Tbx5 and Tbx4 trigger limb initiation through activation of the Wnt/Fgf signaling cascade

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SUMMARY

A tight loop between members of the fibroblast growth factor and the Wnt families plays a key role in the initiation of vertebrate limb development. We show for the first time that Tbx5 and Tbx4 are directly involved in this process. When dominant-negative forms of these Tbx genes were misexpressed in the chick prospective limb fields, a limbless phenotype arose with repression of both Wnt and Fgf genes. By contrast, when Tbx5 and Tbx4 were misexpressed in the flank, an additional wing-like and an additional leg-like limbs were induced, respectively. This additional limb formation was accompanied by the induction of both Wnt and Fgf genes. These results highlight the pivotal roles of Tbx5 and Tbx4 during limb initiation, specification of forelimb/hindlimb and evolution of tetrapod limbs, placing Tbx genes at the center of a highly conserved genetic program.

Key words: Tbx5, Tbx4, Limb initiation, Wnt, Fgf, Chick

INTRODUCTION

T-box (Tbx) genes play key roles during organogenesis and pattern formation in both vertebrate and invertebrate embryos. They encode a group of transcription factors characterized by a highly conserved DNA-binding motif (T-box) and its unusual mode of DNA recognition (Kispert and Herrmann, 1993; Muller and Herrmann, 1997). From a combination of embryological and genetic approaches, a picture is emerging that Tbx genes belong to highly conserved genetic networks (Papaioannou and Silver, 1998; Smith, 1999; Ruvinsky and Muller and Herrmann, 1997). From a combination of embryological and genetic approaches, a picture is emerging that Tbx genes belong to highly conserved genetic networks (Papaioannou and Silver, 1998; Smith, 1999; Ruvinsky and Herrmann, 1997). From a combination of embryological and genetic approaches, a picture is emerging that Tbx genes belong to highly conserved genetic networks (Papaioannou and Silver, 1998; Smith, 1999; Ruvinsky and Herrmann, 1997).

Recent studies have shown that members of the fibroblast growth factor (Fgf) family play key roles in the limb initiation (Martin, 1998; Martin, 2001). When applied locally in the lateral plate mesoderm, several Fgfs induce an ectopic limb (Cohn et al., 1995; Ohuchi et al., 1995; Crossley et al., 1996; Vogel et al., 1996; Ohuchi et al., 1997). In a current model, signaling of two different Fgfs, Fgf8 and Fgf10, is a key for the limb outgrowth (Martin, 2001). Although data highlight the pivotal roles of Fgfs, limb bud formation is initiated in Fgf10 knockout mice, suggesting that another factor(s) may induce limb initiation (Sekine et al., 1999; Min et al., 1998). Interestingly, limb buds of the Fgf10-null mice exhibit robust expression of Tbx5 and Tbx4 in the limb fields, although the limb buds cease to grow and remain flat (Sekine et al., 1999; Min et al., 1998). This observation indicates that Tbx5 and Tbx4 may be constituents of the a priori genetic program that acts upstream of the Fgf signaling cascade.

Recently, tight crosstalk between Fgf and Wnt proteins, another family of secreted factors, has been shown to control limb initiation in the chick embryo (Kawakami et al., 2001). Wnt2b and Wnt8c (which are expressed in the chick forelimb and hindlimb, respectively) are capable of inducing ectopic limbs in the flank. This additional limb formation is mediated through a β-catenin-dependent process and subsequent Fgf10 induction, placing Wnt proteins upstream of Fgf signaling. However, the intracellular events that control expression and signaling of these extracellular factors have not been elucidated. To date, Hox9 genes are known to be expressed in the lateral plate mesoderm and to demarcate the normal limb...
fields and the Fgf-induced additional limbs. This indicates that the Hox code, along the anteroposterior axis of the embryo, is one of the key determinants for the limb bud fields, implying that Hox genes act upstream of these signaling molecules (Cohn et al., 1997). Nonetheless, there is a gap between the Hox genes and the Wnt/Fgf network, in which putative transcription factor(s) act as initiator(s) of limb bud development. To explore this, we performed a series of experiments using both loss-of-function and gain-of-function approaches. Our data highlight the pivotal roles played by Tbx5 and Tbx4 during initiation of limb bud outgrowth.

MATERIALS AND METHODS

Chick embryos

Fertilized chick eggs were purchased from Takeuchi and Yamagishi poultry farms (Nara, Japan). Eggs were incubated at 39°C in a humidified incubator, and embryos were staged as described (Hamburger and Hamilton, 1951). Embryos were fixed in 4% paraformaldehyde (PFA) at 4°C overnight, washed three times, dehydrated with graded methanol, and then stored in 100% methanol at −30°C. To stain the skeletal elements, embryos were fixed in 5% Trichloroacetic acid (TCA) and stained with 0.1% Alcian Blue.

Plasmids construction

The coding regions of chick Tbx5 and Tbx4 were amplified by the PCR, and ligated into a pSLAX 12 Nco vectors (pSLAX-Tbx5 and Tbx4, respectively) (Morgan and Fekete, 1996). To construct the dominant-negative forms, a repressor domain of the Drosophila Engrailed gene (Jaynes and O’Farrell, 1991) was ligated into the pSLAX plasmid (pSLAX-En). Entire sequences of chick Tbx5 and Tbx4 were amplified by the PCR with primers, then ligated to pSLAX-En to create fusion genes (pSLAX-EnTbx5 and pSLAX-EnTbx4). In both cases, En gene was placed at the N termini. These fusion cDNAs were isolated and subcloned into a RCASBP retroviral vector and a modified pCAGGS vector (Niwa et al., 1991; Koshiba-Takeuchi et al., 2000).

Retrovirus preparation and injection

RCASBP-EnTbx5 and EnTbx4 plasmids were transfected into chick embryonic fibroblast cells isolated from SPF chick embryo (line c/o) trunks, then cultured for 1 week to allow the virus to spread. Cells were replaced with low-serum media, and incubated further for virion production. Then, media were collected and spun to concentrate virus particles as previously described (Morgan and Fekete, 1996). Then, media were replaced with low-serum media, and incubated further for virion production. Then, media were collected and spun to concentrate virus particles as previously described (Morgan and Fekete, 1996).

In ovo electroporation

In ovo electroporation was carried out as previously described (Takeuchi et al., 1999; Morose et al., 1999). We modified our in ovo electroporation techniques to obtain efficient expression of the transgenes in the limb buds. Briefly, a CUY-21 electroporator (Gene System, Osaka, Japan) was used. Two platinum electrodes (Gene System, Osaka, Japan) were used. An anode was inserted beneath the embryonic endoderm and a cathode was placed on the ectoderm surface. Then, a DNA solution was injected by a sharp glass pipette into the embryonic tissues. Electric pulses were applied (7-9 V, 60 mseconds pulse-on, 50 mseconds pulse-off, three to five times) during injection of the DNA solution.

Whole-mount in situ hybridization and probe isolation

In situ hybridization was performed as previously described (Wilkinson, 1993). Probes for chick Wnt8c, Fgf10 and Fgf8 were kindly provided by Drs Jane Dodd, Sumihare Noji and Juan Carlos Izpisua Belmonte (Hume and Dodd, 1993; Vogel et al., 1996; Ohuchi et al., 1997). Chick Pea3 was amplified by RT-PCR utilizing the published sequence, then subcloned into the pKRX vector (Schutte et al., 1997). The DNA fragment encoding the Env gene was isolated from the RCASBP retrovirus vector (Mogan and Fekete, 1996), and then subcloned into pBluescript SK(-) vector. Chick Wnt2b was also amplified by RT-PCR, then subcloned into the pKRX vector.

RESULTS

Misexpression of the dominant-negative Tbx5 and Tbx4 induces the limbless phenotype

Data obtained from Fgf10-null mice suggest that Tbx5 and Tbx4 act upstream of Fgf10 signaling (Sekeine et al., 1999; Min et al., 1998). To confirm this in chick embryos, Tbx5 and Tbx4 dominant-negative forms were constructed by fusing the Engrailed suppressor domain to their N termini (EnTbx5 and EnTbx4, respectively) (Jaynes and O’Farrell, 1991). When increasing amounts of these fusion proteins were introduced into cultured limb mesenchyme cells along with the Anf (atrial natriuretic factor) promoter-luciferase (Hiroi et al., 2001; Bruneau et al., 2001) or mouse Fgf10 promoter-luciferase reporter (a kind gift from Dr B. Bruneau), EnTbx5 and EnTbx4 repressed Tbx5- and Tbx4-dependent transactivation of these reporters, respectively, in a dose-dependent manner (data not shown). This suggests that the repressive action of the Engrailed suppressor domain, which was reported to be mediated through interaction with Groucho (Jimenez et al., 1997; Tolkunova et al., 1998), functions in the mesenchyme cells of limb bud. We inserted these fusion constructs into an RCAS retroviral vector and prepared infectious virions from chick embryonic fibroblast cells (Morgan and Fekete, 1996).

When the EnTbx5 retrovirus was infected into the right prospective wing field at stages 7 to 10, a completely wingless phenotype arose at E12 (Fig. 1A). As expected, wing formation was completely disturbed at the shoulder level (Fig. 1A’): the right scapula is missing, leaving hypomorphic ribs underneath (red arrowheads in Fig. 1B). In some cases, severe distal truncation was observed at E9 (Fig. 1B). Embryo skeletal preparations showed truncated wings (W’) with hypoplastic scapular (’s’) and clavicular bones (’c’) (Fig. 1B’).

When EnTbx4 was misexpressed in the right prospective leg field, two phenotypes were obtained. Infection at the early stages (stage 7 to 10) resulted in a completely legless phenotype (red arrowheads in Fig. 1C, C’). In this embryo, hypoplastic ilium and ischium were evident (’i’ and is’ in Fig. 1C, respectively). The pubis was absent, making the right side of the pelvis hypoplastic and deformed (Fig. 1C’).

When the EnTbx4 viral infection was performed at later stages (stage 11 to 13), the legless phenotype was not obtained, instead leg structure truncation occurred (red arrowheads in Fig. 1D). In this case, the right leg was small and severely distorted, as observed by Alcian Blue staining (red arrowhead in Fig. 1D’); a short and thin femur (’f’ in Fig. 1D’) and hypomorphic distal structures (red arrowheads in Fig. 1D’). In addition, the right ilium and ischium were small and deformed (’i’ and is’ in Fig. 1D’, respectively), and the pubis was missing from the pelvis right side (Fig. 1D’). These results indicate that Tbx5 and Tbx4 are directly involved in limb formation processes.
To examine the specificity of *EnTbx5* and *EnTbx4*, we misexpressed these genes in leg and wing buds, respectively. Misexpression of *EnTbx4* in the wing bud and *EnTbx5* in the leg did not induce any morphological abnormality at E8 (Fig. 1E,F, respectively). Whole-mount in situ hybridization for the *Env* gene encoded in the vector revealed expression of transgenes in limbs (Fig. 1E,F). Although signals were observed only on the limb surface, expression of transgenes and the resultant normal morphology suggest that *EnTbx5* and *EnTbx4* specifically abrogate development of the wing and leg buds, respectively.

**Stage-dependent action of the dominant-negative Tbx5 and Tbx4**

In a series of misexpression studies, the resultant phenotypes are dependent on viral infection timing. To explore further, we injected dominant-negative Tbx viruses at various developmental stages that included stages 7 to 13 (Table 1).

Interestingly, limbless phenotypes were obtained only when the viruses were injected between stages 7 and 10. Viral injection at later stages (10 to 13) failed to induce any limbless alterations, setting up a short critical period. As there is a lag between viral injection and full transgene expression in the RCAS vector, viral injection between stages 7 and 10 results in the full expression of *EnTx5* and *EnTbx4* in stages 11 to 14 (Mogan and Fekete, 1996). As previously reported, the crosstalk between Wnts and Fgfs and the subsequent limb induction process begin around stage 14 (Kawakami et al., 2001). This implies that early viral injection and subsequent expression of dominant-negative Tbx genes may affect processes that precede the Wnts and Fgf signaling cascades, highlighting the putative roles of Tbx genes in limb initiation, rather than in limb outgrowth maintenance. By contrast, limb truncation occurred even in the cases of later viral infection, although the ratio decreases as the limb development proceeds. This suggests that the dominant-negative Tbx expression at later stages only weakly affect the maintenance phase, in which the Wnt and Fgf signaling cascades primarily operate. Nonetheless, we cannot exclude the possibility that lower
Table 1. Stage-dependent actions of the dominant-negative Tbx5 and Tbx4

A Effect of misexpression RCAS-EnTbx5

<table>
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<th>Stage of infection</th>
<th>Total</th>
<th>Number with wingless phenotype</th>
<th>Number with wing truncations</th>
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<td>11</td>
<td>0 (0%)</td>
<td>1 (9%)</td>
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<td>13</td>
<td>10</td>
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<td>1 (10%)</td>
</tr>
<tr>
<td>Totals</td>
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B Effect of misexpression of RCAS-EnTbx4

<table>
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<th>Number with legless phenotype</th>
<th>Number with leg truncations</th>
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</tr>
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</table>

Wingless and legless phenotypes were obtained only when the viruses of the dominant-negative Tbx5 (EnTbx5) and Tbx4 (EnTbx4) were infected between stages 7 and 10. Viral injection at later stages (stage 10 to 13) induced limb truncation at a lower rate, revealing a critical period between stages 7 and 10. By contrast, misexpression of EnTbx5 in the leg and EnTbx4 in the wing did not induce morphological alteration even when infected at early stages.

Misexpression of the dominant-negative Tbx5 and Tbx4 represses the Wnt/Fgf signaling

Our data strongly suggest that Tbx5 and Tbx4 directly control limb initiation processes. To confirm this further, the expression of several genes in the EnTbx5- and EnTbx4-misexpressed limb buds was examined. In this case, the dominant-negative forms of Tbx5 and Tbx4 were misexpressed by in ovo electroporation, as the electroporation enables us to express two plasmids simultaneously. Consequently, we monitored the domains of transgene expression by co-electroporation of an EGFP (enhanced green fluorescent protein) expression plasmid (pCAGGS-EGFP) (Takeuchi et al., 1999; Momose et al., 1999).

When the dominant-negative EnTbx5 was misexpressed in the right prospective wing field, Wnt2b was downregulated in a region where GFP signals were evident (Fig. 2A,A’). Normal expression of this gene was observed in the lateral plate mesoderm of the left wing field (Kawakami et al., 2001). By contrast, when electroporation was performed in the right prospective leg field, Wnt8c expression was normal in a GFP-positive area (Fig. 2B,B’) (Hume and Dodd, 1993), implying that EnTbx5 misexpression specifically affects Wnt2b in the wing field, but not Wnt8c in the leg. When Fgf8 expression was analyzed, this gene was repressed in the right limb buds (red arrowheads in Fig. 2C). Normal expression of Fgf8 was evident in the AER on the contralateral side (Fig. 2C). As the EnTbx5 fusion gene was inserted in the RCAS retrovirus vector, in situ hybridization utilizing an Env probe visualizes the transgene expression domain. Nineteen hours after electroporation, robust Env expression was detected at the prospective wing field (arrowheads in Fig. 2D), indicating successful EnTbx5 misexpression in this area. As shown in serial sections, Fgf10 expression was downregulated in the same region of the embryo (arrowheads in Fig. 2E). Next, chick Pae3 expression was examined, as Pae3 encodes an Ets-type transcription factor that acts downstream of Fgf signaling (Roehl and Nusslein-Volhard, 2001; Raible and Brand, 2001). As expected, Pae3 expression was repressed (red arrow in Fig. 2F), whereas normal expression was visible in the entire prospective left wing region. These observations are in accordance with the hypothesis that Tbx5 is involved in early processes before the onset of the Wnt and Fgf protein interaction.

Next, the expression patterns of the same set of markers were examined in the EnTbx4-misexpressed embryo. In the EnTbx4-misexpressed leg buds, Wnt8c expression was repressed in a domain where GFP signals were observed (Fig. 2G,G’). Contrary to this, when EnTbx4 was misexpressed in the prospective wing, Wnt2b was normally expressed, although extensive GFP signals were evident (Fig. 2H,H’). This indicates that EnTbx4 represses Wnt8c in the leg, but not Wnt2b in the wing, implying that EnTbx4 specifically affects Wnt8c. Fgf10 expression was repressed (Fig. 2I), whereas the mesodermal cells on the left side showed normal expression patterns. When the EnTbx4 construct was electroporated in the leg field, clear repression of Fgf8 and Pae3 was evident (red arrowheads in Fig. 2K,L) where robust Env expression was observed (Fig. 2J), indicating that EnTbx4 also abrogates the early processes of limb initiation. In the limbs where the dominant-negative Tbx genes were misexpressed, apoptotic cell death was not detected by the TUNEL method (data not shown).
Fig. 2. Expression of the dominant-negative Tbx5 (EnTbx5) and Tbx4 (EnTbx4) induces repression of Wnt and Fgf genes, and Pea3. (A) When EnTbx5 was misexpressed in the prospective wing field by in ovo electroporation, Wnt2b expression was repressed 24 hours after electroporation (arrowheads), whereas this gene was normally expressed in the left side. (A’): Robust fluorescent signals derived from the co-electroporated EGFP gene were evident where Wnt2b was repressed. (B) By contrast, Wnt8c expression was normal 14 hours after electroporation of EnTbx5 in the prospective leg field. (B’): Robust GFP fluorescent signals were observed in the leg field. (C) Expression of Fgf8 was repressed in the hypoplastic right wing buds (red arrowheads). Normal expression of Fgf8 was evident in the left wing buds. (D) Robust Env expression indicated successful EnTbx5 misexpression in the prospective wing field 19 hours after electroporation (arrowheads). In such embryos, expression of Fgf10 (E) and Pea3 (F) was repressed, whereas the normal expression was evident on the opposite side of the embryo. (G) When EnTbx4 electroporation was performed in the prospective leg field, Wnt8c expression was repressed (arrowheads) where robust GFP signals were observed (G’). (H) When EnTbx4 was misexpressed in the prospective wing field, Wnt2b expression was unaffected, although this area showed strong GFP signals (H’). (I) Expression of Fgf10 was repressed in the hypoplastic leg buds (red arrowheads). Normal expression of this gene was evident in the left leg buds. (J) Thirty hours after electroporation, robust env expression was evident in the leg field (red arrowheads). In its serial sections, expression of Fgf8 (K) and Pea3 (L) was repressed (red arrowheads). (M-O) Top row shows wings and bottom row shows legs. Arrowheads indicate repression. (M) When a dominant-negative form of Lef1 (dnLef1) was misexpressed, expression of Fgf10 was weakly repressed in both dnLef1-misexpressed wing and leg fields 24 hours after electroporation. At this stage, expression of Tbx5 and Tbx4 was normal. (N) Repression of Fgf10 became evident 30 hours after electroporation. At this stage, expression of Tbx5 and Tbx4 was also weakly repressed. (O) Repression of Tbx5 and Tbx4 became evident 36 hours after electroporation.
Dominant-negative Lef1 does not affect Tbx5 and Tbx4 expression

As previously reported, Wnt signaling regulates Fgf10 expression in both the presumptive wing and leg buds, placing Wnts signaling upstream of Fgf10 (Kawakami et al., 2001). Although our data suggest that both Tbx5 and Tbx4 control expression of Wnt2b/Wnt8c, our data suggest that both Tbx5 and Tbx4 control expression of Fgf10. Although our data suggest that both Tbx5 and Tbx4 control expression of Wnt2b/Wnt8c, Fgf8 and Fgf10, misexpression data obtained from the EnTbx5 and EnTbx4 constructs do not clarify whether Tbx genes lie upstream of Wnt protein signaling, or vice versa. To elucidate this, an expression construct (CAGGS-dnLef1) for a dominant-negative form of Lef1 (Kengaku et al., 1998), the direct nuclear target of Wnt signaling, was constructed. In this experiment, a pCAGGS vector that has been shown to drive rapid and robust expression of transgenes in tissues was used (Niwa et al., 1991; Koshiba-Takeuchi et al., 2000). This construct was electroporated into both the prospective wing and leg fields at stages 9 to 11, and Tbx5 and Tbx4 expression was then examined. Twenty-four hours after electroporation, both Tbx5 and Tbx4 were normally expressed in the prospective wing and leg fields, respectively (Fig. 2M). At this stage, Fgf10 expression was evident in both the wing and leg fields, although weak repression was observed (Fig. 2M). Repression of Fgf10, Tbx5 and Tbx4 became evident 30 hours after electroporation (Fig. 2N). Thirty-six hours after electroporation, repression of Tbx5 in the wing and Tbx4 in the leg became obvious (Fig. 2O). As the Wnt signaling controls Fgf10 and Fgf8 expression, we speculate that the dnLef1 repressed Fgf10 and Fgf8, thereby indirectly downregulating Tbx5 and Tbx4 expression. Taken together, our data again suggest that Tbx5 and Tbx4 lie upstream of both the Wnt and Fgf signaling in limb induction. In these experiments, pCAGGS-EGFP was co-electroporated to visualize the domain of misexpression and GFP signals in the electroporated areas (data not shown).

Tbx5 misexpression in the flank induces an additional wing-like limb

If Tbx5 and Tbx4 are involved in the early processes of limb initiation, forced misexpression of these genes in the flank would be expected to induce the formation of additional limb buds, as observed in implantation of Fgf- or Wnt-expressing cells. As Tbx5 and Tbx4 specify the wing and leg identity of limb buds, respectively, Tbx5 and Tbx4 misexpression should induce an additional wing and leg, respectively. Chick wing is covered by feathers, whereas leg has scaled digits with claws and feathers in the proximal part. Based upon these morphological differences, we can identify which type of limb is formed by misexpression. Implantation of Wnt-expressing cells, for example, Wnt2b in the restricted flank region, induces a mosaic additional limb (Fig. 3A,A′) with wing-like morphology on the anterior side (W′) and leg-like structure on the posterior (L′) (Fig. 3A′). This additional limb formation is accompanied by the expression of Tbx5 (red arrow) and Tbx4 (blue arrow) on the anterior and posterior sides, respectively (Fig. 3B).

To confirm the identity of Wnt2b-induced additional limb buds, we checked expression of Hox9 genes. As reported previously, Hoxb9 and Hoxc9 are expressed in the leg, whereas Hoxd9 is expressed in the wing (Nelson et al., 1996; Cohn et al., 1997; Takeuchi et al., 1999). As expected, leg-specific Hoxb9 and Hoxc9 genes were expressed in the posterior side of the additional limbs, leaving the anterior side negative (Fig. 3C,C′). By contrast, wing-specific Hoxd9 was expressed mainly in the anterior part, although this gene did not show a clear boundary of expression (Fig. 3C′). Consequently, these observations are consistent with the mosaic nature of the Wnt2b-induced additional limbs (Fig. 3D).

For the misexpression experiments, Tbx5-EGFP and Tbx4-EGFP fusion genes were inserted into the pCAGGS expression vector (CAGGS-Tbx5-EGFP and CAGGS-Tbx4-EGFP). As previously reported, Tbx5-EGFP and Tbx4-EGFP fusion genes exhibit the same biological functions as the unmodified Tbx5 and Tbx4, respectively. In addition, the pCAGGS vector drives rapid transgene expression, making this system ideal for analyzing early developmental stages (Niwa et al., 1991; Koshiba-Takeuchi et al., 2000). When the CAGGS-Tbx5-EGFP expression construct was electroporated into the flank, a wing-like limb was formed (red arrowhead in Fig. 3E). Although this additional limb is smaller than the normal wing, its digits were extensively covered by feather buds. When this embryo was stained with Alcian Blue, skeletal patterns of this additional limb were similar to the normal wing (data not shown).

When Wnt2b expression was examined, induction of this gene was clearly observed in the flank, where the GFP was evident (Fig. 3FG). Fgf10 gene was induced in the small additional limb bud (red arrowhead in Fig. 3H). Sixty hours after electroporation, an additional limb was clearly observed at the posterior side of the normal wing (red arrowhead in Fig. 3I). In this additional limb, distinct expression of Fgf8 was induced at the anterior side (red arrowhead in Fig. 3J). To confirm the wing identity of this additional limb, expression of several markers was examined. A mosaic nature of the additional limb whose posterior margin (Fig. 3K). By contrast, wing-specific Hoxd9 was induced in the additional limb predominantly possesses the wing identity, with the wing-like expression of Hox9 genes, albeit partially.

Tbx4 misexpression in the flank induces an additional leg-like limb

When the expression plasmid CAGGS-Tbx4-EGFP was electroporated into the flank at stage 10, an additional limb was induced at E10 (red arrowheads in Fig. 3M). Contrary to the Tbx5 misexpression, this limb showed leg-like morphology in its distal half: separated digits were covered by scales, and claw formation on the distal part was suggested by the suppression of feather formation. When expression of several markers was examined, Wnt8c induction was observed (Fig. 3J) in an area where EGFP was detected (Fig. 3L). Expression of another leg-specific Hoxc9 became very faint (Fig. 3K). By contrast, wing-specific Hoxd9 was induced in the additional limb (Fig. 3L). These results indicate that the Tbx5-induced additional limb predominantly possesses the wing identity, with the wing-like expression of Hox9 genes, albeit partially.

When expression of Hox9 genes was examined, leg-specific Hoxb9 and Hoxc9 genes were induced in the additional limb (Fig. 3RS, respectively). By contrast, expression of wing-specific Hoxd9 was suppressed, especially in the posterior half.
Tbx5 and Tbx4 initiate limb development

These results suggest that the Tbx4-induced additional limb mainly possesses the leg identity, with partial wing-like appearance.

**Stage-dependent action of Tbx5 and Tbx4**

As observed in Table 1, the actions of dominant-negative Tbx genes and the resultant phenotypes are dependent on the developmental stages, where stages 7 to 10 are highly susceptible phases. To complement these loss-of-function approaches, stage-dependent actions of Tbx5 and Tbx4 in gain-of-function experiments were examined. For this purpose, we electroporated pCAGGS-Tbx5-EGFP and pCAGGS-Tbx4-EGFP plasmids at various stages (summarized in Table 2). Interestingly, in both cases, induction of additional limb formation was obtained only when these expression plasmids were electroporated between stages 8 and 12. From stage 13 onwards, complete induction of additional limb formation was never observed, although we observed robust fluorescent signal derived co-electroporated pCAGGS-EGFP (data not shown). This is in clear contrast to the Fgf-induced additional limb buds in the flank, which can be induced when Fgf is applied during stages 13 to 15 (Crossley et al., 1996; Vogel et al., 1996). Local application of Fgf at earlier or later stages never induces additional limb buds, setting up a brief sensitive window. This also suggests that competence to the Fgf signaling is strictly controlled in a stage-dependent way. Contrary to this, our misexpression experiments have shown that the formation of additional limb buds is observed only when Tbx genes are...
misexpressed in the flank between stages 8 and 12, before the mesoderm cells become competent to respond to Fgf signaling. Thus, the Tbx-sensitive period begins and ends before the stages at which Wnt-expressing cells can induce additional limb buds. These lines of evidence again indicate that Tbx3 and Tbx4 act upstream of Wnt/Fgf signaling (Fig. 4).

DISCUSSION

In vertebrates, a correct pattern of organ formation is crucially dependent on interactions between different embryonic tissues. For initiation of limb outgrowth, two different inductive signals, Fgf and Wnt, establish tight regulatory loops to ensure the correct transduction of signals and morphogenesis (Kawakami et al., 2001). Prior to limb initiation, Wnt2b is expressed in the intermediate and the lateral plate mesoderm of the prospective wing field. In the prospective leg region, Wnt8c is expressed in the caudal region of the lateral plate mesoderm. Through a β-catenin-dependent pathway, these Wnt molecules induce Fgf10 in the prospective wing and leg fields. Consequently, Fgf10 induces Fgf8 in the surface ectoderm through the Wnt3a/β-catenin-dependent pathway. Hence, orchestrated influx and efflux of morphogenetic signals between different embryonic tissues are pivotal for the correct pattern formation of vertebrate limb buds. As reported previously, Tbx5 and Tbx4 genes are activated in Fgf-induced ectopic limbs in the flank (Ohuchi et al., 1998; Gibson-Brown et al., 1998; Issac et al., 1998; Logan et al., 1998). Our study has shown that Tbx genes induce Fgf genes in the flank, suggesting that the influx and efflux of signals operate in the Tbx/Fgf cascade.

When the dominant-negative forms of Tbx5 and Tbx4 were misexpressed in the prospective limb fields, complete limbless phenotypes arose with disruption of shoulder and pelvis formation (Fig. 1). Scapula was not formed, and ribs were hypomorphic. In the leg region, ilium and ischium were hypoplastic without pubis formation, implying that scapula is hypomorphic. In the leg region, ilium and ischium were not formed, and ribs were hypomorphic.

Table 2. Stage-dependent actions of Tbx5 and Tbx4 in limb initiation

<table>
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<tr>
<th>Stage of injection</th>
<th>Total</th>
<th>Number of limb-like extra limbs</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>8</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>9</td>
<td>15</td>
<td>4 (27%)</td>
</tr>
<tr>
<td>10</td>
<td>12</td>
<td>5 (42%)</td>
</tr>
<tr>
<td>11</td>
<td>18</td>
<td>6 (33%)</td>
</tr>
<tr>
<td>12</td>
<td>16</td>
<td>2 (13%)</td>
</tr>
<tr>
<td>13</td>
<td>12</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>14</td>
<td>10</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>15</td>
<td>10</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Totals</td>
<td>92</td>
<td>17</td>
</tr>
</tbody>
</table>

(A) Tbx5 was misexpressed by in ovo electroporation at various developmental stages. Wing-like additional limbs were induced only when electroporation was performed at stages 8 to 12.

(B) Likewise, formation of leg-like additional limbs was observed only when Tbx4 was misexpressed at stages 9 to 12, setting up a short critical period from stages 8 to 12.

morphological changes observed in chick and mouse are alike. Nonetheless, induced alterations were severer in chick embryos, probably because the dominant-negative Tbx genes affect both the Wnt and Fgf cascades.

To construct the dominant-negative forms of Tbx genes, we used the Engrailed suppressor domain. It has been reported that the repressor activity of this domain is mediated through interaction with Groucho (Jimenez et al., 1997; Tolkunova et al., 1998). Although vertebrate Groucho-related genes are widely expressed in developing embryos (Fisher and Caudy, 1998; Chen and Courey, 2000), their activities in the limb are unclear. Nonetheless, when analyzed in primary cultures of chick limb mesenchyme cells, both EnTbx5 and EnTbx4 abrogate efficiently the transcriptional activation of the Anf and Fgf10 promoters by Tbx5 and Tbx4, respectively (data

Fig. 4. Schematic representation of the signal cascade. In early stages of limb development (stages 10 to 12), Tbx5 and Tbx4 activate the Wnt2B/Fgf and Wnt8C/Fgf signals, respectively. Once activated, the Wnt/Fgf cascades feedback on to Tbx5 and Tbx4 genes to establish a tight positive regulatory loop. At later developmental stages, the Wnt and Fgf signaling cascades interact to make a positive feedback loop and maintain outgrowth of limb buds. During later stages, Tbx5 and Tbx4 exert distinct actions to make different limb structures: wing and leg, respectively.
not shown). This suggests that the Engrailed acts as an efficient suppressor in the chick limb buds. Recently, it has been reported that functional knockdown of zebrafish tbx5 resulted in a failure of fin bud initiation and the complete loss of pectoral fins (Ahn et al., 2002), indicating that the antisense oligonucleotide-mediated knockdown and misexpression of EnTbx5 resulted in similar morphological alterations. Hence, exploiting different techniques in different species is important to understand the common mechanism of limb initiation.

Although the extracellular events have been analyzed extensively, intracellular mechanisms that regulate gene expression and limb initiation have remained unsolved. To date, Hox9 genes are known to be expressed in the lateral plate mesoderm. Expression of these genes demarcates the fields of both the normal limb and the additional limbs induced in the flank. This indicates that Hox genes act upstream of these signaling molecules (Cohn et al., 1997). However, a gap exists between the Hox genes and the Wnt/Fgf network. Our data strongly indicate that Tbx5 and Tbx4 operate in this gap. We also have shown that Hox9 genes are controlled by Tbx5 and Tbx4 during wing/leg specification (Takeuchi et al., 1999). These lines of evidence suggest that a regulatory loop between Hox9 and Tbx genes is essential for both limb initiation and the wing/leg identity specification, highlighting the feedback and feed-forward mechanisms in both extracellular and intracellular signaling cascades.

As shown in Fig. 3, misexpression of Tbx5 and Tbx4 in the flank induces wing-like and leg-like limbs, respectively. Nonetheless, these additional limbs did not show the complete wing or leg appearance. Rather, Tbx5- and Tbx4-induced limbs seem to be mosaic with one type predominating over another. This is consistent with the mixed expression patterns of Hox9 genes (Fig. 3). To obtain rapid and robust expression of Tbx genes, we used pCAGGS expression vector. As pCAGGS induces transient expression of transgene, expression of Tbx genes in the flank might have faded out after triggering expression of Wnt/Fgf genes. This would suggest that induced Wnt/Fgf proteins might initiate limb formation even in mesenchyme cells that were not electroproated. In such case, additional limb buds can be composed of mixed cell populations; electroproated Tbx-positive cells and non-electroproated Tbx-negative cells thereby have a mosaic appearance.

Tbx genes regulate pattern formation in both vertebrate and invertebrate embryos. From a combination of embryological and genetic approaches, a picture is emerging that Tbx genes belong to a highly conserved genetic network involving inductive signals (Papaioannou and Silver, 1998; Smith, 1999; Ruvinsky and Gibson-Brown, 2000; Tada and Smith, 2001). In Xenopus, another T-box transcription factor, Brachury (Xbra), directly regulates embryonic fibroblast growth factor (eFgf) and creates a tight feedback loop. Hence, eFgf expression maintains Xbra expression, and Xbra maintains eFgf expression in vivo. Conversely, eFgf inhibition represses Xbra expression and Xbra inhibition represses eFgf expression (Isaacs et al., 1994). In addition, Xbra has been found to regulate the Xenopus Wnt11 gene directly, making another connection to Wnt signaling (Tada and Smith, 2000). In Drosophila, functions of T-box transcription factor optomotor blind (omb) are closely related to Dpp (Decapentaplegic) and Wg (Wingless) signaling cascades. (Pflugfelder et al., 1992; Maves and Schubiger, 1998). These observations strongly suggest that Tbx genes are central in the highly conserved signaling cascades of these inductive signals in both vertebrates and invertebrates.

Although our data indicate that Tbx5 and Tbx4 control Wnt and Fgf genes, how these signals are transduced to the nucleus to control expression of target genes remains to be elucidated. In this sense, we do not exclude the possibility that Tbx proteins act cooperatively with the Wnt and Fgf signaling systems, i.e. in parallel or cooperatively with these cascades. This is compatible with the data published previously (Ng et al., 2002). Because another T-box protein, Tbr1, interacts with CASK, a member of the membrane-associated guanylate kinases (MAGUKs) (Hsueh et al., 2000), Tbx proteins may be targets of extracellular signaling that could change their transcriptional properties depending on the signaling context. This could be related to the stage-dependent action of Tbx genes during limb initiation. As described above, induction of additional limb formation was obtained only when Tbx genes were electroporated between stages 8 and 12. This is in clear contrast to the Fgf-induced additional limb buds in the flank, which can be induced when Fgf is applied during stages 13 to 15 (Crossley et al., 1996; Vogel et al., 1996; Ohuchi et al., 1997; Kawakami et al., 2001). This also strongly suggests that competence to the Tbx misexpression and to the Fgf signaling is strictly controlled in a stage-dependent way. Although we do not know how these differences in the competence are controlled in vivo, the signaling context could modulate Tbx proteins and regulate this critical factor during limb development.

Recently, Anf was shown to be a direct target of Tbx5 (Hiroi et al., 2001; Brunneau et al., 2001). Tbx5 alone activates the Anf promoter efficiently. However, in the presence of another transcription factor, Nkx2.5, Tbx5 is a stronger activator. In the early stages of limb initiation, Tbx5 alone could induce Wnt genes, whereas in the later stages, activated Wnt signaling might modulate Tbx5 activity or induce another transcription factor to act synergistically, forming a positive feedback loop and upregulating Fgf10 to maintain limb outgrowth. To examine this hypothesis, precise biochemical analyses needs to be performed.

Our data reveal that Tbx5 and Tbx4 specifically regulate Wnt2b and Wnt6c, respectively, to initiate limb outgrowth in the early stages of development. In the later stages, Tbx5 and Tbx4 exhibit different actions to form distinct forelimb and hindlimb structures, respectively. These indicate that these genes play distinct roles with distinct specificity. Nonetheless, Tbx5 and Tbx4 are derived from the same ancestral gene (Agulnik et al., 1996; Ruvinsky and Silver, 1997). During evolution, these genes have diversified their biological functions to regulate different Wnt genes and make different limb structures. This is related to our observation that EnTbx5 and EnTbx4 failed to repress Wnt6c in the leg and Wnt2b in the wing. As expected, misexpression of EnTbx5 in the leg and EnTbx4 in the wing did not affect limb development (Fig. 1E,F). This suggests that Tbx5 and Tbx4 have acquired different target specificities during evolution. Although we are still far from a complete understanding of these processes, our data shed further light on vertebrate limb evolution of vertebrate limbs, the functions and evolution of the Tbx gene family.
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