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Tbx5 is essential for forelimb bud initiation following patterning of the limb field in the mouse embryo

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SUMMARY

Transcriptional cascades responsible for initiating the formation of vertebrate embryonic structures such as limbs are not well established. Limb formation occurs as a result of interplay between fibroblast growth factor (FGF) and Wnt signaling. What initiates these signaling cascades and thus limb bud outgrowth at defined locations along the anteroposterior axis of the embryo is not known. The T-box transcription factor TBX5 is important for normal heart and limb formation, but its role in early limb development is not well defined. We report that mouse embryos lacking *Tbx5* do not form forelimb buds, although the patterning of the lateral plate mesoderm into the limb field is intact. *Tbx5* is not essential for an early establishment of forelimb versus hindlimb identity. In the absence of *Tbx5*, the FGF and Wnt regulatory loops required for limb bud outgrowth are not established, including initiation of *Fgf10* expression. Tbx5 directly activates the *Fgf10* gene via a conserved binding site, providing a simple and direct mechanism for limb bud initiation. Lef1/Tcf1-dependent Wnt signaling is not essential for initiation of *Tbx5* or *Fgf10* transcription, but is required in concert with *Tbx5* for maintenance of normal levels of *Fgf10* expression. We conclude that *Tbx5* is not essential for the early establishment of the limb field in the lateral plate mesoderm but is a primary and direct initiator of forelimb bud formation. These data suggest common pathways for the differentiation and growth of embryonic structures downstream of T-box genes.

Key words: Limb, Mouse, T-box, Tbx5, Wnt, FGF, Mouse

INTRODUCTION

The development of vertebrate limbs is an excellent model to study patterning and growth regulation in embryogenesis (Capdevila and Izpisua Belmonte, 2001; Johnson and Tabin, 1997). The limb buds that result in morphologically distinct forelimbs and hindlimbs arise from the lateral plate mesoderm (LPM) at precise locations along the anteroposterior (AP) axis of the embryo. The induction of limb buds at specific locations along the AP axis implies the localized expression of an inductive or competence factor. However, the initial steps in limb bud outgrowth from these fields of patterned LPM and the signals that initiate limb bud outgrowth are not known.

The definition of the fields of LPM that will give rise to limb buds occur possibly as a result of patterning by Hox gene activity along the AP axis of the vertebrate embryo (Cohn et al., 1997; Cohn and Tickle, 1999; Popperl et al., 2000; Rancourt et al., 1995), but it not known what signals are established by these patterning events that lead to limb bud outgrowth. Experiments in chicken embryos have suggested that fibroblast growth factors (FGFs) or other molecules secreted from the intermediate mesoderm (IM), which will give rise to the nephrogenic mesenchyme (NM), may be responsible for initiating limb outgrowth (reviewed by Martin, 1998). These signals are thought to initiate expression of *Fgf10*, which is required for limb bud outgrowth, in the limb field mesenchyme (Min et al., 1998; Sekine et al., 1999). FGF10 in turn activates *Fgf8* expression in the ectoderm overlying the limb field mesoderm (Min et al., 1998; Ohuchi et al., 1997; Sekine et al., 1999). FGF8 and other FGFs secreted from the apical ectodermal ridge (AER) subsequently promote limb bud outgrowth, in part by maintaining *Fgf10* expression (Crossley et al., 1996; Lewandoski et al., 2000; Moon and Capecchi, 2000; Ohuchi et al., 1997; Sun et al., 2002; Vogel et al., 1996; Xu et al., 1998).

FGFs are expressed in the IM and LPM along the entire length of the embryo, so it follows that there must be a

responsive intermediate localized at the level of the limb buds to transduce the signals from the IM. However, contradictory information in both chick and mouse embryos in which NM formation is inhibited has cast doubt on the role of the IM in limb induction, and it has been suggested that there is no requirement for axial signaling to the LPM in limb bud initiation (Fernandez-Teran et al., 1997; Bouchard et al., 2002). Recently, Wnt molecules expressed in the early limb field have been implicated as key regulators of the FGF loop required for limb bud outgrowth (Kawakami et al., 2001). However, mice lacking transcriptional regulators of β-catenin dependent Wnt signaling pathway initiate limb bud growth, although this is not maintained, perhaps because of lack of AER formation (Galceran et al., 1999). What molecule(s) initiate these signaling cascades and thus limb bud outgrowth at defined locations along the AP axis of the embryos is not known.

The T-box transcription factor encoding genes *Tbx5* and *Tbx4* are early markers of the forelimb and hindlimb fields, respectively (Bruneau et al., 1999; Gibson-Brown et al., 1996; Gibson-Brown et al., 1998; Isaac et al., 1998; Logan et al., 1998; Ohuchi et al., 1998; Saito et al., 2002), and have been shown, based on misexpression experiments in chicken embryos, to be involved in regulating limb type (forelimb versus hindlimb) identity (Logan and Tabin, 1999; Rodriguez-Esteban et al., 1999; Takeuchi et al., 1999). Tbx5 is also required in a dose-dependent manner for patterning or growth of the forelimbs, as demonstrated by limb defects of varying degrees caused by dominant *TBX5* mutations in humans with Holt-Oram syndrome (OMIM 142900) or mice that lack one copy of *Tbx5* (Basson et al., 1997; Bruneau et al., 2001; Li et al., 1997). However, the role of Tbx5 in early limb development has not been defined.

In order to investigate the role of Tbx5 in early limb formation, we have analyzed limb bud development in wild-type mice and mice that lack both copies of Tbx5 (*Tbx5*$^{del/del}$ mice) (Bruneau et al., 2001). We show that Tbx5 is required for the earliest signals that initiate limb bud outgrowth from the LPM, including establishment of FGF and Wnt signaling. We propose that Tbx5 initiates limb bud outgrowth following patterning of the LPM by directly activating *Fgf10* in the early limb mesenchyme.

MATERIALS AND METHODS

Mice and in situ hybridization

Mice used were *Tbx5*$^{del/+}$ (Bruneau et al., 2001), *Lef1*$^{+/-}$;*Tcf1*$^{+/-}$ (Galceran et al., 1999) and *Fgfr2*$^{\Delta IgIII/+}$ (Xu et al., 1998). Breeding and genotyping was performed as previously described (Bruneau et al., 2001; Galceran et al., 1999; Xu et al., 1998). Embryos were staged according to Kaufman (Kaufman, 1992), using morphological landmarks to estimate embryonic day (E) of development. All in situ hybridization experiments were carried out on multiple littermates from at least two litters, with similar results. In situ hybridization was performed as described in previously (Riddle et al., 1993). cDNA probes were as described previously (Bellusci et al., 1997; Bruneau et al., 1999; Bruneau et al., 2001; Charite et al., 1994; Crossley and Martin, 1995; Galceran et al., 1999; Lanctot et al., 1999; Smith et al., 1992; Srivastava et al., 1997), or were obtained by RT-PCR on mouse embryo RNA with primers designed using published sequences.

Fgf10 promoter analysis

Fgf10 genomic sequences were obtained from the mouse ENSEMBL database (http://mouse.ensembl.org) and GenBank (http://www.ncbi.nlm.nih.gov). Bacterial artificial chromosomes were identified and obtained from the Centre for Applied Genomics at the Hospital for Sick Children, and a fragment comprising 6.5 kb upstream of the coding region was subcloned in a luciferase expression vector, pXP1. Deletion constructs were made by removing a 600 bp *Afl*II/*Nhe*I fragment (deletion II), a 1.2 kb *Blp*I fragment (deletion III), or a 1.9 kb *Blp*I/*Nhe*I fragment (deletion II/III). Site-directed mutagenesis of TBEa1 was performed by overlap PCR, replacing the sequences GTGTGA by TATAAA, abolishing the putative TBE and introducing a *Ssp*I restriction site for diagnostic purposes. Co-transfections of *Fgf10*-luciferase constructs with a Tbx5 expression construct (Bruneau et al., 2001) and an activated β-catenin expression construct (Miyagishi et al., 2000) were performed in COS-7 cells as described (Bruneau et al., 2001). The results shown are the mean±s.d. of at least two independent experiments performed in triplicate.

RESULTS

Absence of limb buds in *Tbx5*$^{del/del}$ embryos

We examined *Tbx5*$^{del/del}$ mice to elucidate the potential role of Tbx5 in early limb development. *Tbx5*$^{del/del}$ mice had no evidence of forelimb bud initiation at embryonic day (E) 9.5, as evidenced by scanning electron microscopy (Fig. 1). A ridge of LPM was observed in *Tbx5*$^{del/del}$ embryos, but unlike wild-type embryos, *Tbx5*$^{del/del}$ embryos had no detectable limb bud outgrowth, including the formation of an AER (Fig. 1B,D, see Fig. 3B,D). Although limb bud outgrowth was not observed in *Tbx5*$^{del/del}$ embryos, the establishment and patterning of the limb field was intact in these embryos (Fig. 2). Examination of the site of expression of the defective *Tbx5* transcript produced from the *Tbx5*^{del} allele (Bruneau et al., 2001) allowed identification of a patterned forelimb field in *Tbx5*$^{del/del}$ embryos (Fig. 2A,B). *Tbx5* mRNA levels appeared somewhat decreased compared with those in wild-type embryos, perhaps because of fewer *Tbx5*-expressing cells, but the patterning of the domain of *Tbx5* expression was intact. LPM differentiation was also unaffected by the lack of Tbx5, as assessed by expression of the bHLH transcription factor gene *Hand2* (Charite et al., 2000; Fernandez-Teran et al., 2000; Srivastava et al., 1997) (Fig. 2C). Regionalization of the limb field was normal, as the restriction of LPM expression of the Hox gene *Hoxb8* (Charite et al., 1994) and of the ETS transcription factor *Pea3* (Chotteau-Lelievre et al., 2001) to the posterior of the limb field occurred normally in *Tbx5*$^{del/del}$ embryos (Fig. 2D,E). *Pea3* is initially expressed in the LPM up to the posterior of the forelimb field, and is subsequently transcribed in the nascent limb bud mesenchyme (Chotteau-Lelievre et al., 2001). The initial regional LPM expression of *Pea3* at E9 was intact in *Tbx5*$^{del/del}$ embryos, but limb bud expression at E9.5 was lost (Fig. 2E,F). Patterning of the forelimb field is therefore unaffected by loss of TBX5.

Hindlimb markers are not induced in the *Tbx5*$^{del/del}$ forelimb field

It has been hypothesized that TBX5 establishes forelimb identity in part by suppressing expression of genes that define hindlimb identity (Rodriguez-Esteban et al., 1999; Takeuchi et al., 1999). To determine if the loss of Tbx5 resulted in a

conversion from forelimb to hindlimb identity, we examined the expression of *Pitx1* and *Tbx4*, which encode hindlimb-specific transcription factors thought to be involved in establishing hindlimb identity (Lanctot et al., 1999; Logan and Tabin, 1999; Rodriguez-Esteban et al., 1999; Szeto et al., 1999; Takeuchi et al., 1999). No induction of *Tbx4* or *Pitx1* was observed in the presumptive forelimb field of *Tbx5*$^{del/del}$ embryos at E9 (prior to limb bud initiation) or at E9.5 (subsequent to limb bud initiation), suggesting that endogenous TBX5 does not repress hindlimb-specific genes in order to direct forelimb identity (Fig. 2G,H and data not shown).

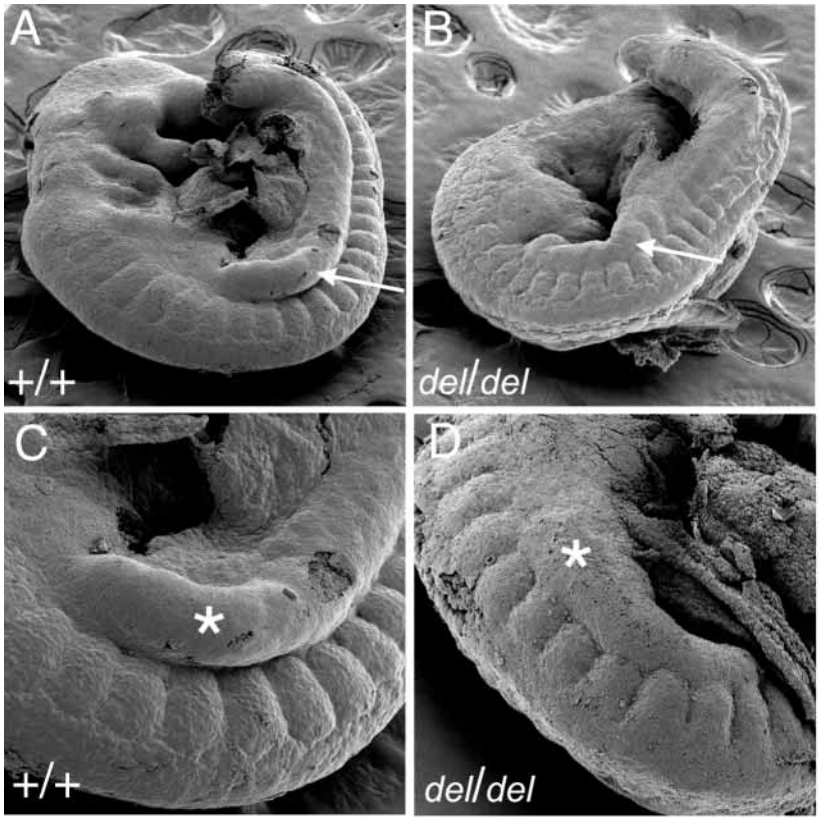


Fig. 1. Scanning electron micrographs of wild-type (A,C) and *Tbx5*$^{del/del}$ (B,D) embryos at E9. Limb bud outgrowth is apparent in wild-type but not in *Tbx5*$^{del/del}$ embryos (arrows in A,B, asterisks in C,D). Lateral plate mesoderm is seen in embryo of either genotype as a ridge along the side of the embryo.

Tbx5 is upstream of FGF signaling

Analysis of genes involved in the initiation of limb outgrowth confirmed that Tbx5 is required for early events in limb bud development. Expression of *Fgf10* and *Fgf8* was not detectable in the forelimb field of *Tbx5*$^{del/del}$ embryos at E9-9.5 (Fig. 3A-E). The expression of *Fgf10* was never initiated in the LPM at E9 (Fig. 3A), but was intact in the intermediate mesoderm and hindlimb field of *Tbx5*$^{del/del}$ embryos (Fig. 3A-C). The observation that hindlimb expression of *Fgf10* can be detected in E9.5 *Tbx5*$^{del/del}$ embryos indicates that undetectable *Fgf10* expression in the forelimb field at this stage is not due to general growth retardation of the embryos, because hindlimb development is delayed compared with that of the forelimb. Furthermore, no *Fgf10* expression is detected in the limb field of E9 *Tbx5*$^{del/del}$ embryos, which are not growth-delayed compared with their littermate controls. The lack of *Fgf10* expression in the forelimb field is therefore a direct consequence of the loss of TBX5 activity in the limb field. Absence of *Fgf8* expression in of *Tbx5*$^{del/del}$ forelimbs is likely to be due to the absence of FGF10 signaling (Min et al., 1998; Sekine et al., 1999). Similarly, although the expression of the early forelimb field marker *Pea3* was intact in the LPM of *Tbx5*$^{del/del}$ embryos at E9, its expression in the putative forelimb bud at E9.5 is absent in *Tbx5*$^{del/del}$ embryos (Fig. 2F), presumably because of the absence of FGF signaling, which is known to regulate *Pea3* transcription in other vertebrate tissues (Raible and Brand, 2001; Roehl and Nusslein-Volhard, 2001). Expression of the FGF-responsive *Snai1* gene, encoding a transcription factor expressed in the early limb bud (Ciruna and Rossant, 2001; Isaac et al., 2000; Nieto, 2002; Sefton et al., 1998), was also undetectable in *Tbx5*$^{del/del}$ embryo putative forelimbs (Fig. 3F), while its expression elsewhere in the embryo was unaffected. In all cases, although growth retardation was observed for *Tbx5*$^{del/del}$ embryos at E9.5, expression of the genes examined was unaffected in tissues other than those destined to become forelimb, indicating that developmental delay is not the cause of the altered gene expression.

Tbx5 expression precedes Fgf gene expression in the LPM

It has been suggested that FGFs from the IM/NM, in particular FGF8, signal to the LPM to initiate limb bud formation, and perhaps to induce *Tbx* gene expression (Crossley et al., 1996; Gibson-Brown et al., 1998; Isaac et al., 2000; Isaac et al., 1998; Logan et al., 1998; Martin, 1998; Ohuchi et al., 1999; Ohuchi et al., 1998; Rodriguez-Esteban et al., 1999; Vogel et al., 1996). However, comparisons of *Fgf* and *Tbx5* expression have been limited to chicken embryos. To establish a potential hierarchy of regulators of limb development in the mouse, we examined the temporal and spatial relationship of expression of *Fgf8*, *Fgf10* and *Tbx5*. We focused on *Fgf8*, because *Tbx5* expression does not initially rely on FGF10 (Sekine et al., 1999).

Tbx5 is expressed initially at E8.0 in a broad region of the LPM corresponding to the cardiac crescent (Bruneau et al., 1999). At E8.5 (5 somites), *Tbx5* expression is still confined to the developing heart (Fig. 4A), but shortly thereafter, at the eight-somite stage, expression of *Tbx5* is robustly detected additionally in a discrete region of the LPM that corresponds to the forelimb field (Fig. 4B). This domain of *Tbx5* expression is continuous with the cardiac domain of *Tbx5* expression. At this stage and until E8.5 (eight somites), *Fgf8* expression is rarely or not detectable in the IM (Fig. 4D) (Crossley and Martin, 1995). *Fgf8* at the eight-somite stage is very weakly detectable in the IM, and this expression is reliably observable in very few embryos at this stage (Fig. 4E). At subsequent stages of development, *Tbx5* remains expressed in the mesenchyme of the limb primordium, although not immediately adjacent to the IM expressing *Fgf8* (Fig. 4C,F). *Fgf10* begins to be expressed at E9 in the limb mesenchyme,

overlapping with the domain of *Tbx5* expression, as well as in the IM (Fig. 3A). Therefore, *Tbx5* expression in the LPM destined to become forelimb precedes expression of *Fgf8* in the IM and *Fgf10* in the LPM.

Tbx5 is upstream of Wnt signaling

Wnt signaling has been implicated in the initiation of limb formation. Wnt2b in the forelimbs and Wnt8c in the hindlimbs of chicken embryos appear to be sufficient to initiate limb bud outgrowth via a β-catenin dependent pathway, including *Fgf10* expression (Kawakami et al., 2001). We could not detect expression of *Wnt2b* or *Wnt8c* in early mouse limb buds (data not shown), indicating that these Wnts, unlike in chick embryos, might not be involved in early stages of mouse limb formation. Although the identity of homologous Wnts in mouse that might be involved in limb bud formation is not known, transcription factors downstream of β-catenin dependent Wnt signaling, LEF1 and TCF1, play a key role in mouse limb bud outgrowth (Galceran et al., 1999). *Lef1* and *Tcf1* were expressed in the early limb field of wild-type embryos, but their transcripts were undetectable in *Tbx5*$^{del/del}$ forelimbs (Fig. 5A,C,E). It is not known whether Wnt signaling is upstream or downstream of *Tbx5*, and there is precedent to suggest that Wnts can directly regulate T-box genes via LEF1 (Galceran et al., 2001; Yamaguchi et al., 1999). To determine the hierarchy of regulation between *Tbx5*, Wnt signaling and *Fgf10* expression, we examined *Tbx5* and *Fgf10* expression in *Lef1*/*Tcf1* double mutant embryos (Galceran et al., 1999). *Tbx5* expression at E9.25 was unaffected in *Lef1*$^{-/-}$;*Tcf1*$^{-/-}$ embryos, while *Fgf10* expression was detectable at weaker levels than wild-type in these mutants (Fig. 5B,D,F). We conclude that *Tbx5* acts upstream of Wnt signaling in the developing limb bud, and Wnt signaling via LEF1 and TCF1 are required to maintain normal levels of *Fgf10* expression.

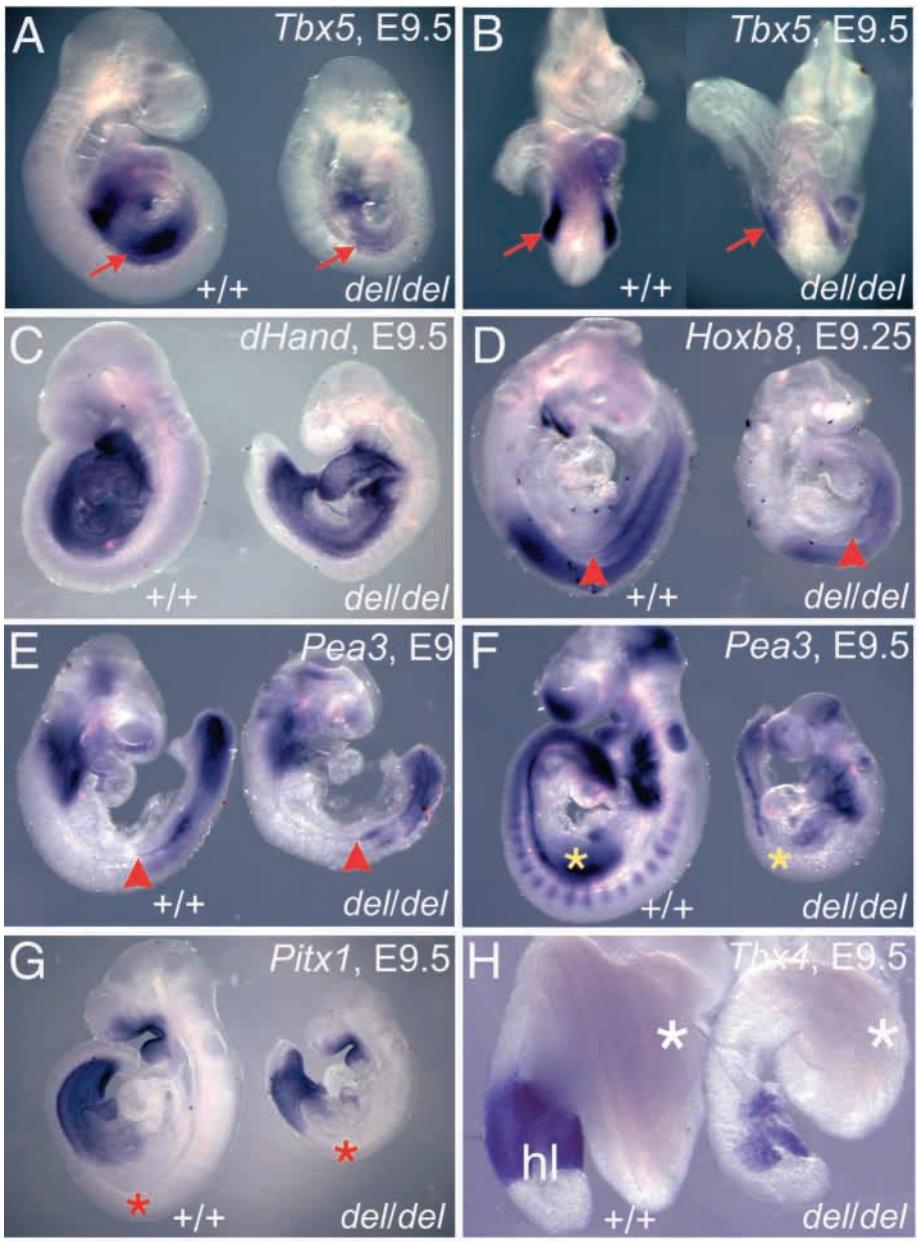


Fig. 2. Intact patterning of the LPM in *Tbx5*$^{del/del}$ embryos, as demonstrated by expression of *Tbx5* mRNA (A,B, arrows) from the wild-type or deleted alleles. LPM differentiation, as shown by *Hand2* expression (*dHand* in figure), is intact in embryos of either genotype (C). Normal patterning of the forelimb field in *Tbx5*$^{del/del}$ embryos is also apparent as demonstrated by expression of *Hoxb8* and *Pea3* mRNA in the LPM up to the posterior region of the forelimb field (arrowheads) in both wild-type and *Tbx5*$^{del/del}$ embryos (D-F). After the initiation of limb bud outgrowth at E9.5, *Pea3* expression is expressed in the forelimb mesenchyme of wild-type embryos, but is not detectable in the forelimb field of *Tbx5*$^{del/del}$ embryos (F, asterisks). Hindlimb markers *Pitx1* (G) and *Tbx4* (H) are expressed normally in the hindlimb field (hl) but not in the forelimb field (asterisks) of wild-type or *Tbx5*$^{del/del}$ embryos.

Establishing hierarchies of signaling in the limb bud

We have shown that in mice lacking *Tbx5*, expression of *Fgf10*, *Lef1*, *Tcf1*, *Snai1*, *Pea3* and *Fgf8* in the presumptive limb buds is abolished. We have further established that Tbx5 is upstream of Wnt signaling. To examine further the relationship between FGF signaling, *Tbx5* expression and expression of potential downstream targets of TBX5, we examined expression of *Tbx5* and *Lef1* in *Fgfr2*$^{\Delta IgIII/\Delta IgIII}$ embryos, in which the entire immunoglobin-like domain III of the Fgf receptor 2 (*Fgfr2*) gene has been deleted (Xu et al., 1998). In these mice, the different isoforms of Fgfr2 that are receptors for FGF8 (FGFR2c) or FGF10 (FGFR2b), and are required

for FGF function in limb bud formation (Martin, 1998; Xu et al., 1998), are deleted. *Tbx5* expression was normally initiated at E9.25 (Fig. 5G), but not maintained by E10.5, in the presumptive limb bud mesenchyme of $Fgfr2^{\Delta IgIII/\Delta IgIII}$ embryos (data not shown). This is similar to what is observed in mice that lack *Fgf10* (Sekine et al., 1999), and presumably reflects a major role for this receptor in transducing FGF10 signals. Expression of *Lef1* was also unaffected in $Fgfr2^{\Delta IgIII/\Delta IgIII}$ embryos at E9.5 (Fig. 5H).

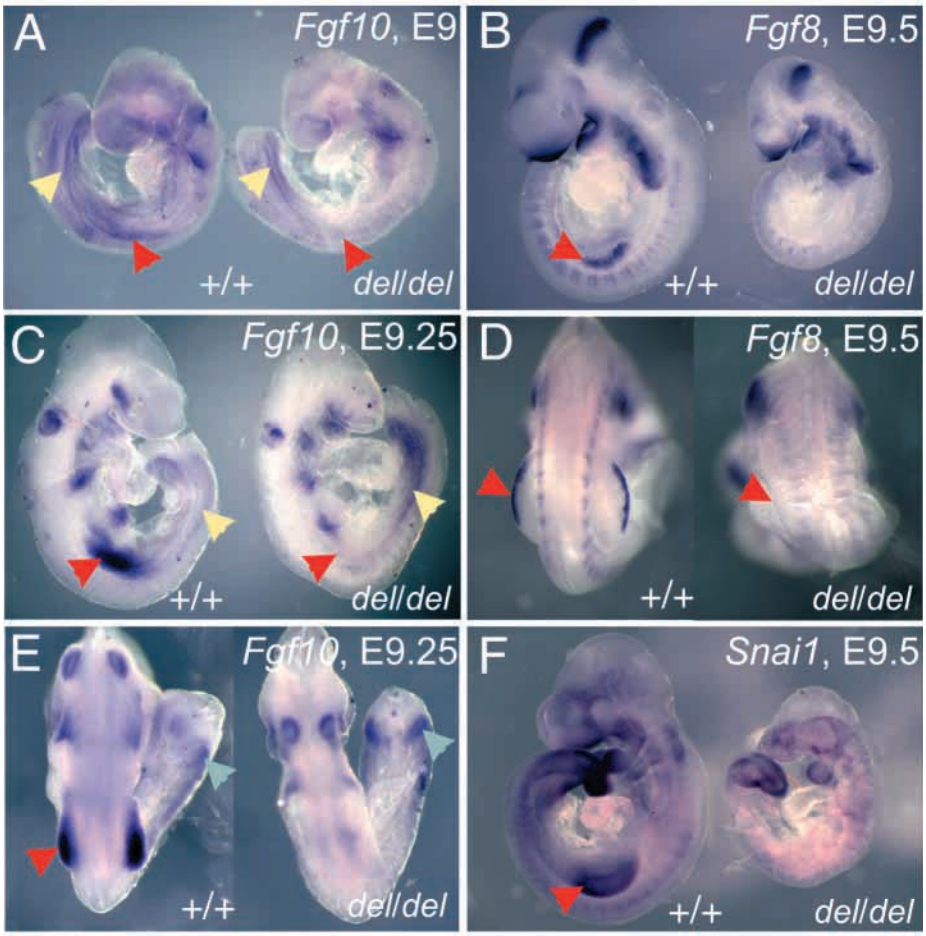


Fig. 3. *Tbx5* is upstream of FGF signaling in the forelimb bud. Wild-type embryos express *Fgf10* (A,C,E), *Fgf8* (B,D) and *Snai1* (F) in the nascent limb buds (red arrowheads). These transcripts are absent in the forelimb field of $Tbx5^{del/del}$ embryos, but expression at other sites in $Tbx5^{del/del}$ embryos is intact, including intermediate mesoderm and hindlimb expression of *Fgf10* (yellow and blue arrowheads, respectively, in A,C,E).

Tbx5 directly activates the *Fgf10* gene

The lack of initiation of *Fgf10* expression in the LPM of $Tbx5^{del/del}$ embryos suggests the possibility that TBX5 may activate the *Fgf10* gene directly. We examined the promoter region of *Fgf10* for potential TBX5 binding sites (TBEs) (Bruneau et al., 2001; Ghosh et al., 2001), by comparing conserved elements of the 7 kb upstream of *Fgf10* in human and mouse genomic sequences; additional confirmation of conserved sites was carried out by comparing mouse and rat genomic sequences. The 7 kb upstream of the *Fgf10*-coding region have been determined to be sufficient for *Fgf10* transcription in limb buds and elsewhere (Sasaki et al., 2001). Three regions of significant homology between mouse and human sequences could be identified (Fig. 6A). Within these regions, three potential TBEs were detected in the genomic sequences of both species [Fig. 6A,B; based on the sites described by Bruneau et al. (Bruneau et al., 2001)]; additional potential TBEs were found outside the regions of homology. Only one site was conserved in human, mouse and rat DNA (asterisk in Fig. 6A). We isolated 6.5 kb of genomic DNA upstream of the *Fgf10* gene, and fused this putative regulatory region to a *luciferase* reporter gene. Co-transfections in COS-7 cells of this *Fgf10* reporter gene with a *Tbx5* expression construct resulted in powerful (up to 120-fold) activation of the reporter gene (Fig. 6C), showing that TBX5 can directly activate the *Fgf10* gene.

The decreased expression of *Fgf10* in $Lef1^{-/-};Tcf1^{-/-}$ embryos indicates a role for Wnt signaling in the regulation of *Fgf10* expression. A single consensus LEF1/TCF1 binding site (TTCAAAG) was identified in mouse and human *Fgf10* regulatory sequences, as shown by 'L' in Fig. 6A. We co-transfected the *Fgf10*-luciferase reporter construct with an activated β-catenin construct into COS-7 cells, with or without the Tbx5 expression construct. This activated β-catenin construct will activate LEF1/TCF1-dependent transcription (Miyagishi et al., 2000). Activated β-catenin at all doses tested activated the *Fgf10*-luciferase reporter gene, with a maximum activation of ~10-fold (Fig. 6D). In combination with *Tbx5*, an additive effect of β-catenin was observed (Fig. 6D).

To identify the regions of the *Fgf10* promoter that were responsive to TBX5 and β-catenin-dependent transcription factors, we performed deletion analysis of the *Fgf10* promoter (Fig. 6E). We deleted a 0.6 kb region corresponding to most of conserved region II (delII), a 1.2 kb region corresponding to most of conserved region III (delIII) or a 1.9 kb deletion that removes all of region II and most of region III (delII/III) (see Fig. 6A). The first deletion removes the conserved TBE (a1), while the second deletion removes two TBEs (a2 and c), as well as the LEF1/TCF1 binding site. Deletion of region III abolished the response of the *Fgf10* promoter to activated β-catenin, which correlates well with the presence of the LEF1-binding site in this region. Activation by TBX5 was not significantly affected by this deletion. However, deletion of region II abolished activation by TBX5 almost completely (1.5- versus 18-fold). Deletion of both regions II and III completely eliminated activation by either activated β-catenin or TBX5. To further delineate the contribution of TBEa1, the putative Tbx5 binding sequences GTGTGA of TBEa1 were mutated to

TATAAA, and the construct assessed for its response to TBX5. Mutation of TBEa1 (muta1 in Fig. 6E) significantly reduced TBX5 activation of the *Fgf10* promoter-luciferase construct to 1.6-fold (compared with 18-fold for the wild-type promoter). The combined data indicate that TBX5 activates the *Fgf10* promoter directly mainly via the evolutionarily conserved TBEa1, and that β-catenin-dependent signaling activates *Fgf10* via a conserved LEF1/TCF1-binding site.

DISCUSSION

We have shown that in mice lacking *Tbx5* there is a complete absence of formation of the forelimb bud, including establishment of the FGF and Wnt signaling pathways that are crucial for limb bud outgrowth. However, patterning of the limb field is intact in these embryos, indicating that TBX5 is responsible for the transition of the limb field to the limb bud. TBX5 appears not to be essential for limb type identity determination, but instead directly initiates limb bud outgrowth by activating the *Fgf10* gene.

Tbx5 and forelimb identity

The absence of *Tbx5* did not result in acquisition of hindlimb identity by the forelimb field. Misexpression experiments in chicken embryos have suggested that forced expression of *Tbx5* in the hindlimb and *Tbx4* or *Pitx1* in the forelimb can induce a conversion of limb type identity (Logan and Tabin, 1999; Rodriguez-Esteban et al., 1999; Takeuchi et al., 1999). *Tbx5* misexpression in the hindlimb was shown to result in the repression of *Tbx4*, suggesting that the endogenous role of Tbx5 in the forelimb is to repress *Tbx4* expression (Takeuchi et al., 1999). Our data showing that lack of *Tbx5* does not lead to expression of *Tbx4* or *Pitx1* in the forelimb field does not support this hypothesis. This is consistent with the observations that limb type identity in chick embryos is determined at stage 9-12, before the induction of *Tbx5* and *Tbx4* in their respective limb fields (Saito et al., 2002). *Tbx5*$^{del/del}$ embryos do not form forelimb buds, so it may be argued that expression of *Pitx1* and *Tbx4* may not be detected in the absence of limb buds, but these genes are expressed in the hindlimb LPM field far in advance of limb bud outgrowth (our data) (Gibson-Brown et al., 1996; Gibson-Brown et al., 1998; Isaac et al., 1998; Logan et al., 1998; Ohuchi et al., 1998; Saito et al., 2002), and expression of *Tbx5* in the forelimb is intact in mice that lack limb buds due to ablation of *Fgf10* (Sekine et al., 1999). Therefore one would anticipate that a molecular conversion of forelimb to hindlimb identity in *Tbx5*$^{del/del}$ embryos would indeed be detected. In addition, examination of *Tbx4* expression in *Tbx5*$^{del/del}$ embryos at E9, prior to the initiation of limb bud outgrowth, did not reveal induction of Tbx4 in the limb field. Furthermore, conditional deletion of *Tbx5* using Prx1-Cre mice, which deletes the *Tbx5* gene in the limb at E9.5, does not result in forelimb to hindlimb conversion either (C. Rallis, B. G. Bruneau, J. Brough, C. E. Seidman, J. G. Seidman, C. J. Tabin and M. P. Logan, unpublished). T-box genes may be involved in later aspects of limb identity, but based on our data and on explant experiments performed in chicken embryos (Saito et al., 2002), they do not seem to be sole regulators of early forelimb versus hindlimb identity.

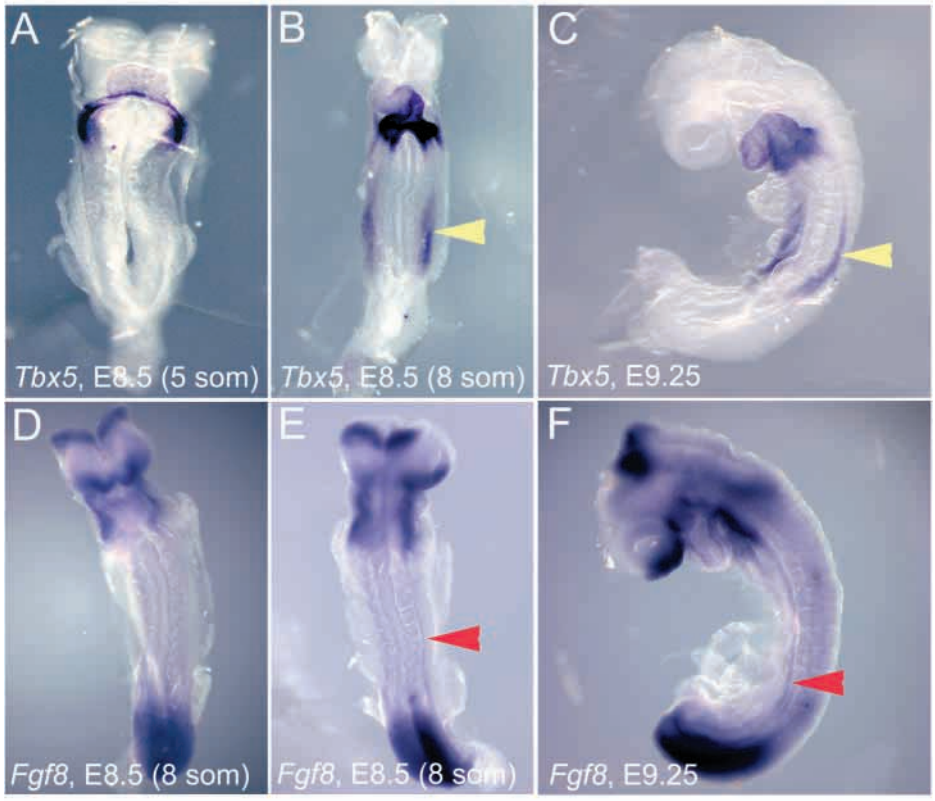


Fig. 4. *Tbx5* expression precedes *Fgf8* expression in the limb field. Expression of *Tbx5* (A-C) is shown at E8.5 (five-somite stage, A), E8.5 (eight-somite stage, B) and E9.25 (C). Expression of *Fgf8* (D-F) is shown at E8.5 (eight-somite stage, D,E) and E9.25 (F). Yellow arrowheads indicate *Tbx5* expression in the forelimb precursors, beginning at the eight-somite stage (B). This expression was readily detectable in all embryos examined at this stage and beyond (B,C). Red arrowheads indicate weak *Fgf8* expression in the intermediate mesoderm, beginning at the eight-somite stage. Note that this weak expression was observable in only a few embryos at this stage (compare D with E), but was reproducibly detected at E9.25 and beyond (F).

Tbx5 and the transition from limb field to limb bud

In *Tbx5*$^{del/del}$ embryos, the establishment of a patterned limb field is intact, while all signs of limb bud outgrowth are absent, including all known regulators of limb bud outgrowth. This is consistent with data showing that limb bud outgrowth at the proper location along the AP axis of the LPM is established at stages 13-15 in chicken embryos, the stage at which *Tbx5* begins to be expressed in the LPM destined to become the forelimb (Kieny, 1969; Pinot, 1970;

Saito et al., 2002; Stephens et al., 1989). It has been suggested that evolutionarily conserved Hox functions are essential for the AP patterning of the LPM (Cohn et al., 1997; Cohn and Tickle, 1999; Popperl et al., 2000; Rancourt et al., 1995). In particular, in the zebrafish *lazarus* mutant, in which all Hox function is abolished, the establishment of the limb fields, including expression of *Tbx5* in the precursors of the pectoral fin, is absent (Popperl et al., 2000). In zebrafish, it has been suggested that Tbx5 controls mesodermal cell migration into the limb field (Ahn et al., 2002). Our observation that the limb field is intact in mouse embryos lacking *Tbx5* shows that this mechanism is not operative in mammals. Therefore *Tbx5* is not essential for establishment of the limb field.

What, then, is the role of *Tbx5* in early limb formation? We propose that *Tbx5* is a primary and direct initiator of limb bud outgrowth following patterning of the limb field. Supporting this possibility is the observation that a limb-specific deletion of *Tbx5* also results in an absence of limb bud outgrowth (C. Rallis, B. G. Bruneau, J. Brough, C. E. Seidman, J. G. Seidman, C. J. Tabin and M. P. Logan, unpublished), and introduction of a dominant-negative TBX5 molecule in the limb field also abrogates limb formation (C. Rallis, B. G. Bruneau, J. Brough, C. E. Seidman, J. G. Seidman, C. J. Tabin and M. P. Logan, unpublished) (J. Takeuchi and T. Ogura, personal communication). Most importantly, *Tbx5* misexpression can cause the induction of an entire limb from the LPM (J. Takeuchi and T. Ogura, personal communication), or an additional limb-like structure from the limb-forming mesoderm (Ng et al., 2002). Furthermore, this role is evolutionarily conserved in all tetrapods, from zebrafish to humans (Ahn et al., 2002; Basson et al., 1997; Garrity et al., 2002; Li et al., 1997; Ng et al., 2002). Therefore *Tbx5* is necessary and sufficient for limb bud outgrowth. *Tbx5* encodes a transcription factor, and is likely to cause limb bud induction by direct activation of downstream targets such as *Fgf10*. Indeed, *Fgf10* expression is never initiated in *Tbx5*-deficient embryos, and we have shown that TBX5 activates the *Fgf10* gene directly via a conserved TBX5-binding site within the *Fgf10* promoter, thus providing a direct and simple mechanism for induction of limb bud outgrowth.

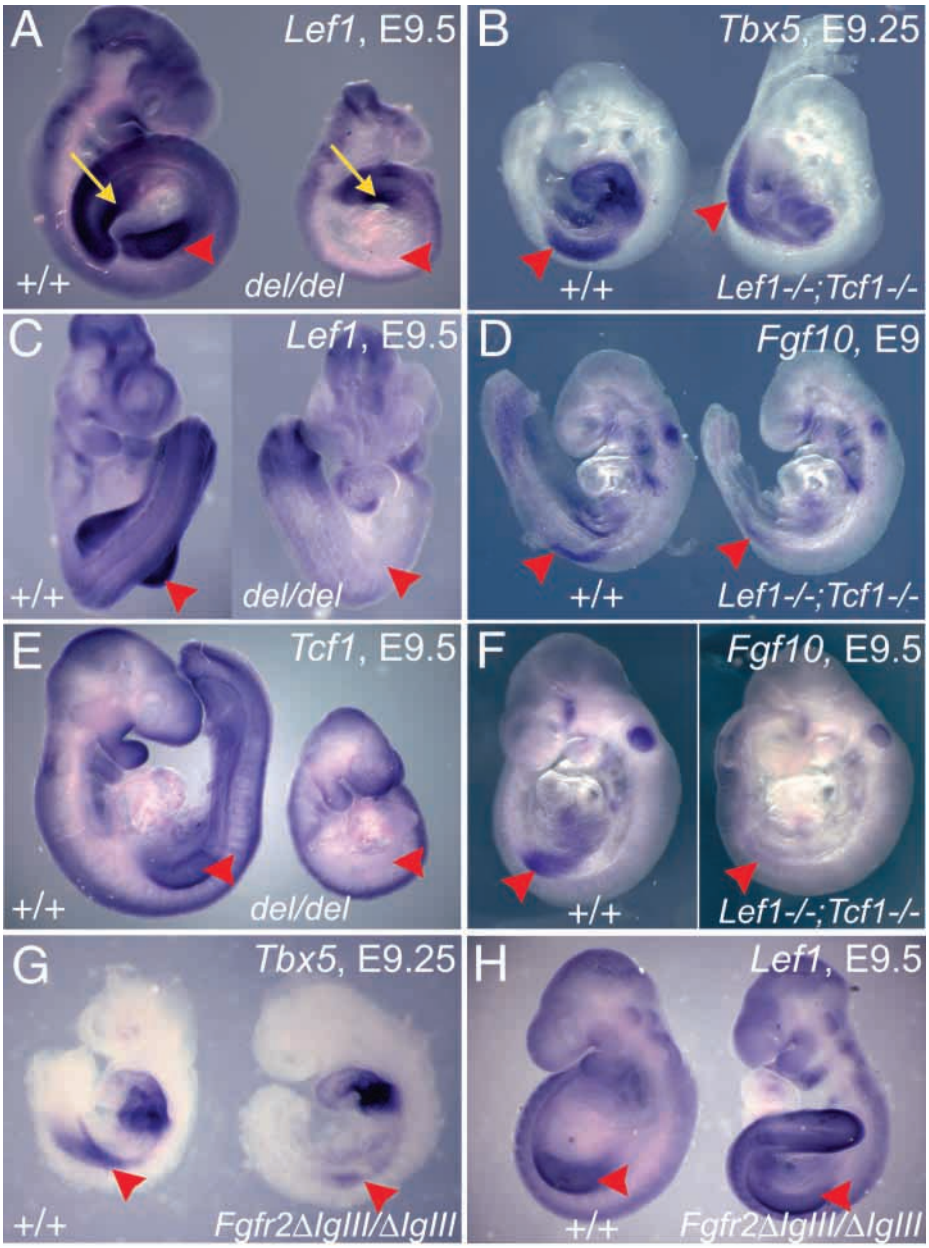
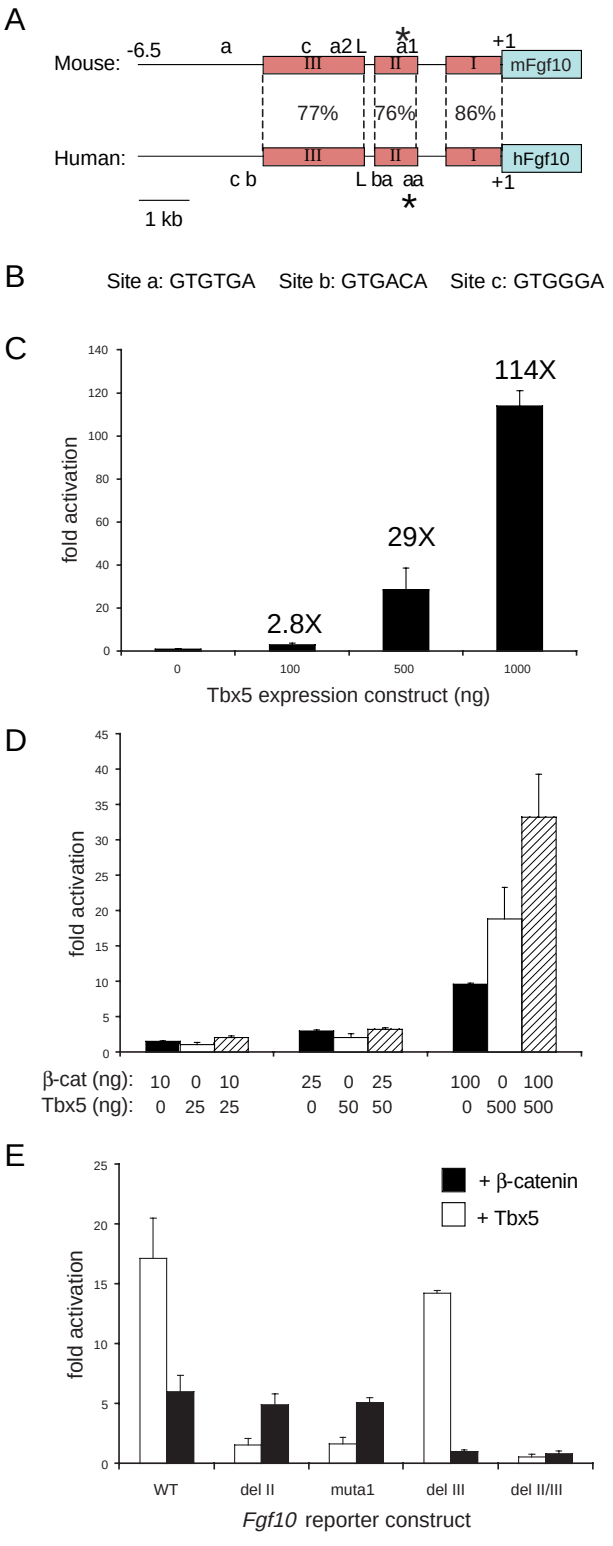


Fig. 5. *Tbx5* is upstream of Wnt and FGF signaling. *Lef1* and *Tcf1* expression is undetectable in the forelimb field of $Tbx5^{del/del}$ embryos (arrowheads in A,C,E), but is intact elsewhere in the embryo, including the hindlimb field (yellow arrows in A). Expression of *Tbx5* is intact in $Lef1^{-/-};Tcf1^{-/-}$ embryos (B). *Fgf10* expression is initiated, but expressed at lower levels than in wild type in the forelimb field of $Lef1^{-/-};Tcf1^{-/-}$ embryos at E9 (D) and E9.5 (F). *Tbx5* (G) and *Lef1* (H) expression is unaffected in $Fgfr2^{\Delta IgIII/\Delta IgIII}$ embryos. Arrowheads in B,D-H indicate the forelimb field.

In contemplating the potential role of TBX5 as initiator of limb bud induction, one must consider that evidence exists to indicate a role for signaling from the NM to the LPM to initiate limb bud outgrowth (Geduspan and Solursh, 1992; Stephens and McNulty, 1981; Strecker and Stephens, 1983). FGFs derived from the NM, particularly FGF8, have been proposed to be the operative molecules in this model (Crossley et al., 1996; Martin, 1998; Vogel et al., 1996). Data exist that dispute this model, and the pieces of the puzzle have not been reconciled (Fernandez-Teran et al., 1997; Martin, 1998).

Indeed, preventing posterior migration of the NM in chicken embryos (Fernandez-Teran et al., 1997) or disrupting differentiation of the NM in mouse (M. Bouchard and M. Busslinger, personal communication) does not lead to abnormal limb formation. We propose that if a role in limb bud initiation exists for axial signaling, it might be to support *Tbx5* expression in conjunction with patterning of the LPM by Hox genes (Popperl et al., 2000) or to confer competence to the LPM for induction of limb bud outgrowth by Tbx5. The role of FGF10 in maintenance but not initiation of *Tbx5* expression in the limb bud indicates that FGFs do play a role in supporting *Tbx5* expression, although they may not be involved in its initiation (Sekine et al., 1999). Supporting this possibility, we have observed that mice lacking FGFR2b/FGFR2c also have decreased *Tbx5* mRNA levels, but initiate *Tbx5* expression normally. A role for FGFs in conferring competence to the LPM would also be consistent with their expression throughout the LPM and IM.



Hierarchies of signaling in limb bud outgrowth

Tbx5 is clearly upstream of FGF and Wnt signaling in the developing limb bud, as shown by the decreased expression of *Fgf10*, *Fgf8*, *Snai1*, *Pea3*, *Lef1* and *Tcf1* in the prospective limb field of *Tbx5* knockout embryos, and the intact expression of *Tbx5* in *Lef1*$^{-/-}$;*Tcf1*$^{-/-}$ and *Fgfr2*$^{\Delta IgIII/\Delta IgIII}$ embryos. It has additionally been demonstrated that *Tbx5* expression is normally initiated in mice that lack FGF10 (Sekine et al., 1999). Furthermore, our genetic and transactivation data indicates that Wnt signals act in concert with TBX5 to activate *Fgf10* fully in the developing limb bud. We have shown in vivo that *Fgf10* requires *Tbx5* for initiation of its expression, whereas Wnt signaling via LEF1 and TCF1 is required to sustain high levels of *Fgf10* expression. This is supported by our in vitro transactivation data, which show powerful activation of the *Fgf10* promoter by TBX5, and lower levels of activation by β-catenin. This is similar to the interaction of VegT and Wnt signaling in *Xenopus* organizer formation (Xanthos et al., 2002). However, it is not clear whether decreased *Fgf10* expression and abnormal limb development in *Lef1*$^{-/-}$;*Tcf1*$^{-/-}$ embryos is due to direct action of Wnts in the

Fig. 6. TBX5 activates the *Fgf10* promoter. (A) Schematic representation of the upstream regulatory sequences of mouse and human *Fgf10*. Regions of homology are indicated in red, and are numbered I, II and III; percent nucleotide identity is indicated between the two sequences. Potential TBX5-binding sites (TBEs) are shown as a, b or c, based on the different types of TBEs (see B). A conserved putative TBE is shown by the asterisk. A conserved putative Lef1/Tcf1-binding site is indicated by 'L'. (B) Delineation of the three types of TBEs in the *Fgf10* promoter. (C) Transactivation by TBX5 of the *Fgf10-luciferase* reporter construct in COS-7 cells. The reporter construct was transfected with increasing concentrations (0, 100, 500 or 1000 ng) of a *Tbx5* expression construct. Mean fold activation is indicated above each bar. (D) Transactivation of the *Fgf10-luciferase* reporter construct by an activated β-catenin (β-cat) construct alone (black bars) or with a *Tbx5* expression construct (white bars) or both (hatched bars). The amount of each plasmid transfected is indicated below the graph. (E) *Fgf10* promoter deletion analysis. Deletion constructs were co-transfected with the *Tbx5* expression construct (white bars) or the activated β-catenin expression construct (black bars). Deletion of region II (del II) or a point mutation of TBEa1 (muta1) did not affect activation by activated β-catenin, but greatly reduced (1.5 times less versus 18 times less) activation by TBX5. Deletion of region III (del III) results in decreased activation by β-catenin, but did not significantly affect activation by TBX5. Deletion of both regions (del II/III) eliminated activation by either construct. All results are expressed as fold increase in luciferase activity compared with the reporter construct alone. Data are shown as mean±s.d. for one representative experiment performed in triplicate.

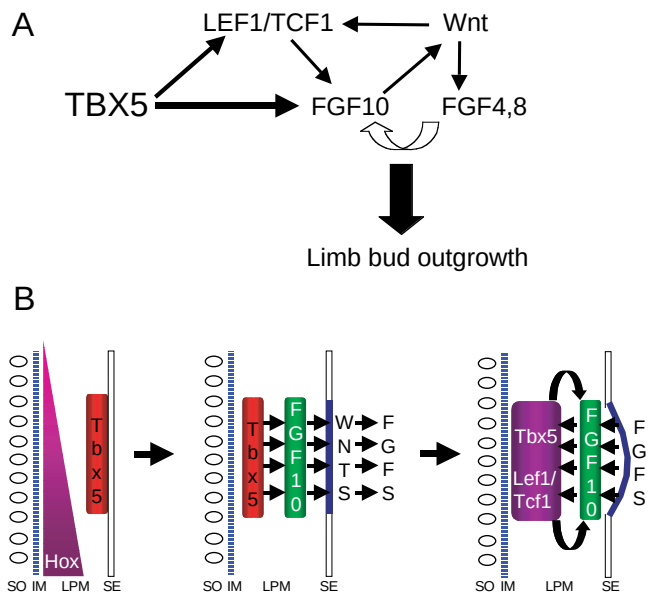


Fig. 7. A model for early stages of limb bud growth. (A) Schematic representation of genetic interactions in early limb bud initiation. The thicker the arrow, the more crucial the interaction. See text for details. (B) Major steps in early limb bud formation. TBX5 in the lateral plate mesoderm (LPM) activates Fgf10, which in turn signals to the surface ectoderm (SE) to activate WNTs and FGFs, which then signal back to maintain *Fgf10* levels, and subsequently *Tbx5* expression. Lef1 and Tcf1 cooperate with TBX5 to sustain *Fgf10* levels. SO, somites; IM, intermediate mesoderm.

mesenchyme, as previously suggested (Kawakami et al., 2001), or whether it reflects an absence of Wnt signaling from the AER (Kengaku et al., 1998), which is defective in the absence of LEF1 and TCF1 (Galceran et al., 1999). Alternatively, FGF signals downstream of *Tbx5* may be essential for mesenchymal activation of Wnt pathways, as shown in the primitive streak of the mouse (Ciruna and Rossant, 2001).

Together, these results suggest that *Tbx5* is upstream of Wnts and *Fgf10* to initiate limb bud outgrowth and initiates the FGF feedback loop required for early limb development by activating expression of *Fgf10*. Although Wnt signaling is not required for initiation of *Fgf10*, Wnt signals act additively in concert with TBX5 to maintain appropriate levels of *Fgf10* expression in the developing limb. The potential hierarchies regulating limb bud outgrowth are summarized in Fig. 7.

Conclusions

In summary, we have shown that *Tbx5* is required for the initiation of limb bud outgrowth after the patterning of the limb field from lateral plate mesoderm. In the developing mouse heart, *Tbx5* is maximally expressed in the posterior segments that give rise to atrium and left ventricle (Bruneau et al., 1999). In *Tbx5*$^{del/del}$ embryos, normal AP patterning is maintained in these segments of the developing heart, while growth of these structures is severely impaired (Bruneau et al., 2001). Therefore, AP patterning of the developing heart is independent of *Tbx5*, while early differentiation and subsequent embryonic growth of specific segments of this organ depends on *Tbx5*. We have shown that similar growth dependency exists in the developing limb, where establishment of the limb field is independent of *Tbx5*, while initiation of proximodistal limb bud outgrowth requires intact *Tbx5* expression. *Tbx5* appears to initiate limb bud formation at least in part by direct transcriptional activation of the *Fgf10* gene.

In Holt-Oram syndrome, which is caused by *TBX5* haploinsufficiency (Basson et al., 1997; Li et al., 1997), limb defects range in severity: more common manifestations are defects of the thumbs or carpal bones, but occasionally phocomelia (severe reduction or absence of zeugopod and stylopod) is observed (Basson et al., 1994; Newbury-Ecob et al., 1996). In these severe cases, it is likely that limb outgrowth signals such as FGF10 and FGF8 are affected because of decreased *TBX5* levels. In fact, mice with an AER-specific deletion of *Fgf8* develop limb abnormalities reminiscent of those found in severe cases of Holt-Oram syndrome (Lewandoski et al., 2000; Moon and Capecchi, 2000). Similar pathways may also be operative in other disorders caused by T-box gene mutations, such as Ulnar-mammary syndrome (OMIM 181450), which is caused by dominant mutations in *TBX3* (Bamshad et al., 1997), or 22q11 deletion syndrome (OMIM), which is caused by *TBX1* haploinsufficiency (Jerome and Papaioannou, 2001; Lindsay et al., 2001; Merscher et al., 2001). Our results place *Tbx5* as the earliest determinant of limb bud outgrowth, establish a firm molecular basis for the implied parallels between limb and heart development, and suggest a common pathway for the differentiation and growth of embryonic structures downstream of T-box transcription factors.

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