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Tbx4 is not required for hindlimb identity or post-bud hindlimb outgrowth

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Tbx4 is a crucial gene in the initiation of hindlimb development and has been reported as a determinant of hindlimb identity and a presumptive direct regulator of *Fgf10* in the limb. Using a conditional allele of *Tbx4*, we have ablated *Tbx4* function before and after limb initiation. Ablation of *Tbx4* before expression in the hindlimb field confirms its requirement for limb bud outgrowth. However, ablation of *Tbx4* shortly after onset of expression in the hindlimb field, during limb bud formation, alters neither limb outgrowth nor expression of *Fgf10*. Instead, post-limb-initiation loss of *Tbx4* results in reduction of limb core tissue and hypoplasia of proximal skeletal elements. Loss of *Tbx4* during later limb outgrowth produces no limb defects, revealing a brief developmental requirement for *Tbx4* function. Despite evidence from ectopic expression studies, our work establishes that loss of *Tbx4* has no effect on hindlimb identity as assessed by morphology or molecular markers.

KEY WORDS: Tbx4, Hindlimb, Limb, T-box, Tbx5, Fgf10, Mouse

INTRODUCTION

Vertebrate limbs consist of paired appendages that emerge from lateral plate mesoderm during embryonic development. The outgrowth of the limb along the proximodistal axis is largely governed by reciprocal fibroblast growth factor (FGF) signaling between the limb mesenchyme and the apical ectodermal ridge (AER), a ridge of specialized epithelium extending along the dorsoventral boundary of the limb apex (Capdevila and Izpisua-Belmonte, 2001; Niswander, 2003). Limb budding is initiated by the expression of *Fgf10* in the limb field mesenchyme, and mutation of *Fgf10* ablates limb initiation (Min et al., 1998; Ohuchi et al., 1997; Sekine et al., 1999). In response to mesenchymal *Fgf10* signaling, the AER upregulates multiple partially redundant FGF genes, *Fgf8* and *Fgf4* being of central importance. Disruption of this mesenchymal-epithelial feedback loop, either by genetic manipulation or physical removal of the AER, results in loss of most limb-specific markers and drastic truncation of the limb. During the process of distal outgrowth, the limb is patterned along the anteroposterior axis by a mesenchymal posterior signaling center called the zone of polarizing activity (ZPA). *Shh* has been shown to be both necessary and sufficient for ZPA activity. The AER and ZPA function similarly in both forelimb and hindlimb and little is known about the regulatory differences that produce the morphological differences between, for instance, the wings and legs of a chicken or the arms and legs of a human. Candidate transcription factors that could coordinate morphogenetic differences throughout limb development have been sought, and attention has centered on the T-box genes *Tbx4* and *Tbx5*.

The T-box family is an evolutionarily ancient family of transcription factors characterized by a shared DNA-binding domain. Several T-box genes are expressed in the limb (Naiche et al., 2005), and heterozygous mutations in *TBX3*, *TBX4* and *TBX5* cause limb defects in humans (Bamshad et al., 1997; Basson et al., 1997; Bongers et al., 2004). In mouse, all of the Tbx2 subfamily (*Tbx2*, *Tbx3*, *Tbx4* and *Tbx5*) have been shown to play roles in limb development (Agarwal et al., 2003; Davenport et al., 2003; Harrelson et al., 2004; Naiche and Papaioannou, 2003). *Tbx5* is expressed specifically in the forelimb, while *Tbx4*, a closely related gene, is expressed in the hindlimb. Both genes are expressed in their respective limb fields well before the morphological appearance of the limb bud and continue to be expressed throughout the limb mesenchyme through late gestation (Gibson-Brown et al., 1996). Due to this differential and early expression, it has been proposed that *Tbx4* and *Tbx5* play central roles in creating the differences between forelimbs and hindlimbs, and several experiments in chick have suggested that ectopic expression of these genes can transform limb fates (Rodriguez-Esteban et al., 1999; Takeuchi et al., 1999; Takeuchi et al., 2003). However, these experiments were complicated by the expression of endogenous *Tbx4* and *Tbx5*. More recent work in which *Tbx5* was replaced with *Tbx4* has suggested that *Tbx4* and *Tbx5* can substitute for each other and serve little or no role in establishing the differences between limbs (Minguillon et al., 2005).

Tbx5 plays a crucial role in the initiation of the forelimb bud. Null alleles of *Tbx5* in mouse and zebrafish result in embryos that show no forelimb bud formation and do not express *Fgf10* in the forelimb field (Agarwal et al., 2003; Garrity et al., 2002; Ng et al., 2002; Rallis et al., 2003). Several lines of evidence have been used to show that *Tbx5* mediates limb outgrowth through direct transcriptional regulation of *Fgf10* (Agarwal et al., 2003; Ng et al., 2002), and experiments with a presumed dominant negative allele of *Tbx5* suggest that loss of this gene during limb development truncates limb outgrowth (Rallis et al., 2003). The high conservation, similar expression pattern and functional redundancy between *Tbx4* and *Tbx5* strongly suggest that *Tbx4* would operate similarly in the hindlimb. However, our previous work has shown that null mutations in *Tbx4* produce a slightly different limb phenotype than *Tbx5* null mutations, in that *Fgf10* expression is initiated in the hindlimb field of *Tbx4* mutant embryos and a morphological bud is formed, but the hindlimb bud does not maintain *Fgf10* expression or grow in explant cultures (Naiche and Papaioannou, 2003). However, embryonic lethality of the *Tbx4* null mutation due to failure of chorioallantoic fusion prevented our examining limb outgrowth in vivo.

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In this study we exploit a conditional allele of *Tbx4* to circumvent allantois failure and extend our data on the phenotype of *Tbx4* null hindlimbs. As *Tbx4* and *Tbx5* have subtly different roles in limb bud initiation, we investigated whether loss of *Tbx4* during later limb development also results in limb truncation, similar to the dominant-negative *Tbx5*, by ablating *Tbx4* gene function at multiple stages of limb development. Our findings indicate that early expression of *Tbx4* is required for maintaining proximal and medial limb tissue, but does not solely regulate *Fgf10* or limb outgrowth. Examination of hindlimbs that had lost *Tbx4* function revealed no role for *Tbx4* in hindlimb identity.

MATERIALS AND METHODS

Generation of *Tbx4* conditional allele

Mice carrying the $Tbx4^{tm1Pa}$ allele containing three *loxP* sites (Naiche and Papaioannou, 2003) were mated with *EIIa-cre* transgenic mice to generate germline mosaic males with all possible recombination products (Xu et al., 2001), which were outcrossed to isolate the conditional allele. Routine genotyping was performed using primer A: GAGGATGTTCCCCAGCTAC and primer B: CAGTCTGAGAGGGTCAGACTC (Fig. 1A).

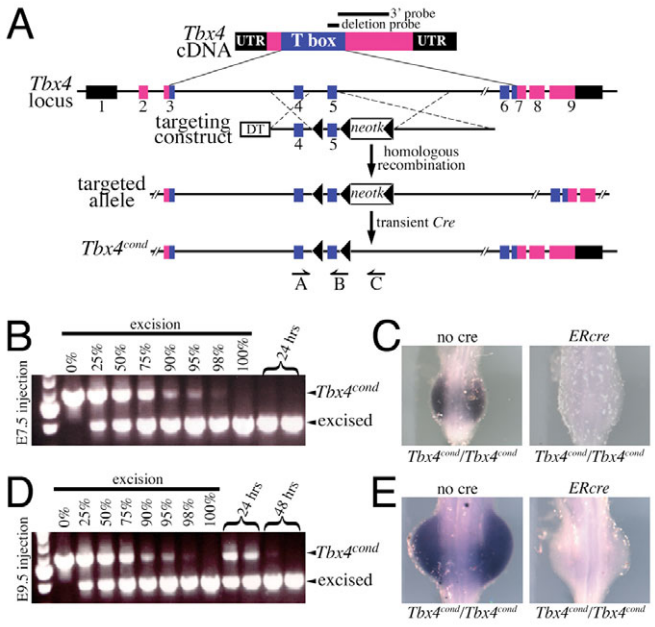

Fig. 1. Creation and excision characteristics of the $Tbx4^{cond}$ allele. (**A**) A *loxP* site and a *loxP*-flanked selection cassette were inserted into the introns surrounding exon 5 (Naiche and Papaioannou, 2003). The selection cassette was subsequently excised via Cre recombination in vivo (see Materials and methods). Numbered boxes, exons; DT, diptheria toxin gene; neotk, neomycin resistance and thymidine kinase selection cassette; triangles, *loxP* sites; half-arrows, genotyping primers. (**B**) Semi-quantitative PCR assessment of $Tbx4^{cond}$ in embryos administered tamoxifen at E7.5 shows no intact $Tbx4^{cond}$ after 24 hours. Embryos (two representative samples) are compared with mixed DNA samples of known composition. (**C**) Dorsal view of the hindlimb field of early E10.5 embryos given tamoxifen at E7.5, hybridized with a probe specific to the deleted region of *Tbx4* (deletion probe in A). No expression is detected in embryos carrying *ERcre*. (**D**) As in B, with embryos administered tamoxifen at E9.5 and recovered after 24 or 48 hours (two representative embryos from each time point). $Tbx4^{cond}$ is roughly 75-90% excised after 24 hours, and 98-100% excised after 48 hours. (**E**) No expression of *Tbx4* is detected in E10.5 embryos carrying *ERcre* and given tamoxifen at E9.5, using the same probe as in C.

Mouse strains, mating and embryo collection

The $Rosa\text{-}ERcre^{T2}$ (de Luca et al., 2005) and *Prx-cre* (Logan et al., 2002) lines were the kind gifts of Thomas Ludwig and Cliff Tabin, respectively. The *EIIa-cre* line was obtained from Jackson Labs (strain #003314). All mice were kept on outbred backgrounds. The dark period was 19.00 to 05.00 h and noon on the day a mating plug was observed was identified as embryonic day (E) 0.5. Yolk sacs were used for genotyping.

ERcre induction via tamoxifen

Tamoxifen (Sigma #T5648, 20 mg/ml in sunflower oil) was administered via intraperitoneal injection. At E7.5, 6 mg (approximately 0.18 mg/g body weight) was administered between 17.30 and 23.30 h. At E9.5 and later time points, 7 mg was administered between 17.30 and 19.30 h.

Embryo genotyping

Embryos from $Tbx4^{cond/cond}$; *Rosa-ERcre* matings were examined for the recombination of $Tbx4^{cond}$ into $Tbx4^{tm1.1Pa}$ (the null allele) using primers A and B (above) in combination with the excision-specific primer C: TCATCTAGGCTTCACAGCC. For *Prx-cre* crosses, all embryos were genotyped for wild type, $Tbx4^{tm1.1Pa}$, and $Tbx4^{cond}$ alleles using primers A, B and C as well as for the presence of *Prx-cre* (using primers CGATGCAACGAGTGATGAGG and GCATTGCTGTCACTTGGTCGT). Quantitation of alleles in Fig. 1 was done using primers A, B and C with a graded series of mixed DNA of known composition prepared identically to sample DNA.

Marker analysis

Whole-mount in situ hybridization was performed according to standard protocols (Wilkinson and Nieto, 1993). Two to five embryos were used for each marker and stage. Alcian Blue and Alcian Blue/Alizarin Red skeletal preparations were performed according to standard protocols (Nagy et al., 2003), with the modification that Alcian Blue stain in the latter protocol was prepared at 150 mg/l in 80% ethanol, 20% acetic acid.

Limb measurements and cell counts

Limb widths were determined by photographing the dorsal aspect of each limb and measuring across the widest part of the limb perpendicular to the proximodistal axis. Cell counts were obtained by dissecting off limbs, dissociating the tissue with 2 μg/ml collagenase for 1 hour at 37°C and counting cells using a hemacytometer. The mitotic index of the progress zone was computed by staining sections with anti-phosphohistone H3 (Upstate), counterstaining with Nuclear Fast Red and counting nuclei in the area 150 μm subjacent to the AER in the center of the limb. Cell death was assessed by incubating live embryos in 5 μmol/l Lysotracker Red (Invitrogen L7528) in Hank's balanced salt solution for 30 minutes at 37°C.

RESULTS

Creation and excision of the *Tbx4* conditional allele

A *Tbx4* conditional allele, $Tbx4^{tm1.2Pa}$, was generated by excision of the selection cassette from the $Tbx4^{tm1Pa}$ allele (Naiche and Papaioannou, 2003), leaving intronic *loxP* sites flanking exon 5 (Fig. 1A). Our previous work has demonstrated that removal of exon 5 produces a null allele (Naiche and Papaioannou, 2003). Animals homozygous for the conditional allele, hereafter referred to as $Tbx4^{cond}$, were viable and fertile and showed no phenotype. Homozygous $Tbx4^{cond}$ females were mated to homozygous $Tbx4^{cond}$ males that also carried an optimized tamoxifen-inducible *cre* gene expressed ubiquitously under the *Rosa26* locus, $Rosa\text{-}ERcre^{T2}$ (de Luca et al., 2005). Pregnant females were administered a single injection of tamoxifen at various stages between E6.5 and 11.5. When females were injected at E7.5, embryonic excision of $Tbx4^{cond}$ was complete within 24 hours (Fig. 1B), and a probe directed against the deleted region showed no intact *Tbx4* transcript in the hindlimb field (Fig. 1C). When females were injected at E9.5, embryos showed 70-95% excision of $Tbx4^{cond}$ within 24 hours, greater than 98% excision after 48 hours (Fig. 1D), and complete excision at later

time points (not shown). Despite incomplete excision at the DNA level, *Tbx4* transcripts were undetectable by in situ hybridization after 24 hours (Fig. 1E).

Ablation of *Tbx4* before hindlimb development

In experiments with the *Rosa-ERcre* transgene, we compared $Tbx4^{cond/cond}$ embryos (control) to $Tbx4^{cond/cond}$; *Rosa-ERcre/+* embryos (hereafter referred to as *ERcre* embryos) to ensure that the effects we saw were not due to either the $Tbx4^{cond}$ allele or to tamoxifen administration. During these experiments, we noted that Cre activity from the *Rosa-ERcre* caused apoptosis and fetal lethality, which will be described elsewhere, so we also examined $Tbx4^{cond/+}$; *Rosa-ERcre/+* embryos to ensure that the observed phenotypes were due to the loss of *Tbx4* rather than the effects of Cre activity. To first verify that excision of $Tbx4^{cond}$ produced the expected phenotype, we administered tamoxifen at E6.5, approximately 24 hours before expression of *Tbx4* is seen in any tissue. *ERcre* embryos (n=19) were indistinguishable from *Tbx4* null embryos and died at E10.5 due to failure of chorioallantoic fusion (data not shown).

We then administered tamoxifen at E7.5 to excise *Tbx4* after the initial formation of the allantois, but before expression appeared in the hindlimb field. Tamoxifen injections at E7.5 resulted in complete excision of *Tbx4* within 24 hours (Fig. 1B), well before hindlimb expression appeared at E9.5. Approximately half of the *ERcre* embryos recapitulated the null phenotype, while the remaining *ERcre* embryos underwent allantoic fusion and survived past E10.5. In these embryos, a visible limb bud was formed but was degenerating by E11.5 (Fig. 2A,B). Early limb markers in *ERcre* embryos injected with tamoxifen at E7.5 were identical to those of *Tbx4* null embryos: at E10.0 *Fgf10* was weakly expressed in the hindlimb field (Fig. 2C,D) and sporadic *Fgf8* was seen in the AER (Fig. 2E,F), but *Fgf10* was lost from the hindlimb bud by E10.5 (Fig. 2G,H).

Survival of some *ERcre* embryos given tamoxifen at E7.5 allowed us to examine patterning of *Tbx4*-ablated hindlimbs at later stages. The posterior determinant *dHand* (*Hand2* – Mouse Genome Informatics), which is upstream of *Shh* in the very early limb bud, is normally expressed in the posterior of the hindlimb bud at E10.5, but was diffusely expressed across the ventral margin of the entire *ERcre*

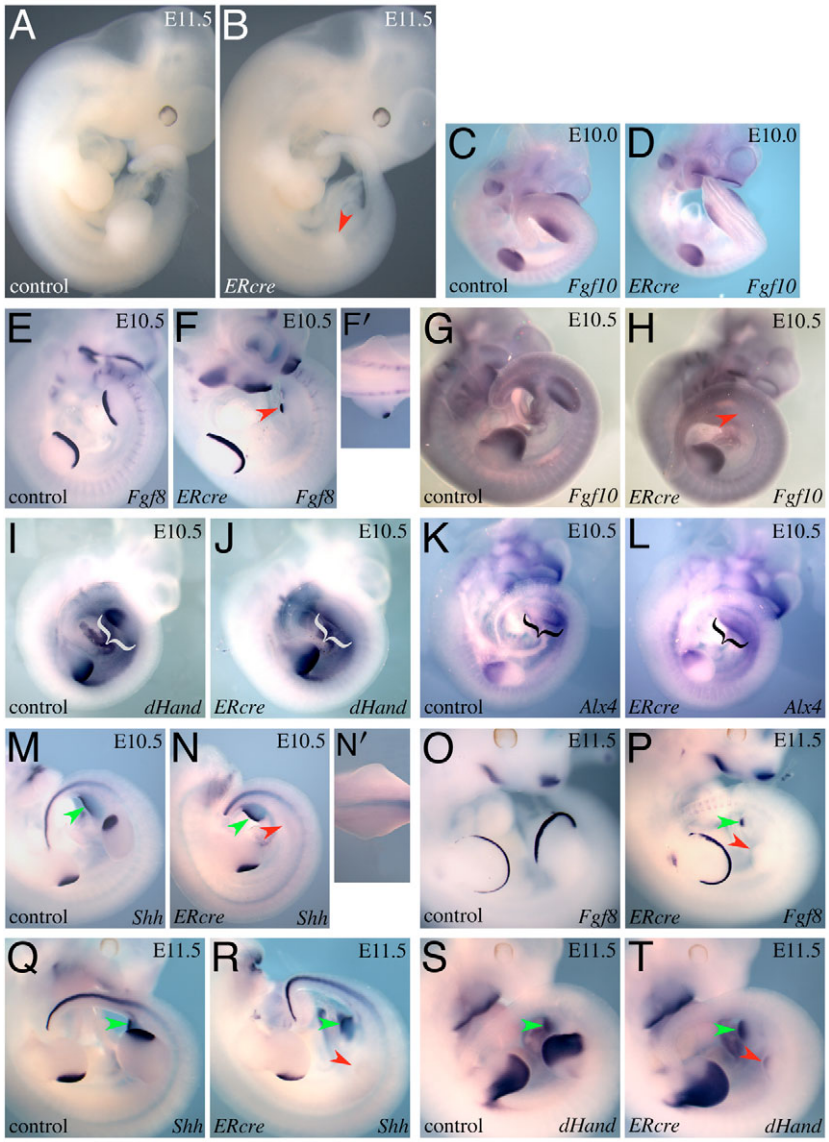

Fig. 2. Phenotype of $Tbx4^{cond/cond}$ embryos administered tamoxifen at E7.5. (**A**,**B**) In *ERcre* embryos, hindlimb development fails at E11.5. (**C**,**D**) *Fgf10* is present at low levels in the hindlimb field of E10.0 *ERcre* embryos. (**E,F**) *Fgf8* is sporadically activated in the AER of *ERcre* embryos. (**F′**) Dorsal view of hindlimb in F. (**G**,**H**) *Fgf10* is normally expressed throughout the hindlimb bud at E10.5, but has been lost from E10.5 *ERcre* hindlimb buds. (**I**,**J**) *dHand* is confined to the posterior third of the hindlimb bud in control embryos but is expanded across the entire ventral margin of E10.5 *ERcre* hindlimb buds. Brackets indicate margins of the hindlimb bud. (**K**,**L**) The anterior hindlimb bud expression of *Alx4* is expanded across the hindlimb bud in E10.5 *ERcre* embryos. (**M**,**N**) *Shh* is not observed in the *ERcre* hindlimb. (**N′**) Dorsal view of hindlimb buds of embryo in N. (**O-T**) At E11.5 *Fgf8*, *Shh* and *dHand* are expressed in AER, posterior margin and posterior mesenchyme domains, respectively, in control embryos (O,Q,S), but have been lost from *ERcre* hindlimbs by this stage (P,R,T). Red arrowheads indicate distal tip of hindlimb bud. Green arrowheads indicate proctodeum. All panels to same scale.

hindlimb (Fig. 2I,J). Conversely, the expression domain of *Alx4*, which normally marks the anterior of the limb bud, was also expanded across the entire hindlimb bud in *ERcre* embryos at E10.5 (Fig. 2K,L). In more advanced E10.5 embryos, *Shh* appeared in the posterior hindlimb, but it was not observed in the *ERcre* hindlimbs (Fig. 2M,N). By E11.5 all limb markers examined, including *Fgf8*, *Shh*, *dHand*, *Fgf10* and *Alx4*, had disappeared from the *ERcre* hindlimb (Fig. 2O-T and data not shown). This confirms that *Tbx4* expression in the hindlimb field is required for hindlimb outgrowth in vivo as well as in vitro. This also demonstrates that the *Tbx4*^{cond} allele in combination with *ERcre* can successfully be used to bypass early lethality caused by the effect on the allantois while still producing null phenotypes in later tissues.

Tbx4 excision during hindlimb outgrowth

Tbx4 is expressed broadly throughout E9.5-12.5 hindlimbs and then progressively distally restricted (Gibson-Brown et al., 1996), in a similar fashion to its putative target *Fgf10*. To test the requirement for *Tbx4* in post-bud hindlimb development and outgrowth, we ablated the gene during stages at which it is widely expressed.

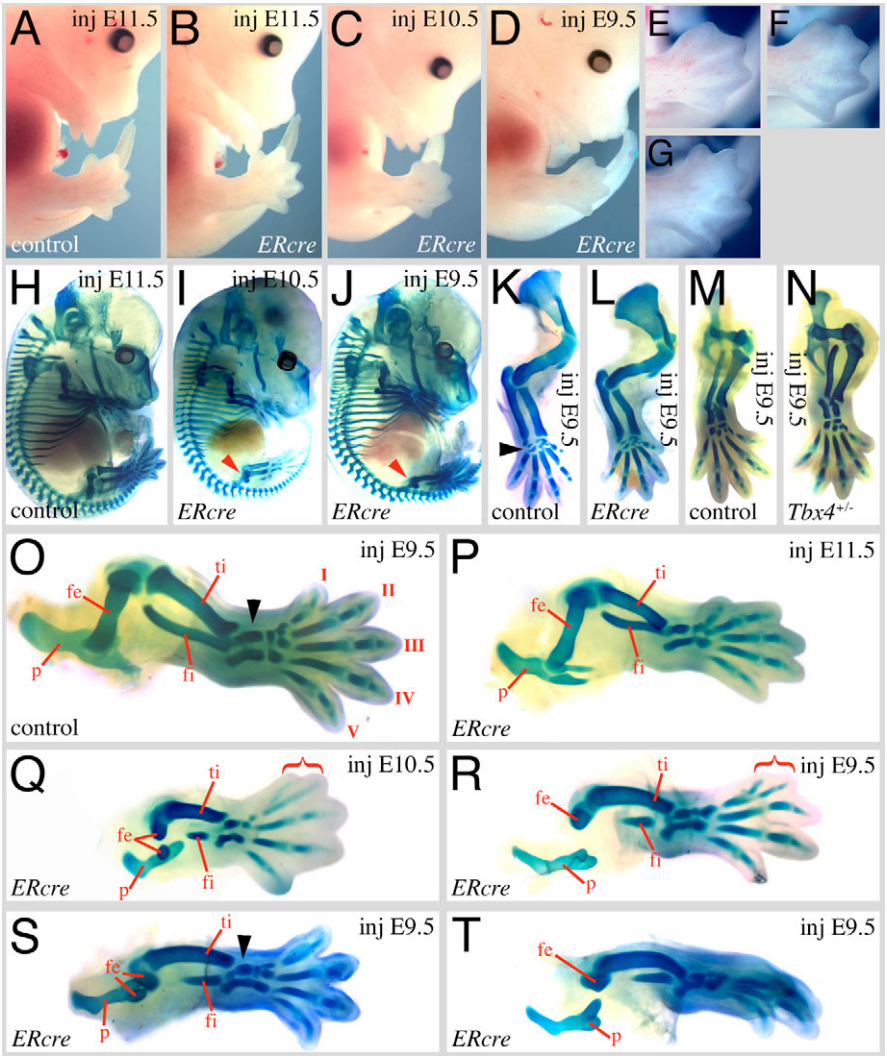

Fig. 3. Phenotype of E14.5 *Tbx4*$^{cond/cond}$ embryos administered tamoxifen at E11.5, 10.5 or 9.5. (**A-D**) Left hindlimbs have been removed for clarity. (A) E14.5 control embryos have five distinct hindlimb digits. (B) *ERcre* embryos injected with tamoxifen at E11.5 resemble controls. (C) *ERcre* embryos injected at E10.5 show soft-tissue fusion of anterior hindlimb digits. (D) *ERcre* embryos given tamoxifen at E9.5 have only four hindlimb digits. (**E-G**) Hindlimbs of additional *ERcre* embryos injected at E9.5, suffering varying degrees of anterior digit fusion. (**H-T**) Cartilage staining of embryos as in A-D. (H-J) Skeletal development is normal overall, but *ERcre* embryos injected at E10.5 and 9.5 have aberrant hip attachments (red arrowheads). (K,L) Forelimbs of *ERcre* embryos given tamoxifen at E9.5 are normal. (M,N) Hindlimbs of embryos carrying the *Rosa-ERcre* allele but heterozygous for *Tbx4* are normal after injection at E9.5. (O-T) Isolated hindlimbs with anterior oriented toward top, except T, which has dorsal oriented toward top. (O,P) Hindlimbs from control embryo and *ERcre* embryo injected at E11.5 have well-developed skeletal elements and five separated digits emerging from the ankle bones. The limb in P is tilted relative to O. (Q) Hindlimb from *ERcre* embryo injected at E10.5 shows a hypoplastic fibula, hypoplastic and discontinuous femur, hypoplastic pelvis, and thin anterior digits (bracket). (R) Hindlimb from *ERcre* embryo administered tamoxifen at E9.5, showing the same defects as in Q. (S) More severely affected hindlimb from an *ERcre* embryo injected at E9.5 with complete anterior digit fusion. (T) Side view of hindlimb in S shows no articulation between the remnants of the femur and the pelvis. I-V, digit identities from anterior to posterior; fe, femur; fi, fibula; p, pelvis; ti, tibia. Black arrowheads indicate carpal bones (forelimb) or the talus and calcaneus bones (hindlimb).

Tamoxifen injection at E9.5, 10.5 and 11.5 did not produce limb truncations in E14.5 embryos, although the hindlimbs of *ERcre* embryos injected at E9.5 and 10.5 were slightly shorter (Fig. 3A-D). Anterior digit fusion was seen in some *ERcre* embryos from E10.5 injections (42%, *n*=24) (Fig. 3C). Anterior digits were abnormal in all *ERcre* embryos injected at E9.5, with 67% having four symmetrical digits and 33% showing fusion of anterior digits (*n*=88) (Fig. 3D-G).

Cartilage stains were done on E14.5 embryos from tamoxifen injections at all of the above stages (Fig. 3H-J). Abnormal pelvic connections were seen in *ERcre* embryos from E10.5 and 9.5 tamoxifen injections (Fig. 3I,J). No defects were observed in the forelimbs of any embryos (Fig. 3K,L) or in hindlimbs of embryos injected at E11.5 (data not shown). Cre-expressing *Tbx4* heterozygous embryos given tamoxifen at E9.5 developed hindlimbs identical to control embryos, demonstrating that Cre activity produced no hindlimb defects when an intact copy of *Tbx4* was present (Fig. 3M,N). However, hindlimbs from *ERcre* embryos injected at E10.5 and 9.5 had hypoplastic pelvises and fibulas, aplastic or severely hypoplastic femurs, which did not articulate with the pelvis, and abnormal anterior digits (Fig. 3O-T). In some *ERcre* embryos from E10.5 injections digit formation was normal, but in some of these embryos the hindlimb digits I and II were thinner than controls and the metatarsal of the first digit appeared to originate near the middle of the metatarsal of digit II instead of near the tarsal bones (Fig. 3Q). The autopod of some *ERcre* embryos injected at E9.5 had thin and partially fused digits I and II (Fig. 3R), similar to E10.5 injections, while others had four-digit autopods, representing either a complete fusion between digits I and II or the loss of digit II (Fig. 3S). In some embryos, soft-tissue fusion appeared to have occurred between digits II and III (Fig. 3G and data not shown), but no fusion between the skeletal elements of digits II and III were ever observed. Despite the obvious abnormalities, all skeletal elements formed in *ERcre* embryos injected at E9.5, including the most distal phalanges, revealing no outgrowth requirement for *Tbx4*.

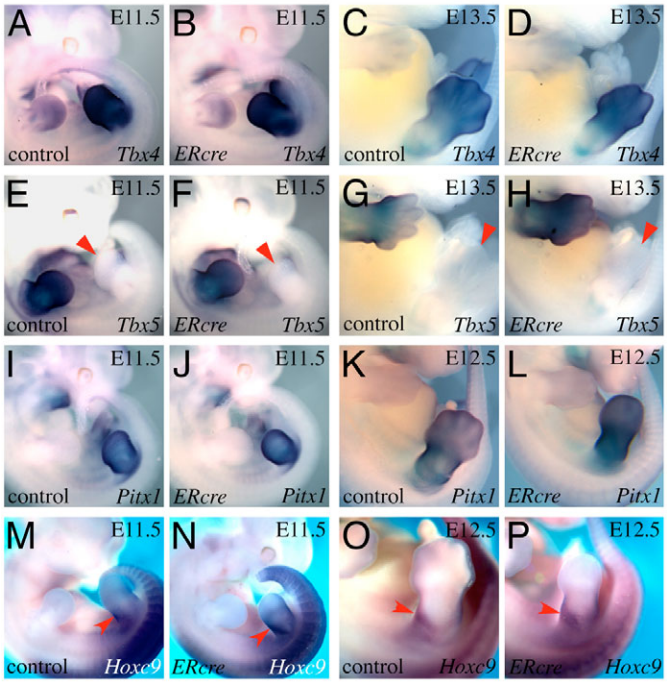

Fig. 4. Limb identity in the hindlimbs of *Tbx4*$^{cond/cond}$ embryos injected with tamoxifen at E9.5 and recovered at indicated stage. (**A-D**) Expression of *Tbx4*, monitored by a probe 3′ of the deleted region (3′ probe in Fig. 1A), is normal in *ERcre* hindlimbs. (**E-H**) *Tbx5* is expressed in the forelimb and excluded from the hindlimb (red arrowheads) in control and *ERcre* embryos. *Pitx1* (**I-L**) and *Hoxc9* (**M-P**) are expressed normally in the hindlimb (red arrowheads) of control and *ERcre* embryos.

Hindlimb identity in *Tbx4*-ablated hindlimbs

Our previous work with the null allele showed that *Tbx4* is not required for the initial expression of hindlimb identity markers at E10.5, but we could not evaluate the maintenance of hindlimb identity in the absence of *Tbx4* due to the failure of hindlimb outgrowth. Using *Tbx4*^{cond} we examined hindlimb identity in *ERcre* embryos injected with tamoxifen at E9.5.

In normal E14.5 embryos, all forelimb skeletal elements reside in the same plane (Fig. 3K), whereas hindlimb skeletal elements rotate such that the femur were nearly perpendicular to the plane of the tibia and footplate (Fig. 3O). In *ERcre* embryos, the relative positions of the pelvis and distal limb elements clearly indicated a hindlimb-like orientation (Fig. 3S,T). Additionally, the carpal bones of the normal forelimb are short (Fig. 3K), while the homologous tarsals in the normal hindlimb form two noticeably longer bones, the talus and the calcaneus. *ERcre* hindlimbs showed evident formation of the talus and calcaneus (black arrowheads in Fig. 3O,S).

Tbx4 itself is a marker of hindlimb identity, so the expression of this gene was examined using a probe 3′ of the deleted region (3′ probe, Fig. 1A). *Tbx4* was expressed robustly throughout the hindlimbs of *ERcre* embryos at all stages examined (Fig. 4A-D and data not shown). *Tbx5* is a marker of forelimb identity, and some evidence suggests that *Tbx4* and *Tbx5* function antagonistically to exclude each other from their respective limbs (Takeuchi et al., 1999). However, *Tbx5* was maintained exclusively in the forelimb of *ERcre* embryos at least 3 days after *Tbx4* function had been lost (Fig. 4E-H). Recent evidence suggests that *Pitx1* is a major determinant of hindlimb identity (Logan and Tabin, 1999; Minguillon et al., 2005), and this gene was maintained in *ERcre* hindlimbs at all stages examined (Fig. 4I-L). A more downstream reporter of hindlimb fate, *Hoxc9*, was also maintained in embryos that had lost *Tbx4* gene function (Fig. 4M-P). By both molecular and morphological markers, we found no evidence for a role for *Tbx4* in determining hindlimb identity.

Limb patterning in *Tbx4*-ablated hindlimbs

Our data show that *Tbx4* plays a major role in formation of the skeletal elements of the limb, and both anterior and posterior skeletal elements (digits and fibula, respectively) were missing from E14.5 *ERcre* embryos injected with tamoxifen at E9.5. We therefore investigated earlier limb patterning in *ERcre* embryos injected at E9.5 to determine the source of these defects.

The timing of the appearance and growth of the hindlimb buds at E10.5 did not vary between control and *ERcre* embryos (data not shown). To determine the presence of the ZPA, we first examined the expression of *Shh* in the limb. *Shh* was expressed in posterior mesenchyme of E10.5 and 11.5 hindlimbs and was not appreciably different in *ERcre* embryos (Fig. 5A-B′). To observe the effects of *Shh* signaling, we examined the expression of *Ptc* (*Ptch1* – Mouse Genome Informatics), a *Shh* response gene. *Ptc* appeared in its normal domain in both E10.5 and 11.5 *ERcre* hindlimbs, but by E11.5 this domain encompassed a larger proportion of the hindlimb (Fig. 5C-D′). We also examined *dHand*, which is both required for

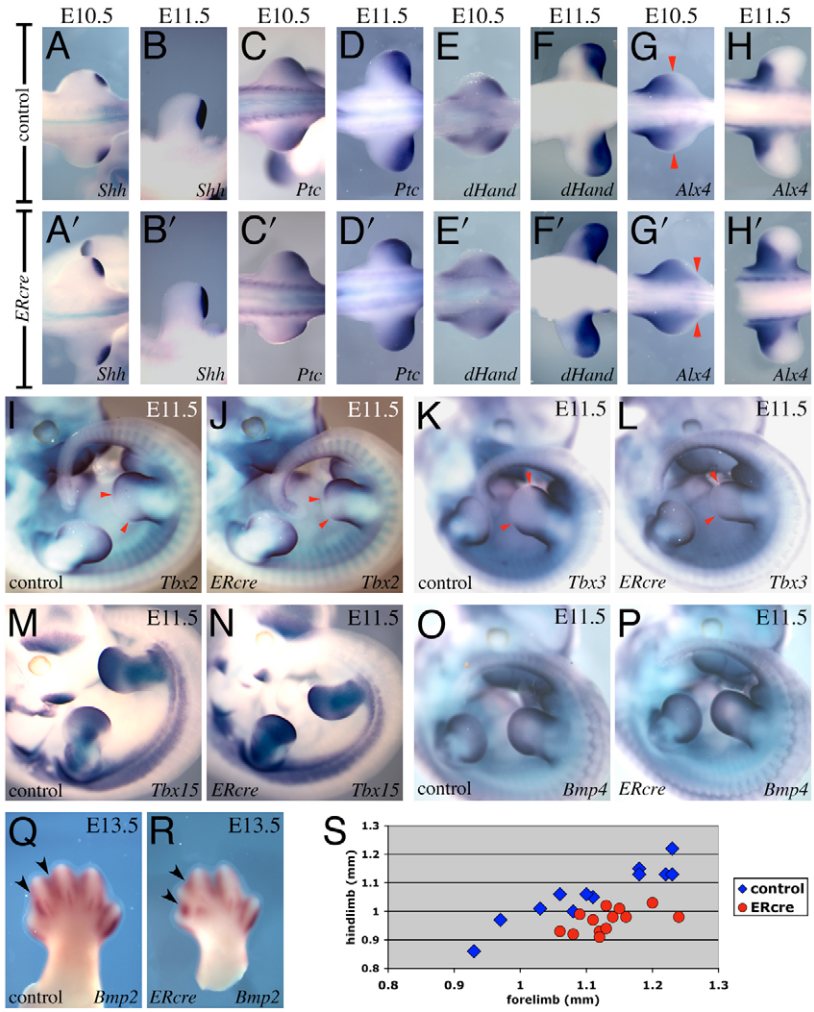

Fig. 5. Expression of limb patterning genes in *Tbx4*$^{cond/cond}$ embryos injected with tamoxifen at E9.5 and recovered at indicated stage. Posterior is to the right. (**A-H′**) Dorsal views of hindlimbs. (A-B′) *Shh* is expressed in the posterior margin of both control and *ERcre* hindlimb at E10.5 and 11.5. (C-D′) *Ptc* is expressed in similarly sized domains in the posterior of control and *ERcre* hindlimbs, but this domain takes up a larger proportion of the smaller *ERcre* hindlimb at E11.5. (E-F′) *dHand* is expressed along the posterior margin of both control and *ERcre* hindlimbs, but, as with *Ptc*, this domain takes up more of the *ERcre* limb at E11.5. (G-H′) *Alx4* is expressed in the anterior of control hindlimb buds but is aberrantly upregulated in the posterior limb bud in *ERcre* embryos. Red arrowheads indicate anteroposterior limits of expression domains. (**I-L**) *Tbx2* and *Tbx3* mark anterior and posterior hindlimb margins, but the gap between these margins, indicated by red arrows, is smaller in *ERcre* embryos. (**M**,**N**) *Tbx15* is expressed in the center of the limb in control embryos and this domain is not appreciably thinner in *ERcre* embryos. (**O**,**P**) *Bmp4* is expressed in the periphery of control and *ERcre* hindlimbs. The AER has been removed. (**Q**,**R**) At E13.5 *Bmp2* is expressed in control and *ERcre* hindlimb interdigital regions but is restricted to a smaller and more distal domain in *ERcre* anterior digits (black arrowheads). (**S**) Scatter plot of width of hindlimbs and forelimbs of E11.5 control (blue) and *ERcre* (red) embryos at widest point.

and dependent on *Shh* signaling, and found a similar result, with normal expression in E10.5 *ERcre* embryos and a normal-sized expression domain in E11.5 *ERcre* embryos, but with that domain encompassing more of the hindlimb (Fig. 5E-F′). The expression domains of *Ptc* and *dHand* at E12.5 were also normally sized but comprised a greater proportion of the hindlimb in *ERcre* embryos than in controls (data not shown).

Having observed an apparent expansion of the posterior limb domain, we also examined anterior patterning. *Alx4* marks the anterior half of the E10.5 hindlimb bud and is restricted to an anteroproximal domain by E11.5 (Fig. 5G,H). In *ERcre* embryos, there is a dramatic expansion of *Alx4* expression, comprising nearly the entire hindlimb in E10.5 embryos (Fig. 5G′) and a broad expanse of the proximal limb bud in E11.5 embryos (Fig. 5H′). Previous work has suggested that *Alx4* represses *Shh* expression and is repressed by *dHand* (Qu et al., 1997; te Welscher et al., 2002), but both these genes showed expression that overlapped that of *Alx4*. A second anterior marker, *Pax1*, was examined and found to be normal in E10.5 *ERcre* hindlimbs (data not shown).

As both anterior and posterior limb markers are present in *ERcre* hindlimbs, we examined the limb margins using *Tbx2* and *Tbx3*. Both these genes were expressed normally in E10.5 *ERcre* embryos (data not shown). At E11.5, *Tbx2* and *Tbx3* were expressed in both the anterior and posterior margin of the limb mesenchyme of control embryos (Fig. 5I,K). In *ERcre* hindlimbs, these expression domains were present and normally sized, but the space between them was obviously narrower, suggesting that there is less tissue in the medial core of the limb (Fig. 5J,L). This was confirmed by cell counts, which showed that the hindlimbs of *ERcre* embryos contained significantly fewer cells ($2.8\pm1.0\times10^6$, n=12) than control hindlimbs ($4.3\pm1.1\times10^6$, n=13) with a confidence interval of P<0.015, while forelimbs showed no significant difference ($4.2\pm1.0\times10^6$ and $5.2\pm1.2\times10^6$ cells, respectively). In addition, forelimb width was similar between E11.5 control and *ERcre* embryos, but the ratio of hindlimb to forelimb width was significantly lower in *ERcre* embryos, with a confidence interval of P<0.0001 (Fig. 5S). This loss of limb core tissue explains the apparent expansion of *Shh* responsive genes across the limb, as posterior signaling will reach proportionately farther across a thinner limb.

Tbx15, the loss of which has been shown to decrease proliferation in the limb core (Singh et al., 2005), was normal in *ERcre* embryos, indicating that the loss of limb core in *Tbx4* mutants occurs along a different pathway (Fig. 5N,O). Bone morphogenetic proteins (Bmps) have multiple roles in limb development, including AER maintenance, skeletal formation and apoptosis of interdigital regions (Capdevila and Izpisua-Belmonte, 2001), and Bmps are known to interact with T-box genes in several systems (Papaioannou and Goldin, 2003). Because post-bud *Tbx4* ablation causes aberrant hindlimb digit formation, we looked at both early and late Bmp expression. The *Bmp2* and *Bmp4* expression domains were normal in *ERcre* embryos at E10.5 and 11.5,

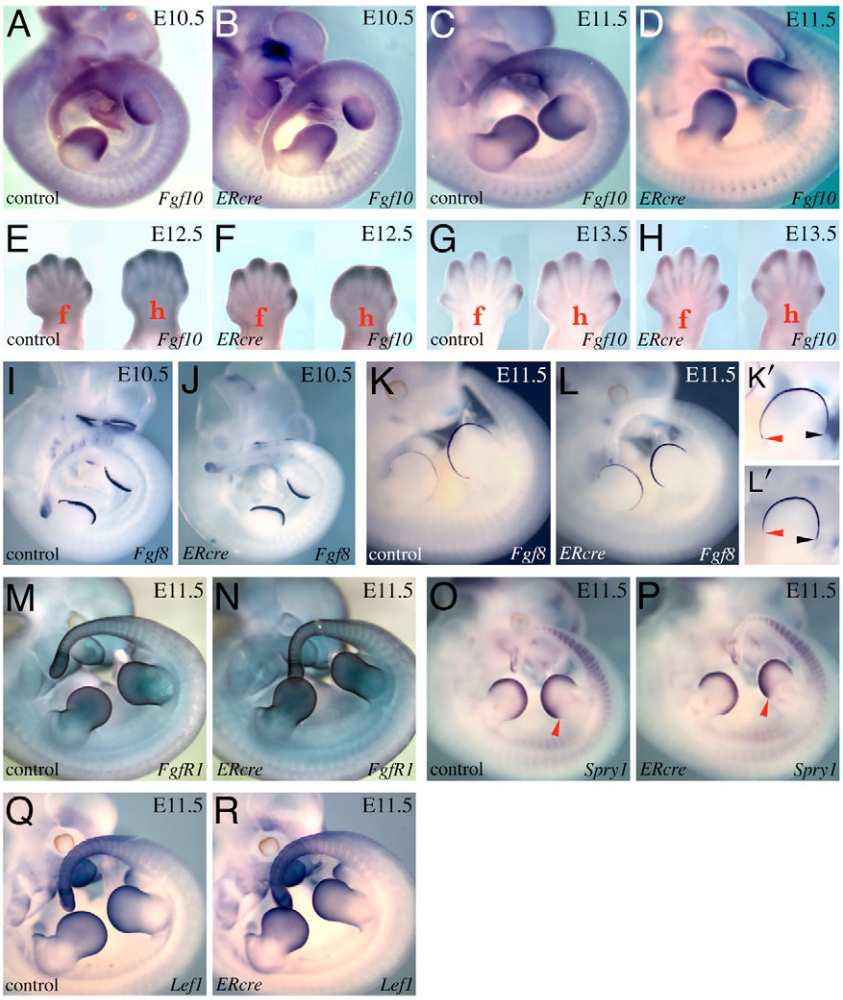

Fig. 6. Expression of FGF pathway genes in *Tbx4*$^{cond/cond}$ embryos injected with tamoxifen at E9.5 and recovered at indicated stage. Posterior is to the right in all panels. (**A-F**) *Fgf10* is expressed throughout the distal mesenchyme in control (A,C,E) and *ERcre* (B,D,F) embryos at E10.5-12.5. f, forelimb; h, hindlimb. (**G**,**H**) At E13.5 *Fgf10* is only seen in four digit tips in the *ERcre* hindlimbs. (**I-L**) *Fgf8* is expressed in the AER of both control and *ERcre* embryos. (K′) Detail of hindlimb in K. *Fgf8* expression normally extends more proximally on the anterior margin (red arrowheads) than the posterior margin (black arrowheads) of the limb bud. (L′) Detail of hindlimb in L. *Fgf8* expression domain is truncated on the anterior margin. (**M**,**N**) *FgfR1* is expressed throughout the limb mesenchyme in both control and *ERcre* embryos. (**O**,**P**) The anterior margin of *Spry1* expression (red arrowhead) underlying the AER is slightly truncated in *ERcre* embryos relative to controls. (**Q**,**R**) *Lef1* is expressed throughout the distal mesenchyme in both control and *ERcre* embryos.

although they also suffered from a loss of medial non-expressing tissue (Fig. 5O,P and data not shown). In E13.5 *ERcre* embryos, interdigital expression of *Bmp2* was distally restricted in the anterior of the hindlimb compared with control embryos, but remained present between each digit, suggesting that the digital fusions observed are due to a change in Bmp regulation rather than the absence of *Bmp2* expression (Fig. 5Q,R).

To explain the loss of limb core tissue observed with these various assays, we examined the mitotic index in control and *ERcre* hindlimbs progress zones, but found no significant differences (3.00±0.75 in controls, 2.86±0.73 in *ERcre* hindlimbs). Cell death was difficult to assess due to the heightened background of cell death caused by *Rosa-ERcre* activity.

Limb outgrowth in *Tbx4*-ablated hindlimbs

Our previous work showed a requirement for *Tbx4* in both outgrowth and maintenance of *Fgf10* expression shortly after limb initiation. Since our current evidence showed no loss of limb outgrowth after *Tbx4* ablation at a later stage, we examined *ERcre* embryos injected with tamoxifen at E9.5 for genes known to be involved in the FGF feedback loop. Despite previous indications that *Fgf10* is a direct transcriptional target of *Tbx4*, we found substantially normal expression of *Fgf10* in *ERcre* embryos at E10.5, 11.5, 12.5 and 13.5 (Fig. 6A-H). The only perturbation of *Fgf10* was associated with digit fusion at E13.5 (Fig. 6H).

Fgf8 is the first, and only non-redundant, FGF ectodermal response to *Fgf10* limb signaling and is normally expressed along the entire AER. *Fgf8* expression was present in the AER of *ERcre* embryos at E10.5 and 11.5 (Fig. 6I-L), but close examination revealed an anterior truncation of the *Fgf8* domain (Fig. 6K′-L′). This anterior loss is consistent with the anterior bias of digit loss observed in E14.5 embryos.

FGF receptor *FgfR1* is known to be key in the limb FGF signaling loop (Ciruna et al., 1997; Verheyden et al., 2005), and loss of its expression immediately after hindlimb bud formation leads to a similar phenotype to that of *Tbx4* (Li et al., 2005). Expression of *FgfR1* was nonetheless robust throughout the hindlimbs of *ERcre* embryos (Fig. 6M,N). *Spry1* is a mesenchymal response to *Fgf8* signaling and is thought to act as a negative regulator of FGF signaling. *Spry1* was observed in its normal domain immediately underlying the AER in *ERcre* hindlimbs, although that domain was anteriorly truncated, mirroring the truncated expression domain of *Fgf8* (Fig. 6O,P). Wnt signaling is thought to be part of the FGF limb feedback loop and regulated by *Tbx4* and *Tbx5* in limbs, but the precise Wnt genes involved are not known in mouse (Agarwal et al., 2003; Kawakami et al., 2001; Takeuchi et al., 2003). As a proxy, *Lef1* expression, which is activated in response to Wnt signaling, was examined. *Lef1* expression was present throughout the distal limb in both control and *ERcre* embryos. Thus, all elements of the FGF limb feedback loop examined in *ERcre* embryos injected at E9.5 were either expressed normally or with minor perturbations in the anterior hindlimb.

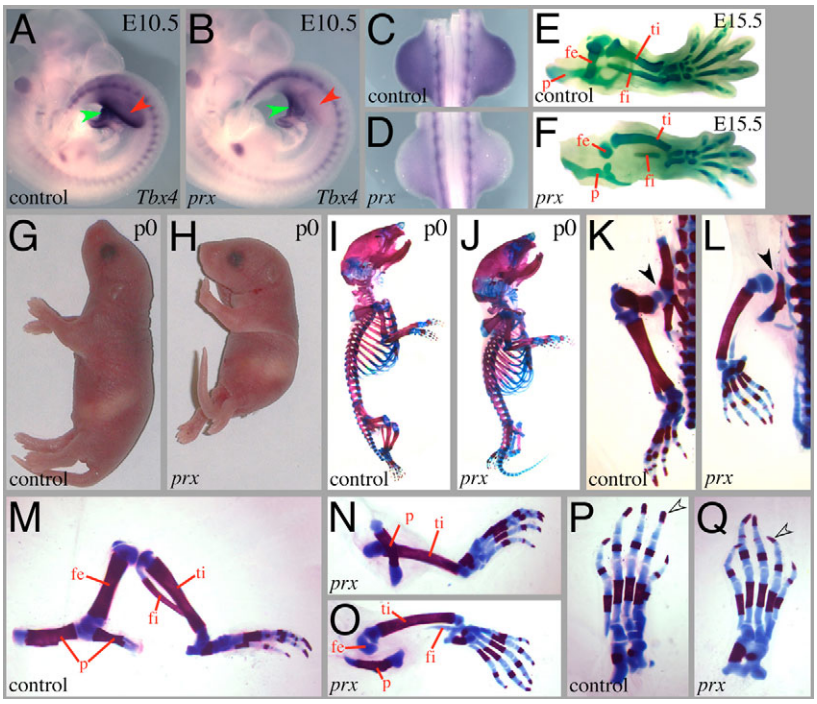

Fig. 7. Phenotype of *Tbx4*$^{cond/cond}$ embryos with (*prx*) or without (control) the *Prx-cre* transgene. (**A-D**) Expression of intact *Tbx4* transcript, monitored with a deletion-specific probe (Fig. 1A). Embryos were also hybridized with *Pax1* probe, which marks the somites and anterior forelimb. (A,B) In late E10.5 control embryos *Tbx4* is expressed throughout the hindlimb (red arrowheads) and the proctodeum or allantois region (green arrowheads) but is partially lost from the hindlimb of *prx-cre* embryos. (C,D) Dorsal views of the hindlimbs in A and B, respectively. (**E**,**F**) Cartilage staining of E15.5 control and *prx-cre* hindlimbs. *prx-cre* embryo has five digits, but severely hypoplastic fibula, femur and pelvis. (**G**,**H**) *Prx-cre* neonates show abnormally turned hindlimbs and small hips. (**I-Q**) Skeletal preparations of neonates. Red, ossified bone; blue, cartilage. (I,J) *prx-cre* neonates have small and abnormally turned hindlimbs. (K,L) Ventral views show that in normal embryos the femur is articulated with the pelvis, while in *prx-cre* embryos there is a large gap between femur and pelvis (black arrowheads). (M,N) Lateral views of hindlimbs. The lower limb elements of *prx-cre* hindlimbs are abnormally oriented relative to the femur. (O) Dorsal view of a *prx-cre* hindlimb. The fibula, femur and pelvis are severely hypoplastic and the fibula and femur are not ossified. (P,Q) Dorsal views of left hindfoot. *prx-cre* hindfoot has partially fused anklebones and mildly reduced second digit (open arrowhead). fe, femur; fi, fibula; p, pelvis; ti, tibia.

Limb-specific deletion of *Tbx4*

Prx-cre drives *cre* expression in forelimbs and hindlimbs (Logan et al., 2002) and in combination with the conditional *Tbx5* allele results in pups wholly lacking forelimbs (Rallis et al., 2003). To bypass the fetal lethality caused by the *Rosa-ERcre* allele, we produced a limb-specific deletion of *Tbx4* by mating *Tbx4*$^{cond/cond}$ females with *Tbx4*$^{tm1.1Pa/+}$(null allele); *Prx-cre*/+ males to generate *Tbx4*$^{cond/tm1.1Pa}$; *Prx-cre*/+ embryos (*prx-cre*) and *Tbx4*$^{cond/+}$; +/+ embryos (control). We dissected early limb bud embryos to observe the kinetics of *Tbx4* excision in the presence of *Prx-cre*. Intact *Tbx4*, as measured by the deletion-specific probe (Fig. 1A), was significantly downregulated but not entirely lost in advanced E10.5 *prx-cre* embryos (Fig. 7A-D), indicating that *prx-cre* hindlimbs express *Tbx4* for longer than *ERcre* hindlimbs administered tamoxifen at E9.5. Cartilage staining of E15.5 embryos revealed that the hindlimb in *prx-cre* embryos had a hypoplastic pelvis and fibula, severely hypoplastic femurs and mild or nonexistent anterior digit fusions, consistent with the phenotype of *ERcre* embryos given tamoxifen at E10.5 (Fig. 7E,F). The ilium and ischium were identifiable, but the pubic rami were missing. At E11.5, we did not observe any major difference in apoptotic cells in the hindlimb between *prx-cre* embryos and controls (data not shown).

prx-cre pups were viable but had abnormally turned hindlimbs and abnormal pelvic regions (Fig. 7G,H). Skeletal preparations showed normal skeletal development in *prx-cre* pups (Fig. 7I,J), but the hindlimbs were turned nearly backwards and not articulated with the pelvis (Fig. 7I-L). It is unclear whether the abnormal turning of the hindlimb in *prx-cre* embryos is a primary defect due to the loss of *Tbx4* or secondary to the loss of the proximal tissues as leverage points.

Compared with control hindlimbs, neonatal *prx-cre* hindlimbs showed relatively normal tibia growth and ossification, while only scraps of cartilage remained of the fibula and the femur. The hypoplastic pelvis had ossified, but the absence of a central cartilaginous region suggests that this represents the fusion of the remaining ilium and ischium (Fig. 7M-O). Foot development was only mildly affected in *prx-cre* embryos, which had partially fused tarsals and a reduced second digit (Fig. 7P,Q). These defects all appeared to be the direct outcome of defects observed at E14.5 in both *prx-cre* and *ERcre* embryos injected at E10.5, suggesting that *Tbx4* has no further role in limb development between E14.5 and birth. Comparison of the *prx-cre* and *ERcre* phenotypes also demonstrates that all of the defects seen in the *ERcre* hindlimbs are specific to the loss of *Tbx4* from hindlimb mesenchyme and that hypothetical interacting or migrating tissues play no role in producing the observed defects.

DISCUSSION

Hindlimb formation requirements for *Tbx4*

We have demonstrated that, despite widespread hindlimb-specific expression, *Tbx4* is only required in a limited developmental window from its earliest time of expression, at E9.5, through to early

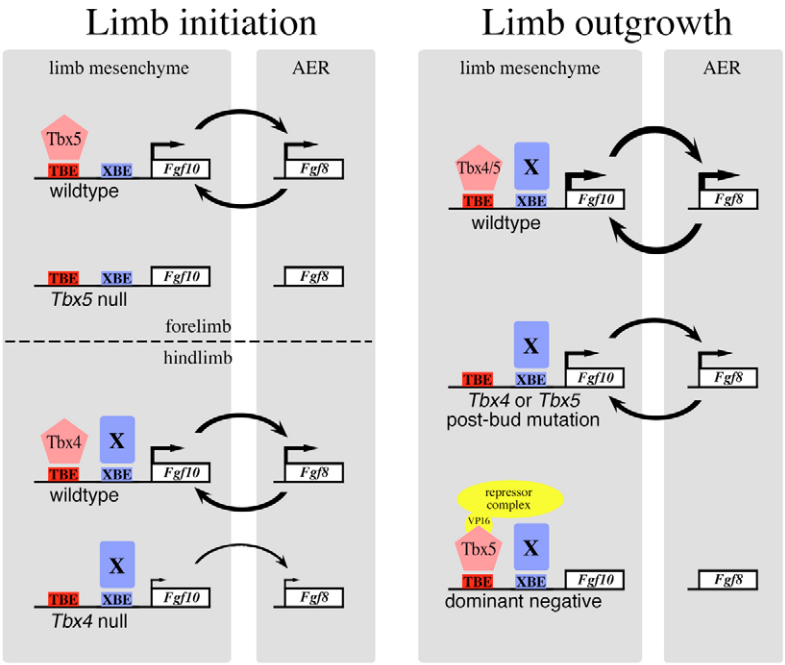

Fig. 8. A proposed model of *Tbx4* and *Tbx5* function in the limbs. In the forelimb initiation stages, *Tbx5* is sufficient to drive threshold levels of *Fgf10*, which upregulate *Fgf8* in the AER, but in the absence of *Tbx5*, no *Fgf10* expression is seen. In the hindlimb, both *Tbx4* and protein X contribute to *Fgf10* expression, so in the *Tbx4* null, sub-threshold levels of *Fgf10* are seen. During the process of limb outgrowth, *Tbx4* or *Tbx5* contribute to robust expression of *Fgf10* in their respective limbs, along with protein X and feedback from the AER, and protein X is sufficient to maintain *Fgf10* expression at lower levels in the absence of *Tbx4* or *Tbx5*. Dominant negative alleles block *Fgf10* expression by recruiting co-repressor complexes.

limb formation, at E11.5. Loss of *Tbx4* in the early part of this window halted limb outgrowth during limb bud formation, while loss of *Tbx4* during bud formation or early limb formation did not affect limb outgrowth, but did result in a dramatic loss of proximal skeletal elements and a more modest loss of distal skeletal elements. During early limb development these limbs showed a consistent loss of core tissue, leading to the relative expansion of *Shh* responsive genes across the limb. Because these differences were already clear at E11.5 and ablation of *Tbx4* after E11.5 led to no additional phenotype, the early loss of limb core tissue was almost certainly the origin of the skeletal malformations observed at E14.5. This suggests that the limb malformations seen in human heterozygous mutations of *TBX4* (Bongers et al., 2004), which cause mild malformation of pelvis, patella size and toe placement (Small Pateela Syndrome, OMIM 147891), are determined in early development rather than during later limb outgrowth.

Gene ablation and lineage tracing has shown that digit I is *Shh*-independent, and digit II is induced by neighboring *Shh* signaling, while digits III, IV and V arise from tissue that once expressed *Shh* (Harfe et al., 2004). In the post-initiation *Tbx4*-ablated hindlimb, the reduction of limb core tissue and the relatively greater area of *Shh* expression reduced the tissue available to form digits I and II, resulting in thin digits, or partial transformation of all remaining tissue to a digit II-like fate.

Our previous work showed no role for *Tbx4* in the initial establishment of hindlimb identity (Naiche and Papaioannou, 2003), and our current work shows no requirement for *Tbx4* in maintenance of hindlimb identity during later stages of limb development. This confirms that other transcription factors, such as *Pitx1* (Minguillon et al., 2005), are responsible for coordinating hindlimb-specific transcriptional regulation and morphological formation.

Tbx4 and FGF signaling

Limb outgrowth is controlled by reciprocal signaling between *Fgf10* in the mesenchyme and *Fgf8*, *Fgf4* and other Fgf genes in the AER (Niswander, 2003). Previous work has suggested that *Tbx5*, and by implication *Tbx4*, is a direct regulator of *Fgf10* in the limbs (Agarwal et al., 2003; Ng et al., 2002). Although hindlimbs that never express *Tbx4* initiate low levels of *Fgf10* expression, this expression fails by early E10.5. However, as shown by our experiments with the conditional allele, the loss of *Tbx4* at approximately E10.5 has no apparent effect on *Fgf10* expression. Thus, our work conclusively shows that *Tbx4* is not required for *Fgf10* expression in limb development after E10.5.

There nevertheless appear to be problems with FGF signaling in the absence of *Tbx4*. Several genetic manipulations that produce a partial loss of FGF signaling also produce limb phenotypes remarkably similar to that observed in post-bud loss of *Tbx4*. Loss of *FgfR1* immediately after the initiation of hindlimb budding, approximately the same stage at which we have ablated *Tbx4*, causes anterior digit fusions producing a symmetrical four digit autopod (Li et al., 2005). Likewise, the loss of *Fgf8* from the AER produces hindlimbs with hypoplastic femurs and fibulas and loss of anterior digits (Lewandoski et al., 2000). Other FGF perturbations have phenotypes that are more or less severe, reflecting different degrees and timing of FGF pathway disruption (Li et al., 2005; Moon and Capecchi, 2000; Sun et al., 2002; Verheyden et al., 2005), or that produce similar phenotypes in the forelimb (Moon and Capecchi, 2000). Partial loss of FGF signaling can also produce a similar spectrum of gene expression changes to loss of *Tbx4*, including loss of anterior *Fgf8* expression and expansion of *Alx4* (Li et al., 2005; Sun et al., 2002; Verheyden et al., 2005). Narrowing of the non-expressing domain of *Tbx2* and *Tbx3* is also evident in figures (Li et al., 2005). It is probable that the reduction we have observed in *Fgf8* is indicative of a partial loss of FGF signaling in *Tbx4*-ablated hindlimbs, a difference probably so slight that it is not observable by in situ hybridization for FGF pathway components.

Shh is thought to be repressed in the normal limb by *Alx4* (Qu et al., 1997). *Shh* also induces *dHand* expression, which in turn is thought to repress *Alx4* (te Welscher et al., 2002), forming mutually exclusive domains of anterior and posterior limb signaling. However, ablation of *Tbx4* either before limb development or during early limb development resulted in overlapping areas of *Alx4*, *dHand* and *Shh* expression. As noted above, several other disruptions of the

FGF pathway cause expansion of the *Alx4* domain, suggesting that this may be a general feature of loss of FGF signaling. It is possible that *Alx4-Shh* interactions are dependent on FGF, and consequently *Tbx4*, function.

A model for *Tbx4* and *Tbx5* function in the limb

The relatively minor effect of post-initiation *Tbx4* loss on FGF signaling is inconsistent with a report using a dominant-negative allele of *Tbx5*, showing that post-initiation loss of *Tbx5* halts FGF signaling and limb growth in the forelimb (Rallis et al., 2003). Recent results from the same lab show that post-initiation ablation of *Tbx5* function using a conditional allele does not result in limb truncations or loss of *Fgf10* (Hasson et al., 2007). Instead, reduction of the forelimb along the anteroposterior axis is seen early in forelimb development, analogous to the thinner hindlimbs seen in our corresponding *Tbx4* study. This suggests that *Tbx4* and *Tbx5* behave similarly in the limb, but that dominant-negative alleles produce a different phenotype than conditional ablation.

In order to explain this difference, we propose that regulation of *Fgf10* in the limb is regulated by (at least) two modules, one (TBE) that is responsive to *Tbx4* and *Tbx5* and one (XBE) that is responsive to an unidentified transcription factor (Fig. 8). In the early limb field, this hypothetical transcription factor is either absent and unnecessary (in the forelimb) or insufficient to drive threshold levels of *Fgf10* expression (in the hindlimb). Thus both limbs are dependent on *Tbx4* or *Tbx5* for establishment of the FGF feedback loop. Once the FGF signaling feedback loop has been successfully set up, *Tbx4* or *Tbx5* and the hypothetical transcription factor have additive effects on total FGF signaling, so the loss of *Tbx4* or *Tbx5* produces relatively mild FGF hypomorphic phenotypes. A dominant negative, where *Tbx4* or *Tbx5* is fused to a transcriptional repression domain, is capable of reducing *Fgf10* transcription below threshold levels, halting limb outgrowth. This model also agrees with in vitro data according to which *Tbx5* alone is capable of driving expression of an *Fgf10* reporter (Agarwal et al., 2003; Ng et al., 2002). The differential requirement for T-box genes between initiation and maintenance of FGF signaling also explains the disparity in *Tbx4* and *Tbx5* conditional phenotypes when combined with the *Prx-cre* allele, as this transgene has been observed to start expression relatively later in the hindlimb than in the forelimb (Kmita et al., 2005; Logan et al., 2002), and does not seem to ablate *Tbx4* gene function until after an FGF feedback loop has formed.

There are several candidates for this proposed *Fgf10* regulator. *Sall4* can drive *Fgf10* limb expression and activates an *Fgf10* reporter synergistically with either *Tbx4* or *Tbx5*, but *Sall4* expression is dependent on T-box regulation and is a poor candidate for an independent regulator of *Fgf10* (Harvey and Logan, 2006; Koshiba-Takeuchi et al., 2006). *Pitx* transcription factors are known to interact directly with T-box genes (Lamolet et al., 2001), and double mutation of *Pitx1* and *Pitx2* produces a phenotype very similar to the post-initiation loss of *Tbx4* (Marcil et al., 2003), but loss of these genes also dramatically downregulates *Tbx4*, suggesting that the observed phenocopy is due to the downstream loss of *Tbx4*. A better candidate is *Snai1*, which is expressed in the hindlimb at a relatively earlier stage than in the forelimb, as predicted by our model, and appears to be upstream of *Fgf10* (Isaac et al., 2000). Also as predicted, *Snai1* expression is maintained in the *Tbx4* null hindlimb field (data not shown) and could therefore drive *Fgf10* in the absence of *Tbx4*. Another excellent candidate is *Lef1*, which can directly regulate *Fgf10* in vitro (Agarwal et al., 2003), and is maintained in the hindlimb after *Tbx4* has been ablated.

While previous hypotheses (ours included) proposed that *Tbx4* was a 'master switch' that dictated hindlimb outgrowth and identity, it now appears that *Tbx4* plays a more cooperative role in regulating these functions. *Tbx4* probably coordinates with numerous other transcription factors to guide limb formation, but its importance should not be underplayed. Not only is *Tbx4* crucial for starting hindlimb outgrowth and for the formation of hindlimb skeletal elements, but also it has been conserved as a hindlimb-specific transcription factor since the evolution of cartilaginous fish (Tanaka et al., 2002). Organisms that have subsequently lost hindlimbs have also lost *Tbx4* expression (Cole et al., 2005; Shapiro et al., 2004; Tanaka et al., 2005). This suggests that there are still roles to be discovered for *Tbx4*, possibly with regards to regulation of elements of limb development other than the skeleton.

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