**Tbx5 and Tbx4 Are Not Sufficient to Determine Limb-Specific Morphologies but Have Common Roles in Initiating Limb Outgrowth**

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**Summary**

Morphological differences between forelimbs and hindlimbs are thought to be regulated by Tbx5 expressed in the forelimb and Tbx4 and Pitx1 expressed in the hindlimb. Gene deletion and misexpression experiments have suggested that these factors have two distinct functions during limb development: the initiation and/or maintenance of limb outgrowth and the specification of limb-specific morphologies. Using genetic methods in the mouse, we have investigated the roles of Tbx5, Tbx4, and Pitx1 in both processes. Our results support a role for Tbx5 in the initiation of limb outgrowth, but not for Pitx1, in initiation of limb outgrowth. In contrast to conclusions from gene misexpression experiments in the chick, our results demonstrate that Tbx5 and Tbx4 do not determine limb-specific morphologies. However, our results support a role for Pitx1 in the specification of hindlimb-specific morphology. We propose a model in which positional codes, such as Pitx1 and Hox genes in the lateral plate mesoderm, dictate limb-specific morphologies.

**Introduction**

Vertebrate forelimbs and hindlimbs are serially homologous structures. Although the limb buds from which they are derived are patterned by common signals during embryonic development, they ultimately form morphologically distinct structures. A question that arises is how cells exposed to common signals can respond differentially and give rise to distinct morphologies.

Vertebrate forelimbs and hindlimbs arise from regions of the lateral plate mesoderm (LPM) at defined locations along the rostral-caudal axis of the embryo. Transplantation experiments in the chick have demonstrated that limb-type specification, the process by which cells of the prospective limb-forming territories are instructed to form either forelimb or hindlimb, occurs prior to the initiation limb bud outgrowth (reviewed in Logan, 2003; Saito et al., 2002; Stephens et al., 1989).

Three genes have been identified that fulfill many of the criteria to be candidates to specify limb-type identity. Two T-box transcription factors, Tbx5 and Tbx4, are expressed in the LPM of either the prospective forelimb or hindlimb region, respectively (Chapman et al., 1996; Gibson-Brown et al., 1996). In addition, a paired-related homeodomain factor, Pitx1, is expressed in the prospective hindlimb region but not in the developing forelimb (Lamonerie et al., 1996; Logan et al., 1998; Shang et al., 1997). The limb-type restricted expression pattern of these genes is retained throughout limb development. Misexpression experiments in the chick have suggested that these genes are involved in specification of limb-specific morphologies. Ectopic expression of Tbx5 in the chick leg bud can induce a partial leg-to-wing transformation. Conversely, misexpression of Tbx4 in the chick wing bud is able to cause a partial wing-to-leg transformation (Rodríguez-Esteban et al., 1999; Takeuchi et al., 1999). Similarly, misexpression of Pitx1 in the wing bud leads to the development of limbs with leg-like characteristics (Logan and Tabin, 1999; Szeto et al., 1999; Takeuchi et al., 1999). Accordingly, in Pitx1 mutant mice, hindlimbs show a loss of hindlimb characteristics (Lancot et al., 1999; Szeto et al., 1999).

Gene deletion and knockdown experiments have shown that Tbx5, Tbx4, and Pitx1 are required for the initiation and/or maintenance of limb bud outgrowth. Functional knockdown of zebrafish tbx5 results in a failure to initiate pectoral fin bud formation (Ahn et al., 2002). Similarly, all skeletal elements of the forelimb are missing in a limb-restricted Tbx5 knockout (Rallis et al., 2003). One of the earliest molecular read-outs of limb initiation is the expression of Fgf10 in the prospective limb fields (Min et al., 1998; Sekine et al., 1999). When Tbx5 is inactivated, Fgf10 is never expressed in the prospective forelimb region (Agarwal et al., 2003). Tbx5 is therefore required for the induction of Fgf10 in the LPM at limb bud stages, which leads to forelimb bud initiation. In Tbx4+/− embryos, induction and initial patterning of the hindlimb appears normal, but it fails to develop further, and Fgf10 expression is not maintained in the hindlimb bud mesenchyme (Naiiche and Papaioannou, 2003). Pitx1 mutant mouse hindlimbs also display an outgrowth defect, although much less severe than that observed in either Tbx5 or Tbx4 mutants. Pitx1−/− hindlimbs are smaller than wild-type, yet the skeletal elements, with the exception of the ilium, are present (Lancot et al., 1999; Szeto et al., 1999).

We have used loxP/Cre technology in combination with transgenic methods in the mouse to disrupt and replace Tbx5 function in the forelimb. Our assay involves attempting to rescue the no-forelimb phenotype of the Tbx5 limb-restricted knockout (Rallis et al., 2003) by expressing either Tbx4 or Pitx1, or both genes simultaneously, in the forelimb-forming region where Tbx5 function has been specifically deleted. This genetic assay, which we refer to as the limb-rescue assay, allows us to test the properties of these factors in two processes of limb development: (1) initiation of limb outgrowth and (2) specification of limb-specific morphologies. We show that Tbx4 can replace the function of Tbx5 and rescue limb outgrowth, whereas Pitx1 cannot. In contrast to previous chick misexpression studies, Tbx4-rescued limbs have a forelimb-like phenotype, suggesting that Tbx4 alone is not able to dictate hindlimb-specific morphology and that forelimb characteristics can develop in the absence of Tbx5. To determine whether Pitx1 can
transform a forelimb to a more hindlimb-like character, we analyzed the morphology of Pitx1 transgenic forelimbs (expressing endogenous Tbx5), and we rescued the no-forelimb phenotype of the Tbx5 limb-restricted knockout by supplying both Tbx4 and Pitx1 simultaneously. In both cases, we observed a partial forelimb-to-hindlimb transformation, suggesting that Pitx1 does play a role in the specification of hindlimb-specific morphologies but that other factors may also be required.

Results

Generation and Characterization of Transgenic Lines

Conditional deletion of Tbx5 in the developing limbs leads to the complete absence of all forelimb elements (Rallis et al., 2003). We have exploited this genetic background and developed an assay to test the ability of Tbx4 and Pitx1 to rescue the forelimb defect that results from the absence of Tbx5 function.

To distinguish transgene-derived expression of Tbx4 and Pitx1 from endogenous expression, the chick cDNAs of each gene were placed under the regulation of the Prx1 regulatory element (Martin and Olson, 2000). To enable detection of transgene-derived protein, the cDNAs were tagged with the HA epitope (Figure 1A). Two independent lines were generated for both Tbx4 and Pitx1 and denoted 4.1 and 4.48 and P1.1 and P1.2, respectively. In all four lines, the transgene is expressed in the hindlimb and forelimbs as well as the cranial mesenchyme and body wall (Figures 1B–1E), consistent with previous observations of Prx1-driven transgenes (Logan et al., 2002). Transgene expression in the limbs corresponds to overexpression of either Tbx4 or Pitx1 in the hindlimbs and ectopic expression of these genes in the forelimbs. Western blot analyses and detection with an anti-HA antibody showed differences in the levels of protein expression. The Prx1-Tbx4(4.48) and Prx1-Pitx1(P1.1) lines express higher levels of protein than the Prx1-Tbx4(4.1) and Prx1-Pitx1(P1.2) lines (Figure 1F).

Tbx4, but Not Pitx1, Can Rescue Limb Outgrowth in the Absence of Tbx5

Tbx5 is required for initiation and outgrowth of the forelimb (Agarwal et al., 2003; Ahn et al., 2002; Rallis et al., 2003). Using a conditional allele of Tbx5 and a Prx1-Cre deleter transgenic line, we have previously shown that in the absence of Tbx5 function, the forelimb fails to form (Figure 2B; Rallis et al., 2003). To investigate whether Tbx4 or Pitx1 are capable of replacing the function of Tbx5 in the forelimb, we crossed the Tbx4 and Pitx1 transgenic lines into the genetic background of the conditional deletion of Tbx5 in the limb (Tbx5\textsuperscript{lox/lox};Prx1-Cre). Heterozygote Tbx5 mice (Tbx5\textsuperscript{lox/};Prx1-Cre), which form normal forelimbs (Rallis et al., 2003), serve as controls (Figure 2A). One of the Tbx4 lines (4.48) was capable of rescuing the forelimb defect in the Tbx5\textsuperscript{lox/lox};Prx1-Cre mice (Figure 2C), and a limb formed in the forelimb region. This demonstrates that Tbx4 can replace the function of Tbx5 in limb outgrowth. The Tbx4(4.1) line, which expresses much lower levels of Tbx4 protein (Figure 1F), was not able to rescue forelimb development (Figure 2D), suggesting that insufficient amounts of Tbx4 protein are produced. In contrast, none of the Pitx1-expressing lines were able to rescue limb outgrowth in the absence of Tbx5 (Figures 2E and 2F), although at least one line expresses Pitx1 at higher levels than Tbx4 in the Tbx4(4.48) line (Figure 1F). These data demonstrate that Tbx4, but not Pitx1, can replace the function of Tbx5 in controlling limb outgrowth.

The Tbx4-Rescued Limb Buds Are Normally Patterned

During limb development, a positive feedback loop between Fgf8, expressed in the apical ectodermal ridge (AER), and Fgf10, expressed in the mesenchyme, is essential for proximodistal outgrowth of the limb bud (reviewed in Martin, 1998). Shh expression in cells of the zone of polarizing activity (ZPA) in the posterior limb mesenchyme is required for the precise anterior-posterior patterning of the limb (Riddle et al., 1993). Expression of Fgf8, Shh, and Fgf10 in Prx1-Tbx4(4.48)-rescued
model using genetic techniques in the mouse, we analyzed the limb-type identity of the Prx1-Tbx4(4.48)-rescued limb buds and limbs. In addition to Tbx5, Tbx4, and Pitx1, certain Hox genes belonging to the HoxC cluster have a limb-type restricted expression pattern and have been implicated in limb-type specification (Nelson et al., 1996; Peterson et al., 1994). Hoxc4 and Hoxc5 are expressed in the forelimb, while Hoxc9, Hoxc10, Hoxc11, Hoxc12, and Hoxc13 are expressed in the hindlimb. Surprisingly, the Tbx4-rescued limb has a forelimb-type pattern of gene expression. Following deletion of Tbx5 and replacement with Tbx4, transcripts from the endogenous Tbx5 conditional allele that has been disrupted by Cre recombinase activity are still detected using a probe that recognizes sequences still present in the recombined, nonfunctional transcript (Figure 4G). Hence, signals that normally restrict Tbx5 expression to the forelimb (Figure 4A) are still functioning. Hoxc4 and Hoxc5, which are normally expressed in forelimb buds (Figures 4B and 4C), are also expressed in Prx1-Tbx4(4.48)-rescued limb buds (Figures 4H and 4I). Conversely, genes normally restricted to the hindlimb are not ectopically expressed in the Prx1-Tbx4(4.48)-rescued limbs. Tbx4, Pitx1, and Hoxc10 are expressed in the hindlimbs but not in the Prx1-Tbx4(4.48)-rescued limbs. Tbx4, Pitx1, and Hoxc10 are expressed in the hindlimbs but not in the Prx1-Tbx4(4.48)-rescued limbs (Figures 4J–4L), as seen in control littermates (Figures 4D–4F). In summary, we detect gene expression patterns characteristic of a forelimb in the rescued limb.

To further analyze the identity of the Prx1-Tbx4(4.48)-rescued limbs, we examined the skeletal morphology in newborn pups and compared them to forelimbs and hindlimbs from control littermates. The forelimb-type character of the rescued limb was evident (compare Figures 4N to 4M). Three main limb-type-defining features are noticeable: the presence of a scapula, the relative length of the stylopod and zeugopod bones, and the joint between stylopod and zeugopod elements. The scapula of Tbx4-rescued newborn pups is indistinguishable from that found in control littermates (Figure 4N, arrow). The bones of the stylopod and zeugopod articulate to form an elbow-like joint with the distal end of the humerus-like bone sitting in an apparent trochlear notch at the proximal end of the ulna-like bone (Figure 4N, arrowhead). In addition, the relative size of the stylopod and zeugopod bones is similar. This is most comparable to the arrangement in the forelimb, where the humerus is of equivalent length to the radius and ulna bones while in the hindlimb the femur is smaller than the tibia. Furthermore, both zeugopod bones in the rescued limb are of similar length, resembling the radius and ulna in forelimbs and not theibia and fibula in hindlimbs. Although overall the skeletal morphology of the Tbx4-rescued limb is remarkably similar to that of the forelimb, it is not completely identical. The deltoid tuberosity of the humerus is absent, and the flexure of the wrist is altered such that the hand extends directly from the wrist and fails to turn inward. In summary, both analyses of Tbx4-rescued limbs with limb-type restricted markers at limb bud stages and examination of the limb skeletal morphology in newborn pups demonstrate their forelimb-like phenotype, refuting the postulated role for Tbx4 in specifying hindlimb identity.

To determine whether these contradictory mouse/chick results were due to differences in the expression...
levels of Tbx4, we doubled the dose of the Tbx4 transgene by crossing the line to homozygosity in the Tbx5lox/lox;Prx1-Cre background. In these cases, although normal limb morphology is severely affected, forelimb-like characteristics can be still detected. A trocheal notch and an olecranon process in the ulna-like bone are clearly identifiable (Figures 4P and 4Q).

Forelimb-like Identity of Tbx4-Rescued Limbs after Ubiquitous Deletion of Tbx5

In the Prx1-Cre deleter line, Cre activity is first detected at E9.0–E9.5 (Logan et al., 2002). However, Tbx5 transcripts are first detected at E8.5 (Agarwal et al., 2003). From our previous work (Rallis et al., 2003) and the results presented here for the 4.1, P1.1, and P1.2 lines that were unable to rescue limb outgrowth (Figure 2), we have demonstrated that this transient expression of Tbx5 is not sufficient to initiate forelimb development. However, this observation raises the interesting possibility that a short pulse of endogenous Tbx5 transcript is sufficient to determine forelimb-specific morphology such that, following deletion of Tbx5 and replacement with Tbx4, a forelimb develops. To address this issue, we used the β-actin-Cre transgenic line (Lewandoski and Martin, 1997) to disrupt Tbx5 gene function ubiquitously in the early embryo and tested the ability of the Prx1-Tbx4(4.48) line to rescue limb outgrowth. Although these embryos die around E10.0 due to heart defects (Bruneau et al., 2001), they survive long enough to determine whether the appropriate set of normally limb-type restricted genes are expressed in these Prx1-Tbx4(4.48)-rescued limbs.

Analyses of Tbx5lox/lox;β-actin-Cre;Prx1-Tbx4(4.48) embryos show that Tbx4 is still able to rescue limb outgrowth even when the cells in the prospective forelimb field have never expressed endogenous Tbx5 (Figure 5A). Consistent with our results using the Prx1-Cre deleter line, these Tbx4-rescued limb buds have a forelimb-like gene expression pattern. The normally forelimb-restricted genes Tbx5, Hoxc4, and Hoxc5 are expressed in the Tbx4-rescued limb in a pattern indistinguishable from wild-type (Figures 5B–5D), while the normally hindlimb-restricted markers Tbx4, Pitx1, and Hoxc10 (Figures 5E–5G) are not ectopically expressed. These results demonstrate that Tbx4 is able to rescue limb outgrowth in cells in the forelimb-forming region that have never been exposed to Tbx5 activity and that the resultant limb expresses genes normally restricted to the forelimb. Furthermore, genes normally restricted to the hindlimb are not ectopically induced. This demonstrates that an initial pulse of Tbx5 expression is not able to determine forelimb-specific morphologies.

Pitx1 Can Partially Transform Forelimb to Hindlimb-like Morphologies

Pitx1 is also expressed in a hindlimb-restricted manner and has been implicated in specifying hindlimb-specific morphologies (Lanctot et al., 1999; Logan and Tabin, 1999; Szeto et al., 1999; Takeuchi et al., 1999). Pitx1-expressing transgenic lines were not able to rescue limb outgrowth following deletion of Tbx5. We were therefore unable to address the ability of this gene to influence limb-type identity in the absence of Tbx5. Instead, we have used our transgenic reagents to test the ability of Pitx1 to transform the forelimb-like morphology of the...
Tbx4-rescued limb and the wild-type forelimb expressing endogenous Tbx5.

We generated double-rescued embryos (Tbx5^lox/lox; Prx1-Cre;Prx1-Tbx4(4.48);Prx1-Pitx1(P1.1)) and Prx1-Pitx1(P1.1) transgenics and compared their limb skeletal elements to forelimbs and hindlimbs of control littermates (Figure 6) and to the Tbx4-rescued limb (Figure 4N). Following deletion of Tbx5 in cells of the forelimb-forming region and replacement with Tbx4 and Pitx1, the limb element that forms shares many morphological characteristics with a normal hindlimb. The articulation between the stylopod and zeugopod skeletal elements is strikingly knee-like (Figure 6B, arrowhead), while between the zeugopod and autopod it is ankle-like (Figure 6B, double arrowhead) compared to the normal forelimb (Figure 6A). The heads of the stylopod and zeugopod bones in the double rescue limb have a head-to-head apposition and the heads of each bone are larger and broadened, as is found in the knee (Figure 6F, dashed line). Moreover, the double-rescued limb zeugopodial element has a protrusion or tuberosity (Figure 6F, arrow), similar to that observed in the tibia (Figure 6H, arrow) that is not present in the radius (Figure 6E). Similarly, this head-to-head apposition and an extended tuberosity on the zeugopodial bone is present in the Prx1-Pitx1(P1.1) transgenic forelimb (Figure 6G, dashed line and arrow, respectively). In addition, at this articulation in the Prx1-Pitx1(P1.1) transgenic forelimb, the olecranon process and trochlear notch normally present in the ulna (Figure 6E, arrowhead) are absent (Figure 6G, arrowhead). Digits in the hindlimb are longer than digits in the forelimbs. In the double-rescued and the Prx1-Pitx1(P1.1) transgenic limbs, digit lengths are increased and therefore more similar to those in the hindlimb than the forelimb. To quantify these differences, we compared the ratio of the lengths of the second digit metacarpal/metatarsal and first phalange (yellow continuous versus dashed line in Figures 6I–6L). The ratio of metacarpal:phalange length is lower than 2 in the forelimb (Figure 6I, ratio 1.6), while in the hindlimb the second metatarsal is longer than twice the length of the first phalange (Figure 6L, ratio 2.5). In the double-rescued limb, this ratio is also above 2 (Figure 6J, ratio 2.6). Similarly, in the Prx1-Pitx1(P1.1) transgenic autopod, the ratio is also above 2 (Figure 6K, ratio 2.25). The length of the anterior zeugopodal bone in the double-rescued and Prx1-Pitx1(P1.1) transgenic limbs is longer than the stylopod bone, resembling the difference in femur/tibia length in the hindlimb rather than the similar length of the humerus/radius of the forelimb.

There are also differences between the Tbx4/Pitx1-rescued limb and the control hindlimb as well as the Prx1-Pitx1(P1.1) transgenic limb. The most obvious is the lack of a posterior zeugopodial bone, with the concomitant loss of the two posterior-most digits. This could be the result of gene dosage effects and may
Common Roles for Tbx5 and Tbx4 but Not for Pitx1 during Limb Bud Outgrowth

Our results demonstrate that Tbx4, but not Pitx1, is able to rescue limb outgrowth in the absence of Tbx5 (Figure 2), suggesting that Tbx5 and Tbx4 play identical biochemical roles in forelimb and hindlimb outgrowth, respectively. In Tbx4-rescued limb buds, outgrowth is initiated and the limb bud generated is normally patterned (Figure 3). Consistent with our observations, Tbx4 can induce the formation of an additional limb when misexpressed in the interlimb flank of a chick embryo (Takeuchi et al., 2003). In contrast, Pitx1 is not able to induce ectopic limbs when similarly misexpressed (M.P.L., unpublished), consistent with our observation that Pitx1 alone is not able to rescue limb formation in the Tbx5 mutant. Furthermore, Pitx1 null mice hindlimbs, although smaller, possess all the skeletal elements with the exception of the ilium (Lancot et al., 1999; Szeto et al., 1999), suggesting it has only a minor role in limb outgrowth.

A role for Tbx5 in initiation of limb outgrowth has been demonstrated in a range of species (reviewed in Logan, 2003). However, a similar role for Tbx4 in hindlimb initiation has not been demonstrated. In Tbx4 null mice, hindlimb bud formation is initiated normally, although after E10 further outgrowth is disrupted (Naiche and Papaiannou, 2003). One explanation for the normal initiation of hindlimb development in the Tbx4 null could be that other factor(s) compensate for the loss of Tbx4. We predict that Pitx1 is not such a compensatory factor because it is not capable of rescuing the Tbx5 null limb phenotype.

One of the earliest defects in Tbx5 null mice is the absence of Fgf10 expression in the LPM, which is required for outgrowth of the limb bud. Moreover, it has been shown that the Fgf10 promoter contains T-box binding sites and that Tbx5 is able to directly upregulate Fgf10 expression (Agarwal et al., 2003). The failure to maintain Fgf10 expression in Tbx4 mutant hindlimbs suggests that Tbx4 may also recognize these Tbx binding sites and activate Fgf10 expression (Naiche and Papaiannou, 2003). Consistent with the idea that Fgf10 may be a common target of Tbx5 and Tbx4, both genes are necessary to activate the expression of Fgf10 in the lung mesenchyme (Cebra-Thomas et al., 2003). We predict that in Tbx4-rescued limbs, Tbx4 binds to T-box binding sites in the Fgf10 promoter to activate expression and initiate limb outgrowth.

Discussion

Common Roles for Tbx5 and Tbx4 Maintains Its Forelimb Identity

All embryos are Tbx5<sup>mmy</sup>/p-actin-Cre;Prx1-Tbx4<sup>4.48</sup> at E10 and are shown in dorsal views. Tbx4-rescued limbs are indicated with arrowheads. (A) Rescued limb. (B–D) Endogenous Tbx5 (B), Hoxc4 (C), and Hoxc5 (D) are expressed in the rescued limb buds. (E–G) Hindlimb-restricted Tbx4 (E), Pitx1 (F), and Hoxc10 (G) are expressed in the hindlimb (arrows) but not ectopically expressed in the rescued limb.

reveal a role for Pitx1 and Tbx4 in regulating cellular responses to signals required for correct anterior-posterior patterning of the limb. In summary, the addition of Pitx1 into Tbx4-rescued limbs or wild-type forelimbs causes a partial forelimb-to-hindlimb transformation, confirming a role for Pitx1 in specification of hindlimb-specific morphologies. However, these limbs are not entirely transformed to a hindlimb, suggesting that other factors are required to dictate complete hindlimb characteristics and/or that factors in the forelimb region (other than Tbx5) prevent complete forelimb-to-hindlimb transformation. Interestingly, Hoxc10 is not ectopically expressed in Tbx4/Pitx1-rescued limbs (Figure 6M), indicating that, in this assay, Tbx4 and Pitx1 are not sufficient to induce Hoxc10 expression and that the partial transformation in limb morphology we observe occurs independently of Hoxc10.
Outgrowth and Identity of the Vertebrate Limb

Figure 6. Pitx1 Transforms the Forelimb-like Morphology of a Normal Forelimb or a Tbx4-Rescued Limb to a More Hindlimb-like Morphology

(A–D) Alcian blue/Alizarin red staining of E17.5 control forelimb (A), Tbx4/Pitx1-rescued limb (B), Pitx1 transgenic forelimb (C), and control hindlimb (D) skeletal elements.

(E–L) Higher magnifications of the stylopod/zeugopod joint are shown for the control forelimb (E), Tbx4/Pitx1-rescued limb (F), Pitx1 transgenic limb (G), and control hindlimb (H) and of the autopod region of the control forelimb (I), Tbx4/Pitx1-rescued limb (J), Pitx1 transgenic limb (K), and control hindlimb (L). au, autopod; F, femur; Fi, fibula; H, humerus; ish, ishium; il, ilium; R, radius; Sc, scapula; st, stylopod; T, tibia; U, ulna; zg, zeugopod.

(M) A Tbx4/Pitx1-rescued embryo at E10.5. Hoxc10 is expressed in the hindlimb but not ectopically induced in the double-rescued limb bud (arrow).

Pitx1 Is a Candidate Axial Cue Required for Specification of Hindlimb-Specific Morphology

Pitx1 is expressed in a broad, caudal domain of the embryo prior to expression of Tbx4 in the presumptive hindlimb-forming region (Lamoneira et al., 1996; Logan et al., 1998). This appears to be an ancient arrangement that has been conserved during evolution, since the single, ancestral Pitx gene is also expressed in a caudal domain in amphioxus (Yasui et al., 2000). In our double-rescued embryos, in which Tbx5 is replaced with both Tbx4 and Pitx1, and in our Pitx1 transgenics, the morphology of the resultant limb is significantly more hindlimb-like than the limb element that forms following rescue with Tbx4 alone. The morphology of the limb elements that form following replacement of Tbx5 with Tbx4 and Pitx1 are not identical to those that form following ectopic expression of Pitx1 in a forelimb expressing endogenous Tbx5. However, we cannot conclude that Pitx1 is functioning differentially in the presence of either Tbx4 or Tbx5, since the levels of transgene-derived Tbx4 and endogenous Tbx5 are, most probably, different.
differences in levels of transgene-derived Tbx4 in comparison to endogenous Tbx5 are apparent since limb elements are absent in Tbx4/Pitx1-rescued limbs that do form in the Prx1-Pitx1 transgenic limb. Our results indicate that Pitx1 is a key downstream factor regulating hindlimb identity. This conclusion is consistent with experiments in the chick that demonstrated that misexpression of Pitx1 is capable of transforming the wing to a more hindlimb-like structure (Logan and Tabin, 1999; Szeto et al., 1999; Takeuchi et al., 1999) and gene deletion experiments in the mouse in which hindlimb characteristics are lost in Pitx1 mutants (Lanctot et al., 1999; Szeto et al., 1999).

Our data also suggest that Pitx1 may not be the single positional cue involved in the regionalization of rostral versus caudal domains in the embryo, subterritories of which will ultimately develop into either a forelimb or a hindlimb, respectively. Replacement of Tbx5 with both Tbx4 and Pitx1 or misexpression of Pitx1 in a Tbx5 wild-type background does not produce a complete forelimb-to-hindlimb transformation, presumably due to the absence of other necessary factors or due to the presence of factors that constitute a rostral/forelimb code. Adding support to this model, the limbs that form following replacement of Tbx5 with Tbx4 (Tbx4 rescue) and in Pitx1−− mice have different morphologies despite both expressing only Tbx4 and not Tbx5 or Pitx1. In the Tbx4 rescue, a limb is formed in a rostral (forelimb) territory and has a forelimb-like morphology. In contrast, the limb that forms in a caudal (hindlimb) domain in the Pitx1−− mice lacks any forelimb characteristics. These results indicate that Pitx1 requires additional factors to distinguish limb-specific morphologies.

Other candidates to specify limb-specific morphologies are the Hox genes. Specific combinations of Hox genes expressed in the embryonic LPM correlate well with the type of limb that will develop (Cohn et al., 1997), and changes in Hox gene expression domains are correlated with the absence of forelimbs in snakes (Cohn and Tickle, 1999). We propose a model (Figure 7) in which limb-specific identity and ultimately limb-specific morphology is specified by different combinatorial codes of factors in the LPM at rostral versus caudal positions. These factors may include a particular combination of Hox proteins and Pitx1. In response to an axial cue that triggers Tbx-mediated limb initiation at the prospective forelimb and hindlimb levels, Tbx5 is activated as a result of a combinatorial code of “rostral” Hox genes, whereas Tbx4 expression is initiated by a combinatorial “caudal” Hox code (Ruvinsky and Gibson-Brown, 2000). The activation of both Tbx5 and Tbx4 at the prospective forelimb and hindlimb level, respectively, is responsible for the initiation and outgrowth of the limb bud. We conclude that Tbx5 and Tbx4 are accurate markers of forelimb and hindlimb identity, respectively, but they do not themselves play a significant role in the specification of limb-type identity.

**Evolution of Tbx5 and Tbx4**

Two cognate gene pairs compose the Tbx2/3/4/5 subfamily of T-box transcription factors: the Tbx2/Tbx3 pair and the Tbx4/Tbx5 pair. These gene pairs have evolved from a single ancestral gene by unequal crossing over to form a two-gene cluster that was later duplicated and dispersed in the genome. The evolutionary history of these genes argues for a high degree of functional overlap between cognate genes (Agulnik et al., 1996). Furthermore, the residues that have been shown to be important for DNA binding and dimerization of the T-domain of Xenopus Brachyury (the prototype of the T-box family) are identical in mouse and chick Tbx4 and Tbx5 (data not shown). Therefore, the simplest model would predict that the Tbx4 and Tbx5 proteins share the vast majority of their target genes rather than having specific targets.

Several models have been proposed to explain the maintenance of duplicate genes in the genome after an initial phase of redundancy. Classical models propose that one of the duplicates will normally degenerate due to the accumulation of deleterious mutations. However, on rare occasions one of the copies may acquire a novel function, endowing the organism with a favorable, selected new function (Ohno, 1970). The duplication-degeneration-complementation (DDC) model was proposed to explain the higher number of duplicate gene copies maintained in duplicated genomes (such as the vertebrate genome) than classical evolutionary models would predict. The DDC model predicts that degenerative mutations in the regulatory elements can increase the probability of duplicate gene preservation and, importantly in our model for Tbx4 and Tbx5 function, that the usual mechanism of duplicate gene preservation is the partitioning of ancestral functions rather than the evolution of new functions. We suggest that the function of the Tbx4/Tbx5 gene pair has been partitioned to initiate outgrowth of two sets of serially homologous appendages. Both copies of the gene are then retained.

**Figure 7. A Model of the Mechanisms that Control Limb-Type Identity and Outgrowth of the Vertebrate Limbs**

Broad territories in the flank of the embryo capable of forming either a forelimb or a hindlimb are specified by distinct rostral and caudal combinatorial codes of factors in the LPM. These codes may include a combination of Hox genes in the rostral domain and a combination of Hox genes and Pitx1 in the caudal domain. In response to a putative axial cue, which may be common to both forelimb and hindlimb, cells in the domain that express a rostral code activate Tbx5, which is required for forelimb outgrowth. Cells in the caudal domain, which express a different positional code including Pitx1, respond to this axial cue and activate Tbx4, which is required for hindlimb outgrowth. IM, intermediate mesoderm; LPM, lateral plate mesoderm; NT, neural tube; so, somite.
because each duplicate carries out some of the essential functions that were previously accomplished by the ancestral gene (Force et al., 1999). In this example, both Tbx4 and Tbx5 would be retained, since each is essential for formation of the hindlimbs and forelimbs, respectively. Regulatory changes, rather than structural changes in the coding region (which tend to be deleterious), have been involved in nonlethal and rapid morphological variation and are therefore candidates to be important components of evolutionary changes. Interestingly, Pitx1 and Tbx4 have been involved in macroevolutionary changes related to the morphology of pelvic structures. The cartilage and bone elements of E17.5 mouse embryos and new-born pups were stained with alcian blue and alizarin red, respectively, essentially as described (Hogan et al., 1994).

**Experimental Procedures**

**Generation of Transgenic Lines**

The conditional allele of Tbx5 and Prx1-Cre transgenic have been described previously (Bruneau et al., 2001; Logan et al., 2002). The Prx1-Cre transgenic lines were generated by the NICHD Transgenic Mouse Development Facility operated by the University of Alabama at Birmingham. Prx1-Tbx4 transgenic lines were generated by the Procedural Services section, NIMR. The CDNAs for Tbx4(AF033670) and Pitx1(AF069397) were HA-tagged at their 3′ end. An insulator element (5′HS4) (Chung et al., 1993) was placed at the 5′ end of the construct. The SV40 polyadenylation signal and artificial intron sequence was placed at the 3′ end of the construct.

**Embryos**

Mouse embryos were staged according to Kaufman (2001). Noon on the day a vaginal plug was observed was taken to be E0.5 days of development. Mice carrying the conditional Tbx5 allele, Tbx5lox/H11001/H9252, were employed as previously described. The Prx1-Cre transgene was identified as previously described (Logan et al., 2002). The Prx1-Tbx4 and Prx1-Pitx1 transgenes were identified using a common reverse primer SV40rev and specific forward primers Tbx4wd, Pitx4wd, respectively. The β-actin-Cre transgene was identified using Cre forward and reverse primers. Details available on request. To generate rescued embryos (Tbx5lox/;Prx1-Cre;Prx1-Tbx4), mice were crossed to homozygous Tbx5lox/ mice. To obtain β-actin-Cre-deleted Tbx5-rescued embryos, adult heterozygotes Tbx5+/ mice (Tbx5lox/+;β-actin-Cre) were crossed to Tbx5lox/+;Prx1-Tbx4(4.48) mice. Heterozygote Tbx5 mice (Tbx5lox+/;Prx1-Cre or Tbx5lox/;β-actin-Cre) were used as controls.

**Western Blot Analysis**

Proteins from both forelimb and hindlimb buds of E10.5 transgenic embryos were obtained using a protein extraction buffer (50 mM Tris-HCl [pH 8.8], 0.5 mM EDTA, 1% SDS). Protein concentration was determined by Bradford method (Bradford, 1976) with a Coomassie protein assay reagent kit (Pierce) with bovine serum albumin as standard protein following manufacturer’s instructions. 4 μg of total protein was subjected to standard SDS-PAGE. Proteins were electrotransferred onto the Immob-Blot PVDF membrane (BIO RAD) using a XCell II Blot module (Invitrogen). The membrane was treated with 5% nonfat dry milk in a solution containing 50 mM Tris-HCl (pH 7.4), 0.5 M NaCl, and 0.1% Tween 20 (TBS-T) for 1 hr and then incubated overnight at 4 °C with anti-HA rat monoclonal antibody (Roche) at a 1:5000 dilution. Unbound antibodies were washed out with TBS-T. Incubation with peroxidase-conjugated goat anti-rat IgG (Calbiochem) at a 1:5000 dilution was performed at room temperature for 1 hr. Unbound antibodies were washed out with TBS-T. The SuperSignal West Pico Chemiluminescent Substrate (Pierce) was used for HRP detection. The membrane was subsequently exposed to CL-X posium Film (Pierce).

**Whole-Mount In Situ Hybridization**

Whole mount in situ hybridizations were carried out essentially as previously described (Riddle et al., 1993). All probes have been described previously: cTbx4, cPitx1, mTbx4, mTbx5 (Logan et al., 1998), mFgf10 (Crossley and Martin, 1995), mShh (Echelard et al., 1993), mFgf10 (Belluscii et al., 1997), mHoxc4, mHoxc5, mHoxc10 (Burke et al., 1995), and mPitx1 (Logan et al., 1998).

**Skeletal Preparations**

The cartilage and bone elements of E17.5 mouse embryos and new-born pups were stained with alcian blue and alizarin red, respectively, essentially as described (Hogan et al., 1994).

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