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Tbx5 is dispensable for forelimb outgrowth

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Tbx5 is essential for initiation of the forelimb, and its deletion in mice results in the failure of forelimb formation. Misexpression of dominant-negative forms of *Tbx5* results in limb truncations, suggesting *Tbx5* is also required for forelimb outgrowth. Here we show that *Tbx5* is expressed throughout the limb mesenchyme in progenitors of cartilage, tendon and muscle. Using a tamoxifen-inducible Cre transgenic line, we map the time frame during which *Tbx5* is required for limb development. We show that deletion of *Tbx5* subsequent to limb initiation does not impair limb outgrowth. Furthermore, we distinguish two distinct phases of limb development: a *Tbx5*-dependent limb initiation phase, followed by a *Tbx5*-independent limb outgrowth phase. In humans, mutations in the T-box transcription factor *TBX5* are associated with the dominant disorder Holt-Oram syndrome (HOS), which is characterised by malformations in the forelimb and heart. Our results demonstrate a short temporal requirement for *Tbx5* during early limb development, and suggest that the defects found in HOS arise as a result of disrupted TBX5 function during this narrow time window.

KEY WORDS: *Tbx5*, Holt-Oram syndrome (HOS), Limb initiation, Limb outgrowth, *Prx1CreERT2*

INTRODUCTION

The forelimb (FL) and hindlimb (HL) develop from outgrowths of the lateral plate mesoderm (LPM) at precise positions along the body axis. Although the precise hierarchical relationships are not completely understood, many of the genes that participate in the initiation and subsequent patterning of the limb bud have been identified and their activities analysed. Two T-box transcription factors, *Tbx5* and *Tbx4*, which are expressed in the FL and HL, respectively, have recently been shown to play crucial roles in the formation of each limb type.

Experiments in a range of vertebrate model systems have shown that *Tbx5* is required for FL and heart development (e.g. Agarwal et al., 2003; Ahn et al., 2002; Rallis et al., 2003). Using a conditional knockout allele of *Tbx5*, it has been demonstrated that when the gene is deleted prior to, or during, limb initiation, no FL initiates (Agarwal et al., 2003; Rallis et al., 2003). Similarly, deletion of *Tbx4* in the mouse leads to failure of HL development, although the phenotype is not as profound as that seen in the FL in the absence of *Tbx5* (Naiche and Papaioannou, 2003).

Although the precise hierarchy remains unclear, and may vary between species, Tbx5 and Tbx4 play pivotal roles during FL and HL initiation, respectively, by activating *Fgf10* in the limb mesenchyme (Agarwal et al., 2003; Logan, 2003; Naiche and Papaioannou, 2003; Rallis et al., 2003). Fgf10, in turn, activates *Fgf8* in the apical ectodermal ridge (AER), a distinct strip at the distal extreme of the overlying ectoderm. Fgf8 signaling from the AER to the underlying mesoderm is subsequently required for the maintenance of mesenchymal *Fgf10* expression, thereby creating a positive-feedback loop of FGF signaling between the two tissue layers. *Tbx5* and *Tbx4* appear to play analogous roles in the FL and HL, respectively. Using a gene deletion and replacement strategy, the FL deficiency defect of the *Tbx5* conditional knockout can be rescued by *Tbx4*, demonstrating that *Tbx4* has the ability to carry out the functions of *Tbx5* in FL initiation (Minguillon et al., 2005).

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Interestingly, *Fgf10* mutant mice initiate a limb bud (Sekine et al., 1999), suggesting that Tbx5 may also regulate other targets. In addition, *Tbx5* continues to be expressed throughout later stages of limb outgrowth. Misexpression of a dominant-negative form of Tbx5 in the chick wing bud leads to the downregulation of *Fgf4*, *Fgf8*, *Fgf10*, *Bmp2* and *Wnt3a*, and truncated FLs result (Rallis et al., 2003; Rodriguez-Esteban et al., 1999). These observations have led to the proposal that, in addition to its activity during initiation, *Tbx5* also regulates later FL outgrowth and may be required for the orchestration of the distinct patterning events taking place in the growing limb (Logan et al., 1998; Rallis et al., 2003; Rodriguez-Esteban et al., 1999).

In humans, mutations in TBX5 are associated with the dominant disorder Holt-Oram syndrome (HOS; OMIM 142900) (Basson et al., 1997; Li et al., 1997), which leads to upper(fore)limb and heart deformities. Although haploinsufficiency of TBX5 is fully penetrant, the severity of the limb phenotypes can be variable, ranging from severe deletion deformities of many of the skeletal elements of the limb (aplasia), to more subtle phenotypes such as extended phalangeal elements of the thumbs (Newbury-Ecob et al., 1996). Similarly, mutations in TBX4 are associated with Small Patella syndrome (SPS; OMIM 147891), which is characterised by abnormalities of the lower(hind)limb. The limb skeletal defects in SPS are also characterised by deletion deformities with variable penetrance; however, they are generally less severe than those observed in HOS. Although the results from gene deletion experiments in the mouse are consistent with the phenotypes observed in HOS and SPS, they do not provide any information on the temporal requirement for *Tbx5* and *Tbx4* to form individual elements of the limb at later stages in development.

Here, we test whether *Tbx5* plays a role beyond the initiation stages of FL formation. Using a tamoxifen (TM)-inducible Cre transgenic line (*Prx1CreERT2*), we have deleted *Tbx5* in the nascent FL buds in embryos ranging from 21 somites [early embryonic day (E) 9.5] to E10.5. Surprisingly, although Cre-catalysed deletion of the *Tbx5* conditional allele was apparent in the FL, limb outgrowth was not impaired. These results demonstrate that *Tbx5* is not required for outgrowth of the limb bud and/or patterning of the FL skeleton. Furthermore, these findings have important implications for our understanding of the aetiology of the limb skeletal defects present in HOS.

MATERIALS AND METHODS

Transgenic mice and embryos

Mouse embryos were staged according to Kaufman (Kaufman, 2001). Noon on the day a vaginal plug was observed, was taken to be E0.5. The mouse lines carrying a conditional allele of *Tbx5* (Bruneau et al., 2001), *Rosa26RlacZ* (Soriano, 1999) and a *Prx1Cre* transgene (Logan et al., 2002) have been described previously. Construction of the *Prx1CreERT2* construct was carried out using standard ligation procedures. The *Prx1Cre* backbone was digested with *Eco*RI to excise the Cre ORF, which was then replaced by an *Eco*RI *CreERT2* fragment.

Tamoxifen induction

TM preparation and induction were performed as described on the Joyner laboratory webpage (http://saturn.med.nyu.edu/research/dg/joynerlab/protocols.html). Briefly, mice were gavaged with 6.5 mg (from a 20 mg/ml stock) of TM at the indicated time points.

Quantitative PCR

To follow the rate of *Tbx5* exon 3 recombination, we used primers flanking this exon and monitored the loss of PCR product resulting from its recombination. Briefly, E10.5 FL mesenchymal cells were dissected and the genomic DNA isolated for use in the quantitative PCR analysis. Quantification of the results was carried out using the ABI Prism 7700 Sequence Detection System, User Bulletin 2, Comparative C_T Method. Primers used were: Tbx5 exon3 Fwd, 5′-GGCATGGAAGGAATCAAGGT-3′; Tbx5 int 3-4 Rev, 5′-ATTCCCTCCAATGACTGTCC-3′. The amount of template was normalised using primers that amplify a fragment of the cardiac actin promoter: mCA Fwd, 5′-CCCCCTGGCTGATCCTCTAC-3′; mCA Rev, 5′-TGGTCGCCTTAGCACCAT-3′. All reactions were carried out in triplicate.

In situ hybridisation

Whole-mount and section in situ hybridisation were carried out essentially as described previously (Riddle et al., 1993; Schaeren-Wiemers and Gerfin-Moser, 1993). A minimum of three mutant embryos were analysed with each probe at each stage described. Most of the probes used have been described previously: *Shh* (Echelard et al., 1993), *Fgf10* (Bellusci et al., 1997), *Fgf8* (Crossley and Martin, 1995), *Tbx5* (Rallis et al., 2003), *Tbx5 ex3* (kindly provided by C. Minguillon, NIMR, UK), *Scx* (Schweitzer et al., 2001), *Sox9* (Morais da Silva et al., 1996), *Col2a* (*Col2a1* – Mouse Genome Informatics) (Metsaranta et al., 1991), *Myod* (Davis et al., 1987), *Pax3* (Goulding et al., 1991), *Sall4* (a kind gift of S. Harvey, NIMR, UK), *Tbx3* (kindly provided by C. Goding, Marie Curie Research Institute, UK) and *Tbx15* (Singh et al., 2005).

Histology

The cartilage and bone elements of newborn mouse pups were stained with Alcian Blue and Alizarin Red, respectively, essentially as described previously (McLeod, 1980).

Phospho-Histone H3 and TUNEL analysis

Detection of proliferating or apoptotic cells was carried out as previously described (Rallis et al., 2003). Briefly, mitotic cells were identified using a rabbit anti-phosphorylated histone H3 primary antibody (Upstate Biotechnology) and an HRP-conjugated goat anti-rabbit IgG secondary antibody (Jackson Laboratory). Apoptotic cell death was assayed by TdT-mediated dUTP nick end labelling (TUNEL) according to the manufacturer's protocol (Q-Biogene).

Size quantification analysis

The extent of the anteroposterior axis was determined by measuring the distance between the anterior-most and posterior-most extremes of wild-type (n=15) and mutant (n=12) limbs at their proximal base.

RESULTS

Tbx5 has a pivotal role during initiation of the FL where it activates *Fgf10* expression in the mesenchymal cells of the emerging limb bud (Agarwal et al., 2003; Rallis et al., 2003). Deletion of *Tbx5* leads to the disruption of FL bud formation and all elements of the limb fail to form (Rallis et al., 2003). Such early and profound defects have prevented the study of potential roles of the gene later in limb development. It therefore remains unclear whether *Tbx5* plays any crucial role in regulating outgrowth and coordinating patterning of the bud after its initiation. Expression of *Tbx5* is not restricted to the initiation stages of limb bud development, but rather it is retained throughout the limb outgrowth stages (Fig. 1 and data not shown). Serial sections of the FL at different embryonic stages (E11.5, E12.5 and E15.5) were examined for the expression of *Tbx5* along with markers of the prospective cartilaginous precursors (*Sox9* or *Col2a*), tendons (*Scx*) and muscles (*Pax3*, *Myod* or muscle myosin). At E11.5, *Tbx5* was expressed in all mesenchymal cells of the limb and was co-expressed in domains overlapping with bone, tendon and muscle precursors (Fig. 1A-D). At E12.5, *Tbx5* expression was retained in all mesenchymal cells of the bud, but not as uniformly as that observed at E11.5 (Fig. 1E). *Tbx5* transcripts were detected, but levels appeared reduced along the dorsal and ventral sides of the limb overlapping with the *Myod* and *Scx* expression domains. However, *Tbx5* expression remained elevated in domains overlapping with *Col2a*-expressing cartilage precursors in the centre of the limb (Fig. 1E-H). At E15.5, *Tbx5* expression levels declined and were maintained in only a few cells at the tip of the digits (not shown).

The pan-mesenchymal expression of *Tbx5* in the early stages of limb outgrowth is consistent with the gene playing a role in the maintenance and coordination of limb outgrowth. To confirm this hypothesis, we used a conditional *Tbx5* allele (Bruneau et al., 2001)

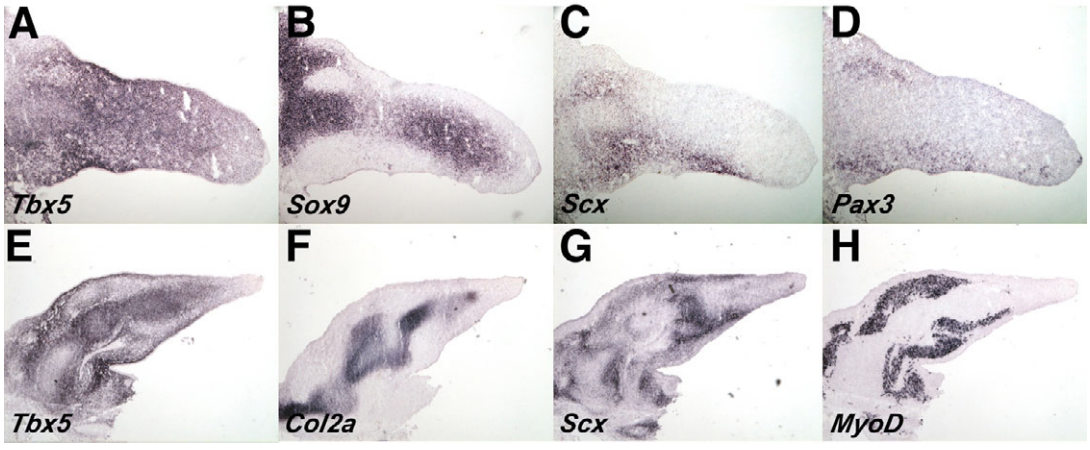

Fig. 1. *Tbx5* is expressed throughout the limb mesenchyme, including the precursors of cartilage, tendons and muscles. Serial section in situ hybridisation using probes for *Tbx5* (**A**,**E**), chondrocyte markers *Sox9* (**B**) and *Col2a* (**F**), tendon progenitor marker *Scx* (**C**,**G**), and myoblast markers *Pax3* (**D**) and *Myod* (**H**). (A-D), E11.5; (E-H), E12.5.

in combination with transgenic mouse lines expressing TM-inducible Cre recombinase (*CreERT2*) (Feil et al., 1997) under the influence of the *Prx1* promoter, leading to expression of CreERT2 in the mouse limbs (*Prx1CreERT2*; Fig. 2). Administration of TM at specific time points and the consequent activation of Cre activity leads to the deletion of *Tbx5* at distinct stages of limb development.

To test the efficacy of the inducible Cre lines that we generated, we compared their activity with that of the previously described *Prx1Cre* transgenic line by crossing *Prx1CreERT2* or *Prx1Cre* mice to the *Rosa26RLacZ* reporter line (Soriano, 1999) (Fig. 2 and data not shown). To induce Cre activity from the onset of *CreERT2* expression driven by the *Prx1* promoter, we tested oral gavage regimes of a single TM dose at E8.5 and a double dose at E7.5 and E8.5. The single dose regime of TM produced comparable levels of recombination as the double dose (data not shown). Gavage regimes at E7.5 or E6.5 resulted in little or no Cre activity, respectively. We therefore used a single oral gavage at E8.5 to detect the earliest Cre activity in the *Prx1CreERT2* lines (see Materials and methods). This comparison reveals that the *Prx1Cre* transgene has more robust Cre activity at early stages than does the *Prx1CreERT2* line. *Prx1Cre* was active in cells of the LPM at the level of the forming bud in the 14-somite stage embryo and, once a bud was visible, Cre activity was evident throughout the mesenchymal cells (Fig. 2A-C). By contrast, during limb initiation stages, *Prx1CreERT2* induced recombination in a relatively small number of sparsely spaced cells (Fig. 2D,E,G,H), and only once the embryo had reached the 21- to 23-somite stage, after initiation of the bud had occurred, was recombination seen throughout the bud (Fig. 2F). From this time point onwards, the *Prx1CreERT2* transgene activity in the reporter was indistinguishable from the *Prx1Cre* transgene (Fig. 2I-M and data not shown). Therefore, the difference between the activities of the *Prx1CreERT2* and *Prx1Cre* deleter transgenes in the FL was the patchy recombination in the FL prospective region in the 14- to 21-somite stage embryos that express the TM-inducible Cre.

To test whether *Tbx5* is required beyond the initiation stages (when the *Prx1Cre* deleter is active), and is necessary for later outgrowth of the FL (when the *Prx1CreERT2* is fully active), we set up crosses to produce $Tbx5^{lox/lox}$; *Prx1CreERT2* embryos. Cre activity was induced by gavaging the mother at E8.5 and harvesting embryos at E10.5. At E10.5, the limb buds are easily detectable and signaling centres such as the AER and zone of polarising activity (ZPA) that control proximodistal and anteroposterior (AP) growth of the limb, respectively, are well established. Unexpectedly, mutant limb buds that are devoid of a functional *Tbx5* allele do form (Fig. 2L,M, Fig. 3). To demonstrate that Cre has been active in these limbs, we carried out the same cross but with a *Rosa26RlacZ* reporter allele also present in the background. Cre was active in all cells of the *Tbx5* mutant limb bud to the same extent as in the wild-type limbs (Fig. 2I-M). To directly follow the amount of recombination of the *Tbx5* conditional allele, we performed

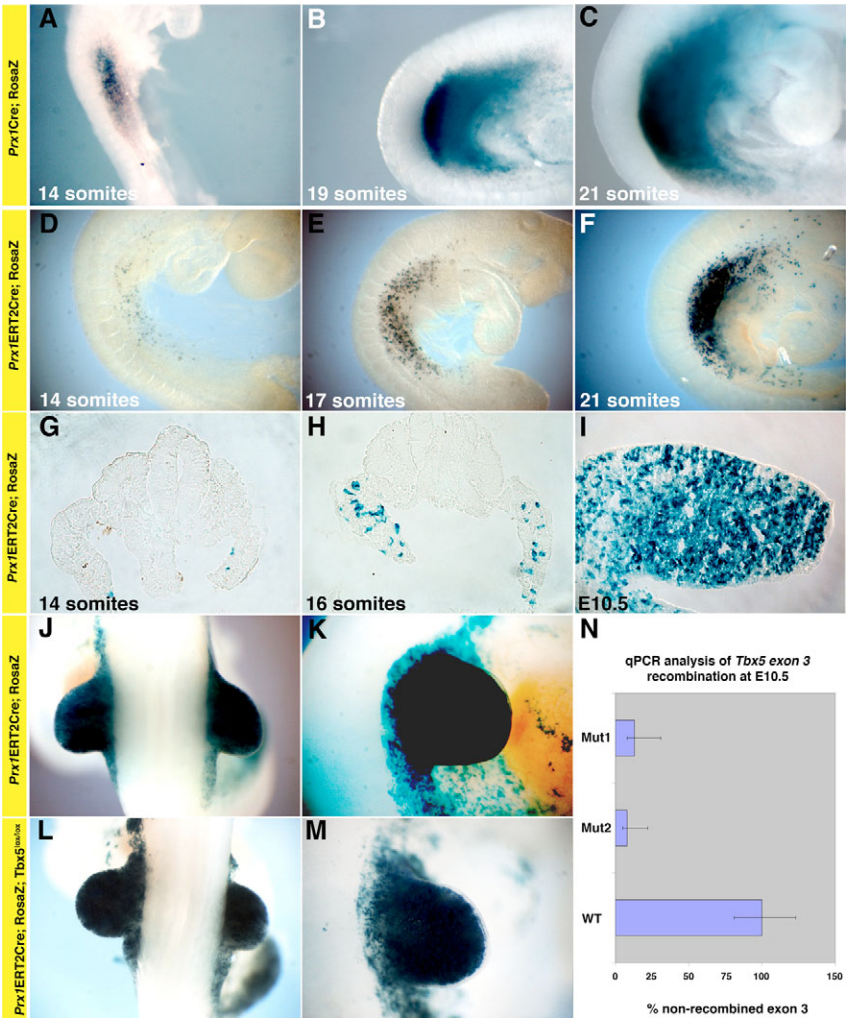

Fig. 2. Comparison of Cre recombinase activity in the developing forelimbs of *Prx1Cre* and *Prx1CreERT2* transgenic embryos using the *Rosa26RlacZ* reporter line. *CreERT2* was activated by gavaging the pregnant females at E8.5. Activation of the reporter by Cre recombination is detected by *lacZ* staining (blue). In *Prx1Cre* embryos, Cre activity was detected throughout the FL-forming region at the 14-somite stage (**A**), and was induced in all cells of the FL of 19- and 21-somite stage embryos (**B**,**C**). In *Prx1CreERT2* embryos, Cre activity was also first detected at the 14-somite stage, but only in a small number of cells (**D**,**G**). Low level activity was still apparent in 16- to 17-somite stage embryos (**E**,**H**), and it was not until the 21-somite stage that Cre activity was detected throughout the limb bud (**F**). By E10.5, the Cre reporter was uniformly activated throughout the limb bud (**I-K**). When the *Prx1CreERT2* transgenic was activated in a background of the *Tbx5* conditional allele ($Tbx5^{lox/lox}$) and Cre reporter, a bud formed despite uniform Cre activity throughout the limb (**L**,**M**). PCR was used to quantitate the levels of *Tbx5 ex3* loss through recombination following TM gavage at E8.5. Recombination of 87%-97% was observed in two independent E10.5 *Prx1CreERT2*; $Tbx5^{lox/lox}$ FLs (Mut1 and Mut2) (**N**). The graph shows the mean values and s.d. obtained from three replicates, normalised to a control; the mean value obtained from the wild-type limb is shown as 100%.

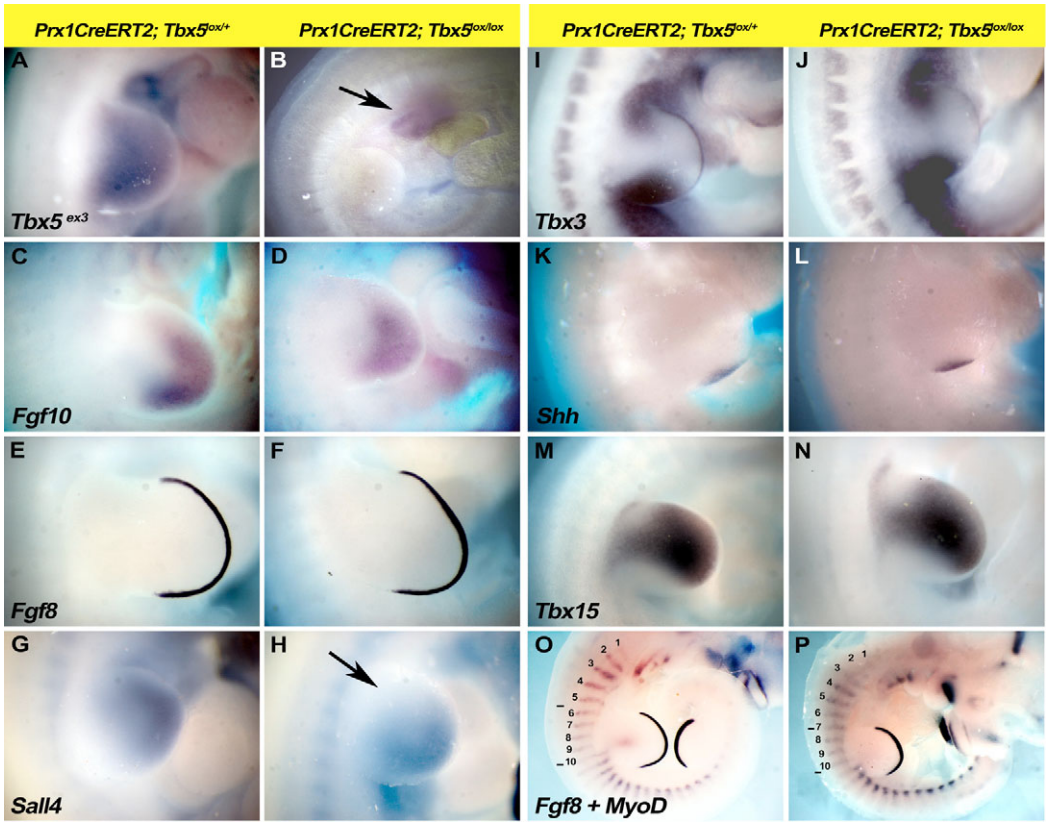

Fig. 3. Following TM administration at E8.5, the limbs of *Prx1CreERT2;Tbx5$^{lox/lox}$* embryos are normally patterned at E10.5. *Tbx5* heterozygous (control) (A,C,E,G,I,K,M,O) and mutant (B,D,F,H,J,L,N,P) embryos from pregnant females gavaged with TM at E8.5, were harvested at E10.5 and analysed by in situ hybridisation for: *Tbx5 ex3* (**A**,**B**); *Fgf10* (**C**,**D**); *Fgf8* (**E**,**F**); *Sall4* (**G**,**H**); *Tbx3* (**I**,**J**); *Shh* (**K**,**L**); *Tbx15* (**M**,**N**); *Fgf8 and Myod* (**O**,**P**). (B,H) Arrows indicate the heart and the anterior domain of the limb, respectively. (O, P) Somites are identified based on *Myod* expression and are numbered in a rostral to caudal direction.

quantitative PCR using genomic DNA extracted from FL mesenchymal cells of E10.5 *Tbx5$^{lox/lox}$*; *Prx1CreERT2* embryos gavaged at E8.5. This analysis confirmed that 87%-97% of *Tbx5 exon3* (*Tbx5 ex3*) (the sequence deleted following the Cre-catalysed recombination to generate the *Tbx5* null allele), had been deleted following TM-dependent Cre induction (Fig. 2N). To further confirm the extent of deletion of the *Tbx5* allele, we performed in situ hybridisation with a probe that recognises *Tbx5 ex3* and thereby identifies cells in which recombination of *Tbx5* has not occurred. In wild-type or heterozygous limbs, this probe mimicked the endogenous *Tbx5* expression pattern and stained both the FL and the heart (Fig. 3A,B and data not shown). In mutant embryos, because the *Prx1* promoter is active in the limbs but not in the heart, expression of the unrecombined, functional conditional allele was detected in the heart (Fig. 3B, arrow). In the limbs, however, no signal was detected, demonstrating that *Tbx5 ex3* had been deleted and that no functional transcripts of *Tbx5* were detectable, reinforcing our observations that Cre is active throughout the mesenchyme of the mutant limb (Fig. 2I,L-N).

These results suggest that the deletion of *Tbx5* in the prospective FL of embryos at the 21-somite stage does not impair further outgrowth of the FL bud. During limb initiation, *Tbx5* is required for the upregulation of *Fgf10* in mesenchymal cells of the bud (Agarwal et al., 2003; Rallis et al., 2003), and in turn Fgf10 induces *Fgf8* in the overlying ectoderm. However, despite the deletion of *Tbx5*, no differences in *Fgf10* expression were detectable (Fig. 3C,D). Consistent with normal Fgf10 signaling, *Fgf8* was expressed in an equivalent domain, and at comparable levels throughout the AER, to that of wild-type or heterozygous littermates (Fig. 3E,F). An orthologue of *Drosophila spalt major*, *Sall4*, has recently been shown in zebrafish and mice to be a target of Tbx5 and to participate in the FGF signaling feedback loop (Harvey and Logan, 2006; Koshiba-Takeuchi et al., 2006). To test whether *Sall4* is also sensitive to *Tbx5* deletion after limb initiation, we performed in situ hybridisation with *Sall4*. *Tbx5$^{lox/lox}$*-deleted limbs had reduced *Sall4* levels when compared with their wild-type counterparts, a phenotype most evident in the anterior domain of the limb (Fig. 3G,H, arrow). To determine whether the AP patterning of the mutant limbs was normal, we analysed the expression patterns of a range of markers expressed in nested domains across the AP axis. *Tbx3* was expressed in anterior and posterior domains, and this pattern was not perturbed in the mutant limb (Fig. 3I,J). *Tbx15* was expressed in an apparently reciprocal pattern to *Tbx3*, comprising a broad medial stripe, and this was unchanged in the *Tbx5$^{lox/lox}$* mutant limb (Fig. 3M,N). The *Shh* expression that marks the ZPA at the posterior of the limb was not disrupted in *Tbx5$^{lox/lox}$*-deleted limbs (Fig. 3K,L). Therefore, the initial expression of *Tbx5* in embryos up to the 21-somite stage is sufficient for two key signaling centres in the limb to be established: the AER and the ZPA.

Although the expression of molecular markers of two key signaling centres, *Shh* (ZPA) and *Fgf8* (AER), were established normally, the appearance of the *Tbx5$^{lox/lox}$*-deleted limbs was not entirely normal. The mutant limbs were narrower in their anterior to posterior extent (Fig. 2J-M). We compared the number of somites that the limbs spanned by analysing the expression of *Fgf8*, which marks the AER, and *Myod*, which marks the somites. Whereas the normal limb spanned approximately five somites (Fig. 3O), the *Tbx5$^{lox/lox}$* limbs spanned only four somites (Fig. 3P). Significantly, although both mutant and wild-type limbs were positioned at the equivalent somite level at their posterior extreme, the anterior extent of the mutant limb was reduced. This anterior bias of the limb phenotype in the *Tbx5* mutant limbs resembles the HOS phenotype,

in which the more anterior structures are most severely affected (see Discussion). Measurement of the mean AP extent of mutant and control limbs at E10.5 indicated that whereas wild-type limbs spanned on average 850 μm, the mutant limbs spanned on average only 700 μm (18% narrower). This reduction in limb size was due to a small, yet detectable, reduction in cell proliferation, and not to an apparent increase in the rate of cell apoptosis in the limb mesenchyme. The number of cells staining positive for phospho-histone H3, a marker for cells undergoing mitosis, was greater in wild-type than in mutant limbs. However, no differences between wild-type and mutant limbs were seen for cells staining positive following TUNEL, a marker of cells undergoing apoptosis (see Fig. S1 in the supplementary material).

Our molecular analysis demonstrated that limb buds lacking a functional allele of *Tbx5* from approximately the 21-somite stage onwards, although slightly smaller, do continue the outgrowth programme, indicating that the gene does not play an essential role during limb outgrowth. To further analyse whether *Tbx5* plays additional roles in later stages of FL outgrowth, we analysed limbs from mice gavaged once at E8.5, E9.5 or E10.5. Induction of Cre activity at these later stages results in recombination in all, or almost all, cells of the FL when embryos were harvested 2 days after gavage (Fig. 4A-C). By E16.5, all cells of the limb were *lacZ*-positive (data not shown), even when embryos were gavaged as late as E10.5. Because females gavaged at E8.5 consistently miscarried at late stages of gestation, we harvested embryos at E14.5. Skeletal elements were analysed by staining E14.5 or E16.5 embryos with Alizarin Red and Alcian Blue. Surprisingly, mutant limbs resulting from TM administration at E8.5, E9.5 and E10.5 contained all skeletal elements (Fig. 4D-I). Furthermore, the overall skeletal patterning was largely normal. Interestingly, only in mutant limbs derived from TM administration at E8.5 were the left limbs more severely affected than the right (data not shown). In the limbs that developed following gavage at E8.5 or E9.5 (n=12), holes were present in the scapula blade and there were obvious notches at the proximal border (Fig. 4E″,G″ and data not shown). The deltoid tuberosity of the humerus was also missing (Fig. 4E,E″,G,G″, arrows). Significantly, the phalangeal elements of digit 1, which are equivalent to the human thumb, were elongated and resembled the tripalangeal defect observed in HOS (Fig. 4E′,G′, arrowheads). In one example, digit 1 was elongated and duplicated in a manner similar to that observed in individuals with HOS (Fig. 4E′, arrowhead) (Newbury-Ecob et al., 1996). The remaining four digits appeared completely normal. These phenotypes were not observed in embryos resulting from TM administration at E10.5, where all the skeletal elements were normal (Fig. 4I). These results are reinforced

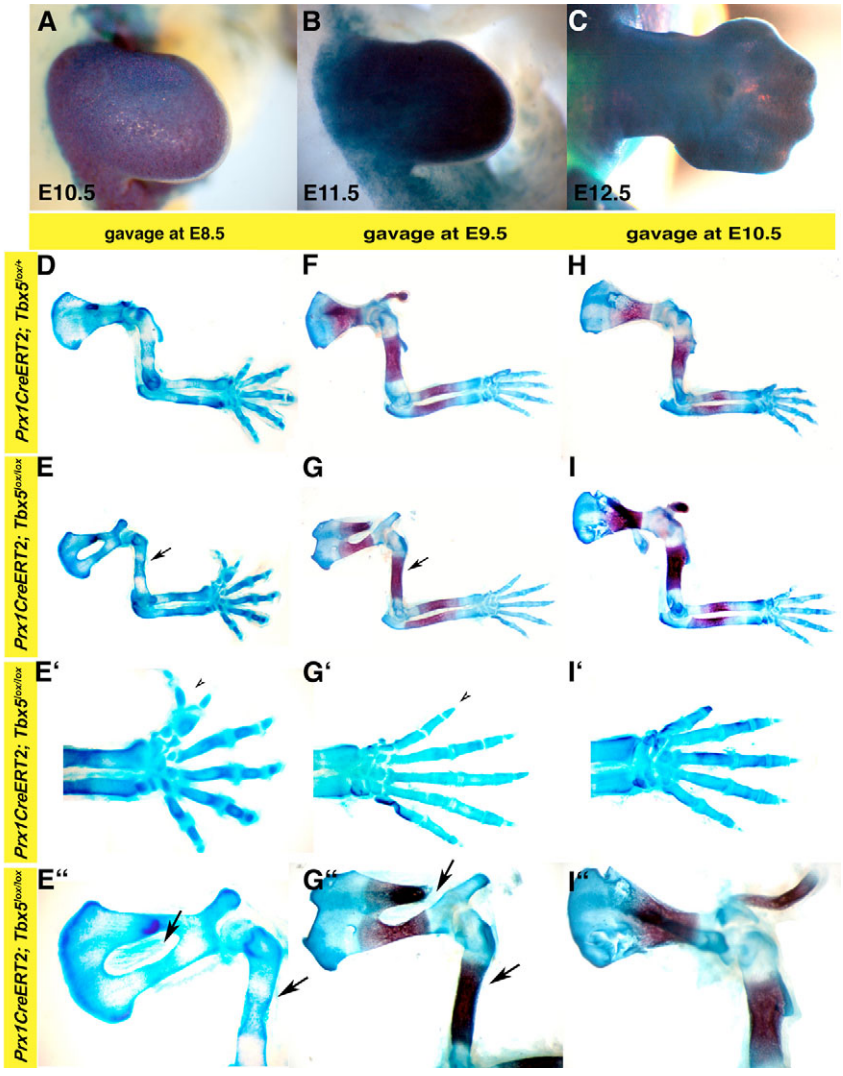

Fig. 4. Outgrowth and skeletal patterning is not impaired in limbs devoid of *Tbx5*. Pregnant females were gavaged with TM at E8.5 (A,D,E), E9.5 (B,F,G) or E10.5 (C,H,I) and harvested 2 days later (**A-C**), at E14.5 (**D**,**E**) or at E16.5 (**F-I**). Embryos were stained for *lacZ* (A-C), or with Alizarin Red and Alcian Blue (D-I). (E,G) Skeletal preparations of FL from *Prx1CreERT2;Tbx5*$^{lox/lox}$ embryos gavaged at E8.5 or E9.5 showed abnormalities comprising a hole in the scapula, lack of the deltoid tuberosity (E,E″,G,G″; arrows) and pronounced elongation of digit 1 (E′,G′; arrowheads). By contrast, skeletal preparations of *Prx1CreERT2;Tbx5*$^{lox/lox}$ embryos gavaged at E10.5 showed normal development (I).

by the absence of abnormal limb phenotypes when a *Col2a-Cre* deleter line (Ovchinnikov et al., 2000) is used to delete *Tbx5* function in the cartilage progenitors of the limb skeleton from E11.5 onwards (P.H. and M.P.O.L., unpublished). These results confirm that *Tbx5* is not required for outgrowth of the FL and demonstrate that *Tbx5* is not required for formation or patterning of the limb skeleton.

DISCUSSION

Mutations in TBX5 are associated with the human dominant disorder HOS that is characterised by multiple upper-limb and heart defects. In vertebrates, *Tbx5* plays a pivotal role in the initiation of the FL by activating *Fgf10* (Agarwal et al., 2003; Ahn et al., 2002; Ng et al., 2002; Rallis et al., 2003), and *Tbx5* knockout mice do not initiate a FL (Agarwal et al., 2003; Rallis et al., 2003). These and other experiments have lead to the placement of *Tbx5* (and its paralogue *Tbx4* in the HL) at the top of the genetic cascade required for limb formation. However, *Tbx5* (and *Tbx4*), continue to be expressed at later stages of limb development (Fig. 1), and misexpression of dominant-negative forms of Tbx5 in the chick leads to limb truncations and the downregulation of genes required for limb outgrowth (Rallis et al., 2003; Rodriguez-Esteban et al., 1999). It has therefore been proposed that *Tbx5* might continue to play a crucial role in limb outgrowth and in later skeletal patterning events in the growing limb (Rallis et al., 2003; Rodriguez-Esteban et al., 1999). Our results, and those of others (Naiche and Papaioannou, 2007), suggest that this is not the case, and that *Tbx5* and *Tbx4* do not have a major role in limb outgrowth and formation of the limb skeleton.

The deletion of *Tbx5* using *Prx1Cre* results in the complete failure of FL formation, whereas deletion with *Prx1CreERT2* does not lead to the same extreme phenotype and early limb markers are expressed relatively normally (Fig. 3). As the resulting mutant limbs do not express an active *Tbx5* gene (Fig. 2N, Fig. 3B), the difference in phenotypes (i.e. limbless versus limb, respectively) is a consequence of the timing of the gene deletion. Our analysis shows that *Prx1Cre* is active in the LPM of 14-somite stage embryos at the level of the forming bud. By contrast, *Prx1CreERT2*, although initially active at the same stage (starting at 14 somites), leads to recombination in a small and sparse number of cells in the bud. Only in 21- to 23-somite stage embryos is activity seen throughout the FL mesenchyme in a manner comparable with that of *Prx1Cre*. The temporal disparity in Cre activity between the two Cre deleter lines, and the distinct phenotypes produced, demonstrate the critical time window within which *Tbx5* is required for FL initiation. This allows us to identify two distinct phases in early limb development: a limb initiation phase that is *Tbx5* dependent (Fig. 5, top), followed by a limb outgrowth phase that is *Tbx5* independent (Fig. 5, bottom). During the initiation phase, Tbx5 activates *Fgf10* expression, and this phase is complete by the 21-somite stage. At this time, key signaling centres in the limb, the AER and the ZPA, are established. Deletion of *Tbx5* after the 21-somite stage (TM administration at stages prior to E10.5) does not impair limb outgrowth, although the limb is not entirely normal. This is consistent with recent observations showing that, in addition to regulating *Fgf10*, Tbx5 also activates *Sall4*. *Sall4* is required for the expression of mesenchymal FGFs, possibly by facilitating establishment of the FGF signaling feedback between the mesenchyme and overlying ectoderm (Harvey and Logan, 2006; Koshiba-Takeuchi et al., 2006), thereby enabling maintenance of *Fgf8* expression in the AER. In humans, mutations in *SALL4* are associated with Okihiro syndrome (OS; OMIM 607323) (Kohlhase et al., 2002), and the limb abnormalities characteristic of OS share many similarities with those found in HOS. Strikingly, the skeletal abnormalities resulting from TM administration at E8.5 and E9.5 are reminiscent of both HOS and OS. It is thus plausible that the phenotypes observed in mice gavaged at E8.5 and E9.5 are due to the reduction in *Sall4* activation by Tbx5 (e.g. Fig. 3H) (Harvey and Logan, 2006; Koshiba-Takeuchi et al., 2006). Crucially, deletion of *Tbx5* function at later stages does not produce HOS-like skeletal phenotypes. This defines the outgrowth phase when the feedback loop between mesenchymal Fgf10 and ectodermal Fgf8 has been established, can operate and is maintained independently of *Tbx5* (Fig. 5, bottom).

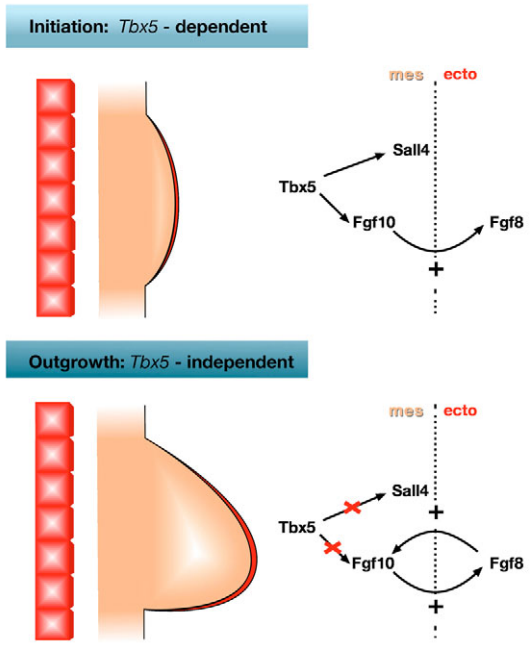

Fig. 5. Model depicting two phases of limb development. An initial *Tbx5*-dependent limb bud initiation phase (top) is followed by a *Tbx5*-independent limb bud outgrowth phase (bottom). See text for details.

Implications for HOS aetiology

Our results demonstrate that deletion of *Tbx5* during distinct phases of limb development leads to phenotypes resembling the clinical manifestations of HOS. When *Tbx5* is deleted during early outgrowth (gavage at E8.5), FL buds are narrower and span the extent of four, rather than five, somites along the main body axis (Fig. 3); this appears to be at least in part due to a reduced rate of cell proliferation, rather than to any increase in cell death (see Fig. S1 in the supplementary material). This phenotype is reminiscent of one of the characteristics of HOS: the loss of anterior structures. These phenotypes are also observed in OS (Kohlhase et al., 2002), in which a reduction in anterior expression is also observed.

Skeletal analysis of *Tbx5* mutant limbs following TM administration up to E9.5 revealed additional phenotypes characteristic of HOS. In individuals with HOS, the left limb is commonly more severely affected than the right (Newbury-Ecob et al., 1996). In embryos generated following a gavage regime at E8.5, but importantly not at later stages of TM administration, the left limb was consistently more severely affected than the right limb.

Furthermore, following gavage at E8.5 and E9.5, the FLs of the resulting embryos had anterior abnormalities, in particular an extended digit 1 (Fig. 4), similar to the thumb abnormalities characteristic of HOS (R. Newbury-Ecob, personal communication) (Newbury-Ecob et al., 1996). Significantly, however, when *Tbx5* was deleted later (gavage at E10.5), no skeletal malformations were observed, demonstrating that HOS skeletal phenotypes are caused by loss of *Tbx5* activity at earlier stages. Furthermore, we did not observe several prominent features of HOS, such as aplasia of skeletal elements. In combination, these results imply that different HOS manifestations can be attributed to distinct stages of *Tbx5* activity.

Recently, suggested roles for *Tbx4* and *Tbx5* in the specification of limb-type identity have been ruled out (Minguillon et al., 2005). Our data and those of others (Naiche and Papaioannou, 2007) suggest that these genes likewise play no role in regulating limb outgrowth, despite their expression being maintained during the outgrowth stages of limb development. It is possible that the activities of Tbx5 and Tbx4 during these later stages of limb development are controlled at a post-transcriptional and/or post-translational level. Evidence for post-translational regulation has come from the description of a PDZ-LIM protein, Lmp4, which is expressed in the limb. When co-expressed in COS-1 cells, Lmp4 is able to bind to Tbx5 and Tbx4, leading to cytoplasmic localisation of the complex via association with the actin cytoskeleton (Krause et al., 2004). It remains to be determined, however, whether Tbx5 and Tbx4 are regulated in this manner during limb development. Similarly, in *Xenopus*, the canonical T-box protein Xbra has been shown to bind Smad1, a binding that modulates its activities (Messenger et al., 2005). Because Smad1 activity is dynamically regulated (Massague et al., 2005), it is possible that a similar situation exists for Tbx5 and Tbx4 during limb outgrowth, and that their activities are controlled via the regulation of co-factors. Our results for Tbx5, and those of others for Tbx4, demonstrate that the activities of these proteins are regulated during limb development. Future work will determine if Tbx5 and Tbx4 are required for other processes during limb development, and whether their activities are regulated at a post-transcriptional or post-translational level.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/1/85/DC1

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