarb, 1993; Niswander and Martin, 1992; Suzuki et al., 1992).

Current models propose that FGF4 mediates mitogenic and morphogenic AER signals. FGF4 may provide a proliferative

signal to the posterior PZ to support limb outgrowth (Niswander et al., 1993; Suzuki et al., 1992). Several lines of evidence suggest that expression and function of *Sonic Hedgehog* (*Shh)*, which mediates the polarizing activity of the ZPA (Riddle et al., 1993), is dependent on FGF4 signaling from the posterior AER (Laufer et al., 1994; Niswander et al., 1994; Vogel and Tickle, 1993). Exogenous FGF4 induces ectopic *Shh* expression in the presence of retinoic acid and maintains *Shh* expression in the ZPA in the absence of the AER (Niswander et al., 1994). PZ cells respond to ectopic SHH by asymmetrically expressing *Bmp2* and 5′ *Hoxd* genes only in the presence of the AER; however, FGF4 can substitute for the AER in this assay (Laufer et al., 1994). Additionally, ectopic SHH can induce *Fgf4* expression in a more anterior portion of the AER than its endogenous expression domain (Laufer et al., 1994). These observations support the hypothesis that a positive feedback loop between FGF4 in the AER and SHH in the ZPA coordinately regulates limb outgrowth and patterning and thus implicate FGF4 in the generation of the normal limb AP axis (Laufer et al., 1994; Niswander et al., 1994).

The limb phenotypes and gene expression patterns described in naturally occurring vertebrate mutants are also consistent with the existence of an FGF4-SHH feedback loop. Lack of *Fgf4* expression in the AER of the murine *limb deformity* mutant is associated with failure to maintain *Shh* expression and abnormal patterning of distal limb elements in these animals (Haramis et al., 1995). Recent studies indicate that the *Formin* gene mutation in *ld* mutants disrupts a signaling relay from *Shh*-expressing mesenchymal cells to the AER (Chan et al., 1995b; Zuniga et al., 1999). *Strong's Luxoid* polydactylous mice display ectopic, anterior expression of *Shh*, *Fgf4* and 5′*HoxD* genes (Chan et al., 1995a). In *limbless* chicks, the posterior mesoderm does not express *Shh* nor does the AER express *Fgf4*. Application of exogenous FGF to *limbless* buds induces low-level *Shh* expression in the posterior mesoderm and subsequent asymmetric mesodermal expression of *Bmp-2*, which is thought to be downstream of SHH (Ros et al., 1996). Chick *talpid*^{2} and *talpid*^{3} polydactylous mutants express *Shh* normally, but *Fgf4* is expressed throughout the AER. This has been attributed to abnormal activation of the downstream SHH signaling pathway throughout the anteroposterior axis in the mesoderm (Caruccio et al., 1999; Francis-West et al., 1995; Lewis et al., 1999).

In total, these observations have been interpreted to indicate that FGF4 performs essential AER roles in normal embryos. However, it is possible that some or all of these functions are normally mediated by other AER factors. FGF2 and FGF8 are also produced by the AER, have similar properties in functional assays (Anderson et al., 1993; Cohn et al., 1995; Crossley et al., 1996; Fallon et al., 1994), and activate some of the same receptor isoforms in vitro as FGF4 (Ornitz et al., 1996; Xu et al., 1999). It is unknown how well these assays reflect in vivo FGF activity and receptor usage, or which FGFs are required for specific AER functions during normal limb development.

Fgf4 null embryos die at E4.5 (Feldman et al., 1995), preventing direct evaluation of FGF4 function in the developing limb using conventional methods of inactivation by gene targeting. To bypass embryonic lethality resulting from disruption of *Fgf4* in the germline, we generated a system to conditionally inactivate *Fgf4* in a developmentally regulated, tissue-restricted manner. *Fgf4* conditional mutants, which contain no *Fgf4* transcripts in the AER of their forelimb buds, were analyzed with regard to limb structure and gene expression at multiple stages. We found that the limbs of the conditional mutants develop normally and that, in contrast to predictions based on the models of FGF4 function described above, expression of *Shh*, *Bmp2*, *Fgf10* and *Fgf8* was normal in these animals. Our findings suggest that other AER factors may normally perform some of the functions currently attributed to FGF4 or may compensate for the absence of FGF4 in the conditional mutants during limb development.

MATERIALS AND METHODS

Generation of mice bearing targeted *Fgf4* alleles and RARCre transgenic lines

To create the *Fgf4* conditional allele, F4^{AP}, a loxP site was inserted in the 5′ untranslated region of the gene at an *Fsp*I site located 59 nucleotides upstream of the translation initiation ATG. A cassette was inserted 89 nucleotides 3′ of the polyadenylation signal that contained: the MC1neo gene (Thomas and Capecchi, 1987), followed by a synthetic polyadenylation site (Levitt et al., 1989) and a pause-termination signal derived from the human complement gene C2 (Ashfield et al., 1991), a loxP site in the same orientation as the 5′ loxP, and the entire coding region of the human placental alkaline phosphatase gene.

To produce the F4^{neo} null allele, a deletion of 141 nucleotides was introduced between an *Xma*III site at nucleotide 286 of exon 1 and an *Xma*III site at position 113 of the first intron. The MC1neo gene was inserted in place of the deleted genomic sequences. The deletion in exon 1 and translational stops in all three reading frames of the MC1 promoter led us to predict that this would be a null allele of *Fgf4*. This was confirmed by failure to detect any F4$^{neo/neo}$ homozygotes among the progeny of heterozygous intercrosses at E9.5, 10.5 and adult (n=85, expected 22 homozygotes).

Targeting vectors were electroporated into RI ES cells and cell lines carrying the desired alterations to the *Fgf4* gene were used to generate chimeras that transmitted the targeted allele.

The RARCre transgene was constructed by placing a 3.5 kb murine RARβ2 promoter fragment (Charité et al., 1994) upstream of coding sequences for Cre recombinase (Gu et al., 1993). The RARCre fragment was microinjected into zygotes by standard methods (Hogan et al., 1986) to generate two transgenic lines.

Genotype determination

DNA was prepared from tails or yolk sacs of adult and embryonic mice, respectively. Southern blot analysis was applied to identify and characterize mice bearing targeted alleles of *Fgf4* or the *RARCre* transgene. Subsequently, PCR assays were utilized to determine the genotype of embryos and adult mice. PCR strategies used for the *Fgf4* conditional and neo alleles are diagrammed in Fig. 1.

Alkaline phosphatase staining

Embryos were dissected in PBS+2mM $MgCl_2$ and fixed overnight in 4% paraformaldehyde in PBS+2 mM $MgCl_2$. After three rinses in PBS+2mM $MgCl_2$, embryos were heated at 65°C for 90 minutes to destroy endogenous alkaline phosphatase activity. Embryos were rinsed once for 15 minutes in 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$, 0.1% Tween 20, 0.5 mg/ml levamisole and stained at room temperature in the dark in BM purple alkaline phosphatase substrate (Boehringer Mannheim Biochemicals) for 2 hours. Embryos to be sectioned were stained overnight, refixed and embedded in paraffin by standard methods. 10 μm sections were counterstained with nuclear fast red.

Whole-mount in situ hybridization and X-gal staining

In situ hybridization on intact embryos was carried out as described (Carpenter et al., 1993) with the following minor modifications: embryos were treated with proteinase K at 10 μg/ml for 5 minutes at 25°C; the anti-digoxigenin alkaline phosphatase-conjugated antibody (Boehringer Mannheim Biochemicals) was used at a dilution of 1:5000; 0.5 mg/ml levamisole was only added to the reaction buffer used to rinse the embryos prior to the enzyme reaction; the substrate was BM purple (Boehringer Mannheim Biochemicals). All experiments were repeated a minimum of three times; littermates were used when feasible and control and mutant embryos were hybridized in the same vial.

X-gal staining was performed as described (Mansour et al., 1993), except that the reactions were carried out at 30°C. Fixation time was 5 to 15 minutes depending on gestational age of embryos.

Skeletal analysis

Skeleton preparations of newborn mice were carried out as described (Mansour et al., 1993).

RESULTS

Elements of the *Fgf4* conditional mutagenesis system

Our system for conditional mutagenesis of *Fgf4* employs three components. Mice carrying either a conditional or a null allele of *Fgf4* were created by gene targeting in ES cells (Thomas and Capecchi, 1987) (Fig. 1) and a transgenic 'switch' was generated to inactivate the conditional allele (Fig. 2). ES clones and chimera offspring containing the *Fgf4* mutant alleles were initially identified by Southern analysis (data not shown). Subsequently, animals were genotyped using the PCR strategy illustrated in Fig. 1.

The $F4^{AP}$ conditional allele contains loxP sites in the 5′ untranslated (UTR) and 3′ flanking regions of *Fgf4* (Fig. 1A). Sequences encoding human placental alkaline phosphatase (AP) were placed downstream of the *loxP* site in the untranscribed 3′ flanking region. This allele is functional prior to inactivation by the Cre-mediated 'switch'; $F4^{AP/AP}$ homozygotes are born at predicted Mendelian frequencies and survive to be fertile adults with normal limbs. Cre-mediated recombination (Baubonis and Sauer, 1993) of this allele deletes all *Fgf4*-coding sequences and generates a null allele of *Fgf4* ($F4^{APR}$). This recombination/deletion event also repositions the AP-coding sequences so that they are expressed and regulated by the *Fgf4* promoter. Thus, AP activity labels those cells in which Cre-mediated recombination has occurred and the *Fgf4* promoter is active.

The $F4^{neo}$ allele is a null allele created by a deletion in Exon 1 of *Fgf4* and insertion of a *neo*^{r} gene (Fig. 1B). No $F4^{neo/neo}$ homozygotes were detected among the progeny of heterozygote intercrosses, a finding consistent with the embryonic lethal phenotype of *Fgf4* null homozygotes previously described (Feldman et al., 1995). $F4^{neo/+}$ heterozygotes were present at expected Mendelian frequencies and were phenotypically normal, fertile adults. We performed conditional mutagenesis in compound heterozygotes carrying an $F4^{AP}$ and an $F4^{neo}$ allele to limit the requirement for Cre-mediated recombination to only one of the *Fgf4* alleles. Use of the $F4^{neo}$ allele also avoids potential problems that could arise from Cre-mediated recombination between $F4^{AP}$ alleles on separate chromosomes since the $F4^{neo}$ allele does not contain loxP sites.

The 'switch' used to regulate inactivation of the conditional *Fgf4* allele was the Cre-recombinase gene expressed under the

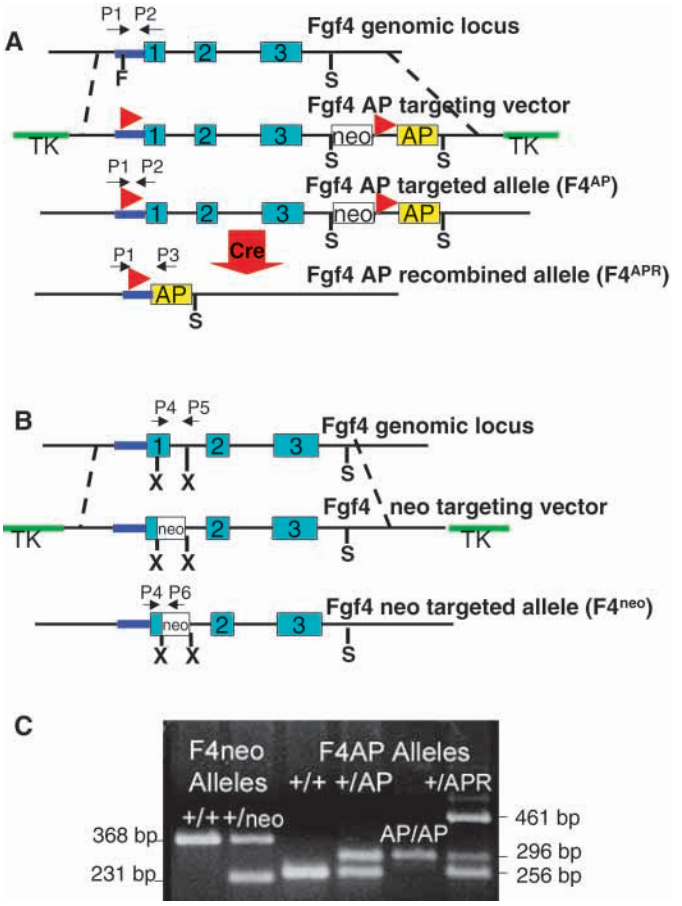

Fig. 1. Conditional mutagenesis of the *Fgf4* locus. (A) The wild-type (WT) *Fgf4* locus is depicted on the first line; exons are represented by blue boxes; untranslated regions are shown as lines (purple, Fgf4 promoter; black, introns and 3′UT). The $F4^{AP}$ targeting vector (second line) was constructed by insertion of a loxP site (red arrowheads) 59 bp upstream of the translation start site. A cassette containing: MC1neo^r (white box), another loxP site in the same orientation as that in the 5′ untranslated region and, human placental alkaline phosphatase-coding sequences and the SV40 polyadenylation signal (yellow box), was inserted 89 bp downstream of the polyadenylation signal. Thymidine kinase (TK, green) genes flank the *Fgf4* homology to permit negative selection against random integrants. Cre-mediated recombination (large red arrow) of the $F4^{AP}$-targeted allele deletes all *Fgf4* sequences between the loxP sites, generating $F4^{APR}$. (B) The $F4^{neo}$ targeting vector contains MC1neo^r between *Xma*III (X) restriction sites at nucleotides 286 of exon 1 and 113 of intron 1. (C) Genotyping at the *Fgf4* locus. PCR primer sets are shown in A and B and fragment sizes are as shown in C. The $F4^{AP}$ and $F4^{APR}$ alleles contain WT sequences in the region examined by the $F4^{neo}$ primer set and vice versa. Each animal was analyzed with both primer sets to obtain a complete genotype. PCR products obtained with P1 and P2 on the WT and $F4^{AP}$ alleles are 256 base pairs (bp) and 296 bp, respectively. The P1/P3 primer set amplifies a 461 bp region of the recombined allele, $F4^{APR}$. The P4/P5 set detects a 368 bp WT product; while P4/P6 detects a 231 bp product from the $F4^{neo}$allele.

control of the Retinoic Acid Receptor-*B*2 promoter (*RARCre*). The function of this promoter has been well characterized in transgenic mice, and includes expression in the intermediate and lateral plate mesoderm as early as E8.5, as well as in the forelimb buds at E9.5 and the AER as it develops at E10.0-10.5 (Charité et al., 1994; Mendelsohn et al., 1991; Reynolds et al., 1991). Thus, *RARCre* should be expressed in all of the relevant tissues that potentially require *Fgf4* for patterning of the forelimb. Two *RARCre* transgenic lines were generated. Expression of *RARCre* was the same in both lines, similar to that previously described in the literature, and consistent throughout our experiments.

Activity of the RARCre transgenic 'switch' in murine embryos

To characterize the production of functional Cre recombinase by the RARCre transgene, we mated *RARCre* transgenic animals to reporter mice carrying the *lacZ* gene targeted to the RNA polymerase II large subunit locus (*P2Bc*). The polII promoter is ubiquitously active but, in these reporter mice, β-galactosidase (β-gal) activity is dependent on Cre-mediated recombination of the locus (O'Gorman et al., 1997). As expected, no β-gal activity was detected in embryos carrying only the reporter *P2Bc* allele (Fig. 2A). Embryos that carry both *P2Bc* and *RARCre* alleles have readily detectable β-gal activity at E9.5 in the forelimb bud and in the developing AER at E10.0. Note that there is very little β-gal activity in the hindlimb at any stage, indicating that few cells express Cre in the hindlimb.

The $F4^{AP}$ conditional allele provides a very sensitive means of assessing the function of *RARCre* (Fig. 2B). AP activity is undetectable in E10.0 $F4^{AP/AP}$ embryos, confirming that there is no 'leaky' expression of the AP gene from the unrecombined $F4^{AP}$allele. In a 31- to 32-somite E10.0 embryo carrying an $F4^{AP}$ allele and *RARCre* ($F4^{AP/+}$; T [RARCre transgene]), AP activity is detectable only at those sites where the *Fgf4* promoter is active and Cre has recombined the $F4^{AP}$ allele; it labels those cells in which functionally relevant recombination has occurred. As expected, only faint staining of the hindlimb AER is evident, whereas staining of the forelimb AER is dark and uniform (Fig. 2B). Cross-sections through the forelimb (Fig. 2B, FL) of the $F4^{AP/+}$;T embryo confirm staining of every cell in the AER, indicating that Cre has recombined the $F4^{AP}$ allele throughout the AER by E10.0. In contrast, the hindlimb AER (Fig. 2B, HL) contains many unstained cells that possess the unrecombined $F4^{AP}$ allele. These results indicate that the $F4^{AP}$ allele is already recombined (and thus a null allele) in RARCre-expressing cells at the time of earliest *Fgf4* expression in the forelimb AER.

Additionally, the AP expression and *lacZ* reporter data demonstrate that the hindlimbs of conditionally mutant animals (where *RARCre* is not uniformly expressed) function as an internal control for the effects of a complete 'knock-out' of *Fgf4* in the forelimb AER.

Fgf4 conditional mutants are viable and fertile

Conditional mutagenesis of *Fgf4* was accomplished by mating mice containing the $F4^{AP}$ allele (genotypes: $F4^{AP/+}$ or $F4^{AP/AP}$) to mice bearing *RARCre* and either null allele ($F4^{APR}$ or $F4^{neo}$, hereafter noted as $F4^{Null}$) in their germline (genotype: $F4^{Null/+}$;T). The results were the same with the use of either null allele. Conditional mutants ($F4^{AP/Null}$; T) were obtained at the expected Mendelian frequencies as embryos and adults, confirming that embryonic lethality had been circumvented. For example, among the 74 progeny of $F4^{AP/AP}$ X $F4^{Null/+}$;T matings examined at E10.0-E11.5, we expected 18 and observed 21 conditional mutants. We generated 50 adults with this mating strategy; we expected 13 and saw 14 conditional mutants. The conditional mutant adults were fertile and produced healthy offspring.

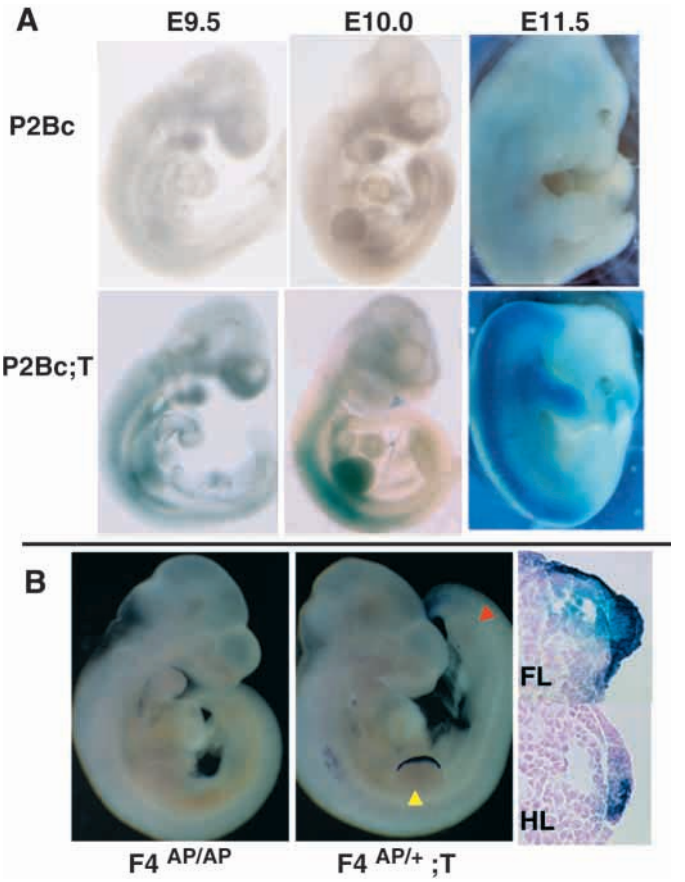

Fig. 2. Activity of the *RARCre* transgene. (A) β-galactosidase activity in offspring of *RARCre* X *P2Bc* reporter mice. E9.5, E10.0 and E11.5 embryos were stained with X-gal; there is no activity in embryos with only the *P2Bc* reporter allele at any stage. Littermates that also carry the *RARCre* transgene (P2Bc/+;T) have detectable β-galactosidase activity, resulting from Cre mediated recombination of the reporter gene, in the forelimb by E 9.5 and in the AER by E 10.0. Note absence of significant hindlimb staining. (B) Alkaline phosphatase (AP) activity requires Cre-mediated recombination of the $F4^{AP}$ conditional allele. E10.0, 30-32 somite, forelimb stage 3 littermates, were stained for AP activity. There is no staining in $F4^{AP/AP}$ mice in the absence of *RARCre*. Cre-mediated recombination of the $F4^{AP}$ allele results in uniform staining of the entire AER in the forelimb (FL, yellow arrowhead) but only partial staining in the hindlimb (HL, red arrowhead) AER. The difference in recombination between fore- and hindlimb is confirmed by examining the AERs in cross-section. The appearance of AP signal in cells outside the AER could be a technical artifact or reflect actual low-level activity of the *Fgf4* promoter in these cells.

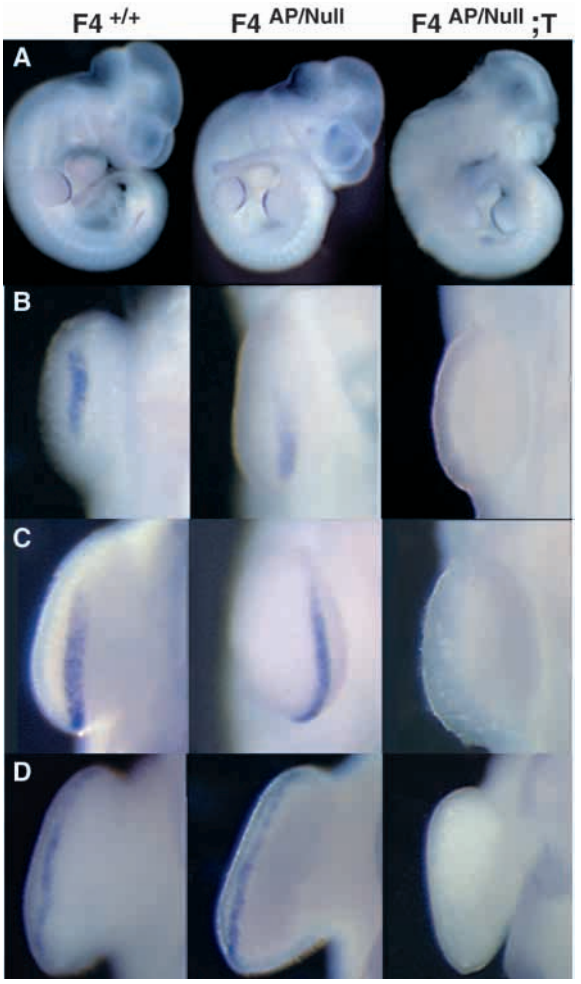

Fig. 3. *Fgf4* expression in conditional mutants and their littermates. Whole-mount RNA in situ hybridization with an *Fgf4* antisense riboprobe containing exon 3 and 3′UTR sequences. (A) E10.5 whole-mount embryos; transcripts are present in hindlimb, but not forelimb AER of conditional mutants ($F4^{AP/Null}$;T), confirming that Cre-mediated recombination of the conditional allele in the context of a null allele completely disrupts *Fgf4* gene function. (B) E10.0 (30 somite) embryos. Close-up view of forelimb AER. Absence of *Fgf4* transcripts correlates with presence of *RARCre* and confirms disruption of *Fgf4* gene function at the earliest stage of normal *Fgf4* transcription. (C,D) Close-up views of the forelimb AERs of E10.5 and E11.5 embryos, respectively.

Fgf4 transcripts are absent in the forelimb AER of *Fgf4* conditional mutants

To demonstrate that *RARCre* activity disrupted *Fgf4* gene function throughout the forelimb AER of *Fgf4* conditional mutant embryos, we performed RNA in situ hybridization on embryos using an antisense *Fgf4* riboprobe (Fig. 3). As expected, at E10.5, there was a strong *Fgf4* signal in the hindlimb AER of the $F4^{AP/Null}$;T conditional mutants, but no *Fgf4* transcripts were detected in the forelimb AER (Fig. 3A,C). Also, as expected, E10.5 $F4^{AP/Null}$ animals without *RARCre* had normal patterns of *Fgf4* expression in the AER of both limbs. The earliest stage that we and others have detected *Fgf4* transcripts is E10.0, or the 30-somite stage when the AER is morphologically distinct (Drucker and Goldfarb, 1993; Niswander and Martin, 1992; Suzuki et al., 1992). When we examined the forelimb AER of *Fgf4* conditional mutants at this stage (Fig. 3B), no *Fgf4* transcripts were detected. Thus, in embryos bearing *RARCre*, the $F4^{AP}$ conditional allele and a null allele, $F4^{AP}$ is recombined throughout the AER prior to the onset of normal *Fgf4* transcription resulting in an AER with no functional *Fgf4* alleles.

Fgf4 conditional mutants have normal forelimbs

Current models of FGF4 function in the developing limb led us to hypothesize that disruption of *Fgf4* in the AER of embryonic mice would result in defective outgrowth of posterior limb elements and altered anteroposterior patterning secondary to disruption of the proposed FGF4-SHH feedback loop. We examined the skeletons of conditional mutants (n=21) in E15.5 embryos, newborns (Fig. 4) and adults, and were extremely surprised to find that patterning and outgrowth of the forelimbs of *Fgf4* conditional mutants were normal, as were external appearance and function in living animals. Embryonic conditional mutants (E10.0-11.5) were also indistinguishable from their littermates with regard to

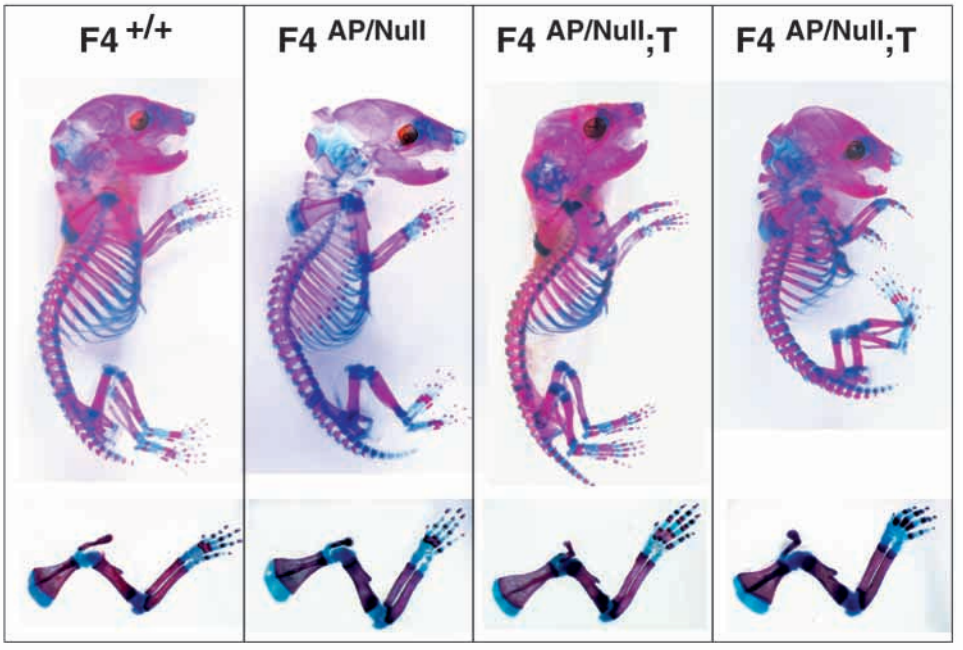

Fig. 4. Skeletal phenotypes of newborns. Alizarin red stains ossified structures and Alcian blue stains cartilage in these preparations. Note that the limbs of *Fgf4* conditional mutants are indistinguishable from wild-type controls.

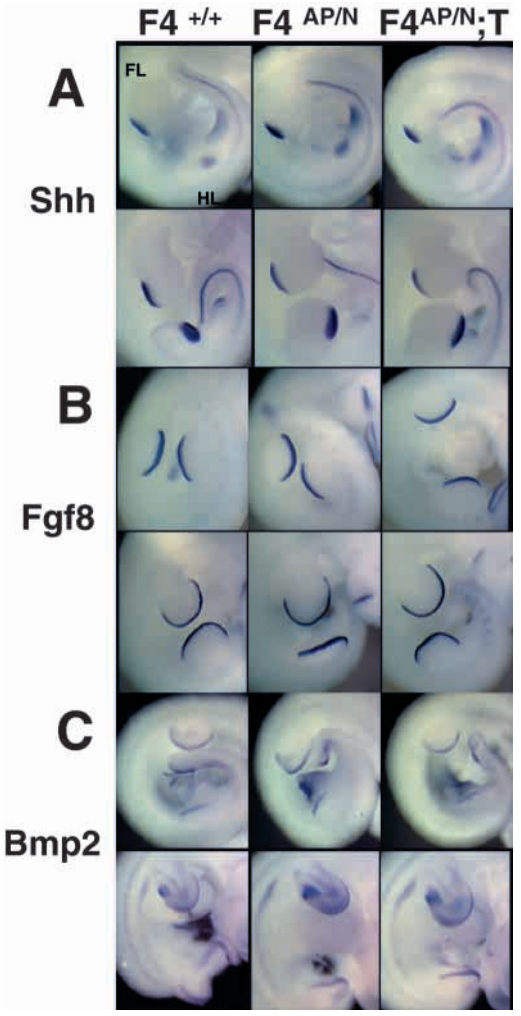

Fig. 5. Molecular phenotypes as assessed by RNA in situ hybridization; normal expression of *Shh* in *Fgf4* conditional mutants. For each probe, top row are E10.5 embryos; bottom row, E11.5. Littermates were used when possible. (A) *Shh* expression in the ZPA. (B) *Fgf8* expression in the AER. (C) *Bmp2* expression in the AER and mesenchyme. Note that each of these genes is expressed normally in *Fgf4* conditional mutants.

initiation, location or size of the limb bud and appearance of the AER.

Sonic Hedgehog is expressed normally in the limb mesenchyme of *Fgf4* conditional mutants

If FGF4 normally maintains *Shh* expression in the ZPA, one would expect decreased *Shh* expression in the forelimbs of *Fgf4* conditional mutants, particularly by E11.5. However, no changes in *Shh* expression were detected (Fig. 5A). Because *Bmp2* is thought to act in opposition to *Fgf4* to control limb outgrowth (Niswander and Martin, 1993) and to be regulated by the FGF4-SHH feedback loop (Laufer et al., 1994), we also expected altered expression of *Bmp2* in the absence of FGF4 (Fig. 5C). Instead, the expression pattern was normal in the mutants. Similarly, there were no detectable alterations in timing, location or intensity of signal when we examined *Fgf8* expression in the AER (Fig. 5B) or *Fgf10* expression in the mesenchyme (data not shown).

DISCUSSION

By applying a system to conditionally mutate *Fgf4* in the forelimbs of developing mouse embryos, we have successfully bypassed the early embryonic lethality that results from mutation of this gene in the germline. A large body of experimental evidence led us to predict that *Fgf4* conditional mutants would have alterations in forelimb bud outgrowth and anteroposterior patterning as a result of altered PZ-AER interactions and disruption of the proposed FGF4-SHH feedback loop. However, our results indicate that *Fgf4* is not required for normal limb development or *Shh* expression in the ZPA. In the absence of FGF4, *Shh* and *Bmp2* are appropriately induced and maintained, consistent with correct anteroposterior forelimb patterning seen in the conditional mutants; *Fgf 8* and *10* are expressed normally in the AER and PZ, a finding consistent with the continued normal forelimb outgrowth observed in these animals.

Besides demonstrating that *Fgf4* gene function is not required for normal limb development, this work also provides new insight into *Fgf4* regulatory elements. Based on expression of *Fgf4* gene fragments linked to *lacZ* in transgenic mice, it has been proposed that the *Fgf4* promoter has minimal *cis*-regulatory activity and that specific sequences in the 3′UTR direct *Fgf4* gene expression in particular expression domains (i.e. AER versus myotome) (Curatola and Basilico, 1990; Fraidenraich et al., 1998). Additionally, in the transgenic system, restriction of *Fgf4* expression to cells in the posterior AER required the presence of ‘inhibitor’ sequences in the *Fgf4* 3′UTR. However, Cre-mediated recombination of the $F4^{AP}$ allele deletes the entire 3′UTR and an additional 89 nucleotides and yet we have found that the *Fgf4* promoter, in its normal 5′ chromasomal context, is active and contains sufficient information to direct AP expression in the AER, myotomes and pharyngeal pouches in the absence of any of the proposed enhancer or *cis*-regulatory sequences contained in the 3′UTR (Fig. 2B and data not shown). The *Fgf4* 5′ promoter fragment used in the transgenic constructs was 1.2 kb, ended 83 bp upstream of the translation start site and may not have contained all the regulatory elements located 5′ of the coding sequences. Notably, the $F4^{APR}$ allele is expressed throughout the AER (Fig. 2B). The broader expression domain is likely the consequence of the AP enzymatic assay being more sensitive than in situ hybridization as a means of detecting low level *Fgf4* expression in the anterior AER at E10.0 (note that wild-type E11.5 embryos have detectable *Fgf4* mRNA throughout the AER, Fig. 3B).

The observation that limbs develop normally in the absence of FGF4 in the AER of *Fgf4* conditional mutants suggests that some of the functions currently ascribed to FGF4 may be performed by other AER factors in vivo. The simplest hypothesis is that another FGF compensates for the absence of FGF4 in the conditional mutants and provides a functionally equivalent signal to the underlying mesenchyme to support continued limb outgrowth and patterning. FGF1, FGF 2 and FGF 8 can each support limb outgrowth in the absence of the AER (Cohn et al., 1995; Crossley et al., 1996; Fallon et al., 1994). FGF2 maintains ZPA signaling in mouse limb microdissectate cultures (Anderson et al., 1993). Similarly, FGF8 can induce and maintain SHH expression in the absence of the AER (Crossley et al., 1996). FGF2, FGF4 and FGF8

display high levels of mitogenic activity when bound to the mesenchymally expressed FGF receptor isoforms 1c and 2c in vitro (Ornitz et al., 1996).

It is extremely unlikely that the normal skeletal and molecular phenotypes of the conditional mutants result from early or persistent *Fgf4* gene function. We used several different methods to prove that *Fgf4* was already disrupted in the forelimb AER of conditional mutants prior to the time that it is normally expressed in this structure: we did not detect *Fgf4* transcripts in the forelimb AER of conditional mutants at E10.0-11.5; AP activity, which requires Cre-mediated deletion of all *Fgf4-coding* sequences from the $F4^{AP}$ conditional allele, is detectable throughout the forelimb AER by E10.0, indicating that not only has recombination of the allele occurred by this stage, but also transcription and translation of the AP reporter. Furthermore, we have confirmed that Cre is expressed in the limb bud ectoderm of E9.5 embryos by detecting AP expression from an allele of *Fgf8* that expresses the AP gene under the control of the *Fgf8* promoter (which is expressed this early in the ventral ectoderm and developing AER) after Cre-mediated recombination of the allele. The limb phenotype in these *Fgf8* conditional mutants is obvious at E10.0. These findings demonstrate that Cre is expressed and active in the ectoderm of the limb bud prior to formation of the AER and *prior to* the onset of normal *Fgf4* expression in the AER. (A. M. M. and M. R. C., unpublished results). In summary, our data confirm that *RARCre* is active sufficiently early and in the appropriate location to recombine $F4^{AP}$ prior to the onset of *Fgf4* expression in the limb and to ensure that the conditional knockout of *Fgf4* is both timely and complete in the forelimb AER.

An important feature of the conditional mutagenesis system that we have described is that it provides a means for evaluating the extent of Cre-mediated recombination in the relevant cells. Since the marker (AP) is expressed under the control of the *Fgf4* promoter only after Cre-mediated disruption of *Fgf4*, labeling is limited to the subset of cells that express *Fgf4* and have a recombined allele. This provides an independent and extremely sensitive assay for the extent of Cre-mediated recombination, which is critical to proper evaluation of the phenotypic consequences.

The power of conditional mutagenesis may not be restricted to modulating a single locus at a time; it should be possible to conditionally disrupt multiple genes simultaneously. Our experience suggests that use of the conditional allele in a null background was not necessary; if Cre is present in a cell, it appears to operate to completion. Recombination between loxP sites at homologous positions on homologous chromosomes also does not appear to be problematic. Thus, simultaneous conditional elimination of more than one *Fgf* in the AER may allow us to examine the potential for functional redundancy among these factors and thus to determine their relative contribution to normal AER activity and limb development.

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