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Pitx1 determines the morphology of muscle, tendon, and bones of the hindlimb

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Abstract

The vertebrate forelimb and hindlimb are serially homologous structures; however, their distinctive morphologies suggest that different mechanisms are associated with each limb type to give rise to limb-type identity. Three genes have been implicated in this process; T-box transcription factors *Tbx5* and *Tbx4*, which are expressed in the forelimb and hindlimb, respectively, and a paired-type homeodomain transcription factor *Pitx1*, expressed in the hindlimb. To explore the roles of *Pitx1* and *Tbx4* in patterning the hindlimb, we have ectopically misexpressed these genes in the mouse forelimb using transgenic methods. We have developed a novel technique for visualising the structure and organisation of tissues in limbs in 3D using optical projection tomography (OPT). This approach provides unparalleled access to understanding the relationships between connective tissues during development of the limb. Misexpression of *Pitx1* in the forelimb results in the transformation and translocation of specific muscles, tendons, and bones of the forelimb so that they acquire a hindlimb-like morphology. *Pitx1* also upregulates hindlimb-specific factors in the forelimb, including *Hoxc10* and *Tbx4*. In contrast, misexpression of *Tbx4* in the forelimb does not result in a transformation of limb-type morphology. These results demonstrate that *Pitx1*, but not *Tbx4*, determines the morphological identity of hindlimb tissues.
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Keywords: *Pitx1*; Limb-type identity; *Hoxc10*; 3-D imaging; OPT

Introduction

During development, the forelimb and hindlimb buds are morphologically uniform, however, as limb development proceeds, unique structures form characteristic of each limb type. For example, in the forelimb, chondrocytes, myoblasts, and tendon cells are directed to form an elbow joint, whereas in the hindlimb, the equivalent cell populations give rise to a knee. The signaling mechanisms that ultimately produce the distinct limb-type morphologies remain unknown. Gaining an understanding of these processes will allow insights into how limb-type identity arises, and in a broader context, how identical cell populations and common signaling cascades are modulated to generate diversity of form.

Three transcription factors that are expressed in a limb-type-restricted manner have been implicated in limb identity. *Pitx1*, a paired-type homeodomain transcription factor, is expressed in a hindlimb-restricted manner (Logan and Tabin, 1999; Logan et al., 1998; Shang et al., 1997; Szeto et al., 1999). In mice lacking *Pitx1*, hindlimb outgrowth is slightly impaired, but evidence of a loss of some hindlimb features supports a role for this factor in determining hindlimb morphology (Lanctot et al., 1999; Marcil et al., 2003; Szeto et al., 1999). T-box transcription factors, *Tbx5* and *Tbx4*, are expressed in the forelimb and hindlimb, respectively, in several species (Chapman et al., 1996; Gibson-Brown et al., 1996; Isaac et al., 1998; Logan et al., 1998; Ruvinsky et al., 2000; Simon et al., 1997; Takabatake et al., 2000). Inactivation of these factors leads to a failure of limb formation, indicating that they are required for initiation of the limb (Agarwal et al., 2003; Ahn et al., 2002; Naiche and Papaioannou, 2003; Rallis et al., 2003).

In the chick, misexpression of *Pitx1* leads to a transformation of forelimb structures to reflect characteristics of hindlimbs, directly implicating this factor in specifying hindlimb identity (Logan and Tabin, 1999). Misexpression of *Tbx5* and *Tbx4* in

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the chick limb has also been reported to result in transformations of the hindlimb and forelimb, respectively (Rodriguez-Esteban et al., 1999; Takeuchi et al., 1999). However, recent work in the mouse has shown that *Tbx4* can replace *Tbx5* function in the forelimb but does not transform the forelimb to a hindlimb, suggesting *Tbx5* and *Tbx4* have a primary role in initiating limb outgrowth, but do not determine limb-type (Minguillon et al., 2005). Replacement of *Tbx5* with both *Tbx4* and *Pitx1*, results in a forelimb with hindlimb-like characteristics, further implicating *Pitx1* in determining hindlimb identity (Minguillon et al., 2005).

To understand the role of *Pitx1* in determining limb-type identity in the mouse, we have investigated its effects using a construct where the transgene is ectopically expressed in the forelimb and over-expressed in the hindlimb using the *Prx1* limb enhancer (Martin and Olson, 2000). As a comparison, we have also investigated the effects of ectopic misexpression of *Tbx4* in the forelimb.

To understand the role of these genes in determining the identity of individual muscles, tendons, and bones of the limb, we have developed a novel method for visualising the complex organisation of these structures in 3D. Using reporter lines, immunohistochemistry, and optical projection tomography (OPT) (Sharpe et al., 2002), we can resolve individual muscles, tendons, and bones and study their shape and position in the forming limb. We have used OPT to generate detailed 3D models and "virtual" sections of limbs that has enabled us to describe changes in attachment and position of muscles and tendons and the identity of skeletal elements resulting from misexpression of *Pitx1*. This is the first use of this approach for studying the forming limb. Our results demonstrate the enormous potential of this technique for future analysis of limb development and the development of other organ systems.

Misexpression of *Pitx1* results in transformation of some skeletal elements, muscles, and tendons of the forelimb to reflect hindlimb characteristics. This includes transformation of the elbow to a knee-like joint, carpal bones of the wrist to tarsal-like bones, and the transformation and translocation of forelimb muscles and tendons to resemble muscles in the hindlimb. In contrast, misexpression of *Tbx4* in the forelimb does not result in similar patterning changes. In *Pitx1* transgenics, hindlimb-restricted factors *Hoxc10* and *Tbx4* are ectopically expressed in the forelimb, whereas no similar changes in gene expression were observed following *Tbx4* misexpression in the forelimb.

Ectopic expression of *Pitx1* in the forelimb also results in downregulation of factors involved in anterior–posterior patterning of the limb, including *Shh* and *Ptc*, and a corresponding loss of posterior digits, whereas over-expression in the hindlimb results in a normal limb.

Materials and methods

Transgenic mice

Pitx1 and *Tbx4* were ectopically misexpressed under the *Prx1* limb enhancer (Martin and Olson, 2000). *Prx1–Pitx1* and *Prx1–Tbx4* transgenic lines were generated as described previously (Minguillon et al., 2005). Hemizygotes of both *Prx1–Pitx1* and *Prx1–Tbx4* lines are viable and fertile and were used for subsequent breeding and embryo harvests. Homozygote pups die at birth. Tendons were visualised with the *Scleraxis(Scx)-GFP* reporter line (R. Schweitzer, unpublished). Cartilage was visualised with the *Collagen2(Col2)-GFP* reporter W. Horton (Cho et al., 2001; Grant et al., 2000) (Shriners Hospital, Portland, Oregon).

Harvesting and genotyping embryos

Mouse embryos were staged according to Kaufman (1992). Noon on the day a vaginal plug was observed was taken as E0.5 day gestation. Embryos used for RNA *in situ* hybridisation were harvested at E10.5 and E12.5. Embryos for immunohistochemistry and OPT analysis were harvested at E14.5 and immediately ex-sanguinated by severing the umbilical cord in medium containing heparin (10 mg/ml, Sigma-Aldrich, U.K.) at 37°C for 1 h. Newborn pups used for skeletal preparations were culled at birth. All specimens were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) with 1% Tween (PBT) at 4°C. E14.5 embryos were fixed for 2 h, and E10.5 and E12.5 embryos and newborn pups were fixed overnight. Specimens were washed in PBT, and E10.5 and E12.5 embryos and pups were dehydrated in graded methanol and stored at 4°C. E14.5 embryos were stored in PBT at 4°C until use.

Prx1–Tbx4 and *Prx1–Pitx1* transgenic embryos were identified by PCR genotyping (Minguillon et al., 2005). To distinguish hemizygote from homozygote embryos at E10.5, real-time quantitative PCR was performed in multiplex mode using two sets of primers and probes for the *SV40* transgene (forward: 5′-GATTCCAACCTATGGAACTGA; reverse: 5′-GGCATT-TCTTCTGAGCAAAAC; probe: 5′-VIC-TGGGAGCAGTGGTGGAATG-CCTTTA-TAMRA), and as a control, the mouse cardiac actin promoter (α*CA*) (forward: 5′-CCCCCTGGCTGATCCTCTAC; reverse: 5′-TGGT-CGCCTTAGCACCATCT; probe: 5′-FAM-CTCCAAGAATGGCCTCAGC-GGTCC-TAMRA) (Tesson et al., 2002). The presence of *Scx-GFP* and *Col2-GFP* transgenes was identified by examination under fluorescent light.

Skeletal preparations

Cartilage and bone of newborn pups was stained using Alcian blue and alizarin red as described (Hogan et al., 1994).

Whole-mount double immunohistochemistry

Limbs were removed from E14.5 embryos and skin was removed using forceps under a light microscope. Samples were blocked in PBS containing 1% bovine serum albumin (BSA), 0.15% glycine, and 0.1% Triton for 2 h at room temperature prior to application of antibodies. Muscles were detected using a monoclonal antibody to fast skeletal muscle myosin (*My-32*, Sigma). Unconjugated *My32* antibody was directly conjugated with Texas red-labeled *Fab* fragments (Zenon™ One Texas Red-X Mouse IgG, Molecular Probes, Oregon, U.S.A.) at 1:800 dilution. GFP was detected using a primary rabbit polyclonal anti-GFP antibody (Molecular Probes), detected with a secondary donkey anti-rabbit antibody conjugated to FITC or Texas Red (Jackson ImmunoResearch Laboratories, Ltd., West Grove, PA, USA), both at 1:400 dilution. Diluted antibodies were applied to specimens in blocking agent (as above) overnight at 4°C. Samples were washed in PBS containing 0.1% Triton for 5 h following application of each antibody, and post-fixed for 30 min in 4% PFA at room temperature.

In situ hybridisation

Whole-mount in situ hybridisations were carried out as previously described (Riddle et al., 1993). All mouse probes have been previously described: Gli3 (Schimmang et al., 1992), Hoxb8 (Charite et al., 1994), Hoxc4, Hoxc5, (Burke et al., 1995), Hoxc10, Hoxc11 (Peterson et al., 1994), MyoD (Davis et al., 1987), Ptc1 (Goodrich et al., 1996), Scx (Schweitzer et al., 2001), Shh (Echelard et al., 1993), Tbx4, Tbx5 (Logan et al., 1998).

Optical projection tomography (OPT)

OPT was performed as described by Sharpe et al. (2002). E14.5 limbs were embedded in 1% low-melting point agarose (LMP Agarose, Invitrogen), mounted on stainless-steel stubs, dehydrated, and cleared using benzyl alcohol-benzobenzoate (BABB, 1:2). Specimens were mounted on a rotating motor and visualised using a Leica *FLIII* microscope fitted with *GFP2* and rhodamine filter sets. Digital images of the specimen were captured in rotation, and these images formed the basis of the 3D and section reconstruction. 3D rendering, virtual section reconstructions, and movies were generated using in-house (MA3Dview) and Amira software (version 3.1, Mercury Computer Systems, Germany).

Identification of skeletal elements, muscles, and tendons

Bones were identified from an anatomical atlas of the embryonic mouse skeleton (Yasuda, 1996). Muscles and tendons were identified using anatomical references for limb structures in the adult rat (Chace Greene, 1963; Hebel, 1986). Structures were positively identified on the basis of the shape, position, and orientation in relation to other elements. Tendons were identified as belonging to specific muscle groups on the basis of myotendinous junctions whereas muscles were identified on the basis of morphology, as well as tendon origin and insertion sites on skeletal elements.

Results

The Prx1 enhancer drives ectopic expression of Pitx1 and Tbx4 during stages of limb morphogenesis

To misexpress hindlimb-restricted genes in the forelimb, transgenic lines were generated using chick cDNAs for *Tbx4* and *Pitx1* placed under the regulation of the *Prx1* limb enhancer element (Martin and Olson, 2000) (see Minguillon et al., 2005). Broad expression of the transgene in the nascent forelimb bud is first detected at E9.0 (16 somites; data not shown). Whole-mount *in situ* hybridisation for *cPitx1* and *cTbx4* mRNA shows uniform expression of the transgene in the mesenchyme of the limb bud at E10.5 (Fig. 1A and data not shown, see Minguillon et al., 2005). Analysis in section at E12.5 demonstrates transgene expression is throughout the limb mesenchyme but is becoming downregulated in the condensing cartilage precursors of the limb skeleton (Fig. 1B). At both stages, expression is also detected in the cranial mesenchyme and body wall (Fig. 1A and data not shown).

Bones of the elbow are transformed in Prx1–Pitx1 mice to reflect characteristics of the knee

Skeletal preparations of newborn *Pitx1* transgenic embryos show a partial transformation of skeletal elements forming the elbow to reflect characteristics of the wild-type hindlimb knee (Figs. 1C–J). In the normal elbow (Figs. 1C and D), the distal head of the humerus articulates with the ulna at the trochlear notch. The olecranon process of the ulna extends proximally from the elbow joint (Fig. 1D). In hemizygote transgenics, the distal humerus is broadened at the elbow (Figs. 1G and H, indicated by black arrow) to resemble the shape of the wild-type femur (Figs. 1E and F). In addition, the head of the radius in the hemizygotes (Figs. 1G and H, indicated by red arrow) is increased in size and is similar to the head of the wild-type tibia. Conversely, the olecranon process of the ulna in the hemizygotes is reduced in size (Figs. 1G and H, indicated by asterisk) compared to the wild-type olecranon process (Fig. 1D).

In the *Prx1–Pitx1* homozygote, which would be expected to express twice the level of transgene-derived *Pitx1* as hemizygotes, transformation of the elbow region is more extreme. To analyse the transformation of the morphology of limb skeletal elements with greater resolution, a *Col2-GFP* reporter, in which the cartilaginous precursors of bone are rendered fluorescent (Cho et al., 2001; Grant et al., 2000), was crossed into the transgenic line and the limbs imaged using OPT. This approach has significant advantages over conventional 2D imaging methods, as it allows the visualisation of the shape and spatial relationship between bones in 3D. In *Prx1–Pitx1* homozygotes the apposition and articulation of the two bones in the elbow region resembles that found in the hindlimb knee (Figs. 1K and L). The distal head of the 'humerus' (Fig. 1L) is more similar to the distal femur (Fig. 1K) and the shape of the proximal head of the single zeugopodal element (Fig. 1J, indicated by red arrow and L) resembles the shape of the proximal tibia (Figs. 1F and K). The proximal head of the tibia has a medial and lateral condyle separated by an inter-condyle fossa (trench). These morphological features are not found at the proximal end of the forelimb zeugopodal elements. In the *Prx1–Pitx1* homozygotes, the proximal head of the single zeugopodal element has condyle elements and an inter-condyle fossa reminiscent of the proximal tibia (compare Figs. 1I and L with Figs. 1F and K). In hemizygous, and to a greater extent in homozygous, *Prx1–Pitx1* transgenic mice, the flexure of the forelimb is altered so that the dorsal surface of the forepaw is rotated outwards from the body, similar to the flexure of the hindpaw, rather than turning inwards as in the wild-type forepaw (data not shown).

In contrast, misexpression of *Tbx4* in the forelimb in *Prx1–Tbx4* transgenic mice does not result in a transformation of skeletal elements to reflect hindlimb morphology. Forelimb and hindlimb skeletal patterning in hemizygous newborn *Tbx4* transgenic mice is not different from wild-type (data not shown). In homozygous *Prx1–Tbx4* embryos, however, skeletal defects are present. Most profoundly, in both the forelimb and hindlimb, the zeugopodal bones are severely truncated with only misshapen cartilaginous remnants remaining (Figs. 1M and N, indicated by black arrows). Overall, these results indicate that misexpression of *Pitx1*, but not *Tbx4*, is capable of transforming some skeletal elements in the forelimb to resemble hindlimb-like features.

Anterior–posterior patterning is disrupted in homozygote Prx1–Pitx1 transgenic forelimbs, but is normal in hindlimbs

In homozygotes, misexpression of *Pitx1* in the forelimb results in a loss of one element of the zeugopod (Fig. 1I, indicated by red arrow), as well as a loss of 2 digits (Fig. 1I, indicated by blue arrow and inset panel). Of the remaining digits, the anterior-most has two phalanges (Fig. 1I, inset, indicated by green arrow) and is elongated, resembling digit 1 of the wild-type hindlimb (Fig. 1E, inset, indicated by green

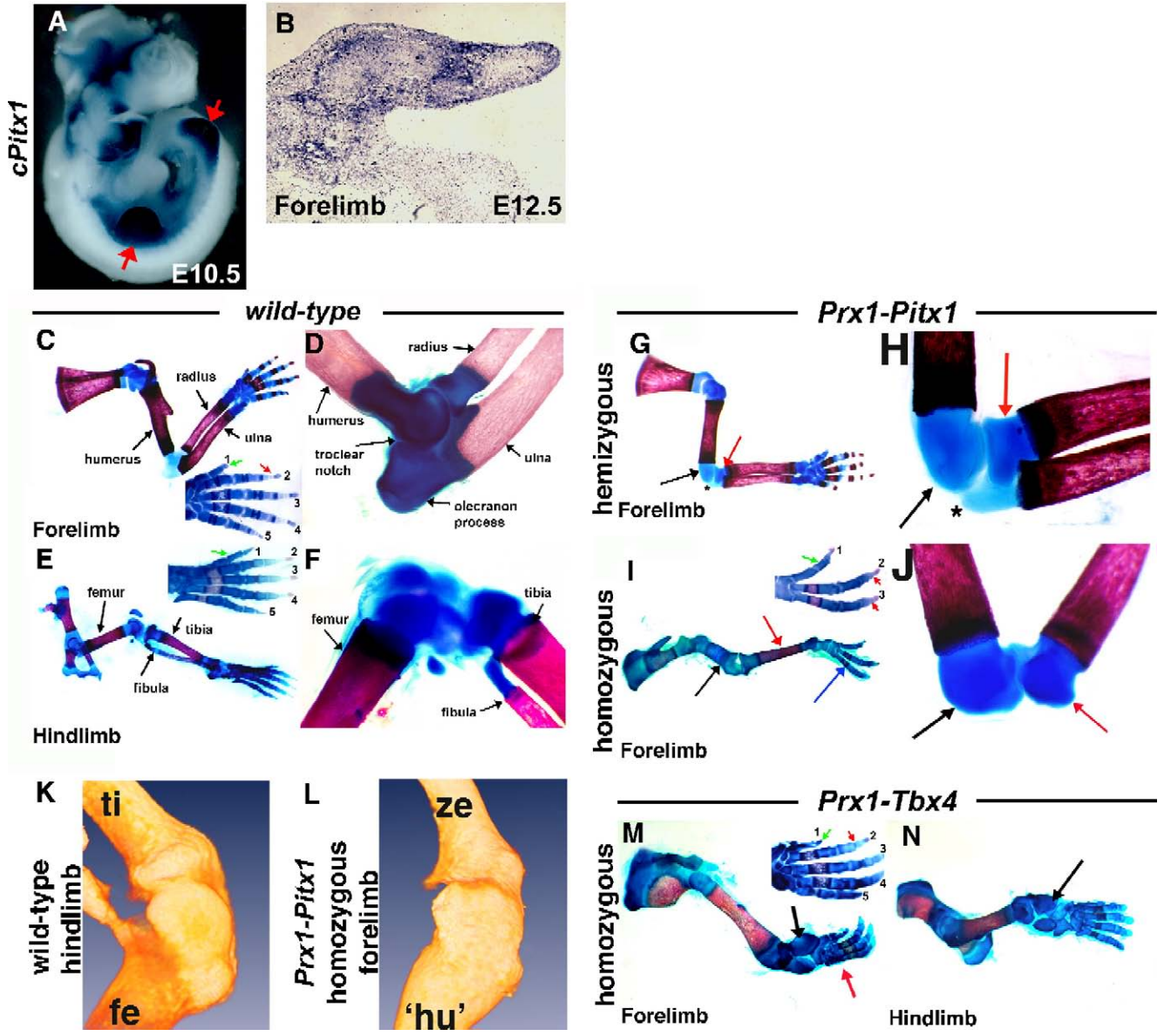


Fig. 1. The *Prx1* transgene is expressed throughout the limb and bones of the elbow of *Prx1–Pitx1* transgenics are transformed to reflect characteristics of the knee. (A) Whole-mount RNA *in situ* hybridisation showing expression of the *Prx1–Pitx1* transgene in the forelimb and hindlimb buds at E10.5 (indicated by red arrows). (B) Section RNA *in situ* hybridisation showing expression of the *Prx1–Pitx1* transgene in the forelimb at E12.5. Transgene expression is becoming downregulated in condensing cartilage precursors. Panels C–J, M, and N show limb skeleton preparations of P0 pups. Panels K and L show volume renderings of OPT-generated images of *Col2-gfp* and *Prx1–Pitx1;Col2-gfp* E14.5 limb skeletons, respectively. (C and D) Wild-type forelimbs. Inset: digit 1 has two phalanges (green arrow), and digit 2 has 3 phalanges (red arrow). (E and F) Wild-type hindlimbs. Inset: digit 1 (green arrow). (G and H) Hemizygote *Prx1–Pitx1* forelimbs have a transformed distal humerus (black arrow) and proximal radius (red arrow), resembling the distal femur and proximal tibia of the wild-type hindlimb (see E and F). There is a loss of the olecranon process (asterisk) of the ulna. (I and J) Homozygote *Prx1–Pitx1* forelimbs have a more extreme phenotype; the distal humerus is transformed (black arrow) to resemble the distal femur of the hindlimb (see panel F). Only one zeugopod bone is present (red arrow), where the proximal end resembles the proximal tibia (see F). Inset: two digits are missing, and of the remaining digits digit "1" has two phalanges and is elongated (green arrow). The two posterior digits ("2" and "3") have three phalanges each (red arrows). (K and L) Volume renderings of wild-type hindlimb knee joint and the equivalent region of the 'elbow' of a homozygote *Prx1–Pitx1* forelimb. Fe = femur, ti = tibia, hu = humerus, ze = zeugopodal bone. (M and N) Homozygote *Prx1–Tbx4* forelimbs do not show a transformation of limb-type identity. Patterning defects are present in the bones of the zeugopod (black arrow), and digits may be missing (red arrow). Inset: digits 1 and 2 lack medial phalanges (green and red arrows, respectively).

arrow). The two posterior digits have three phalanges each (Fig. 1I, inset, indicated by red arrows). Digit patterning is normal in the hindlimbs of homozygotes (data not shown).

In homozygote *Prx1–Tbx4* transgenics, digits 1 and 2 of the forelimb lack medial phalanges (Fig. 1M, inset, indicated by green and red arrows). In some examples, the 5th digit is missing (Fig. 1M, indicated by red arrow). Overall, these results indicate that misexpression of *Pitx1* in the forelimb can severely disrupt normal anterior–posterior patterning, whereas overexpression of *Pitx1* in the hindlimb has no effect. Misexpression of *Tbx4* in the forelimb does not have a similar effect on altering anterior–posterior patterning.

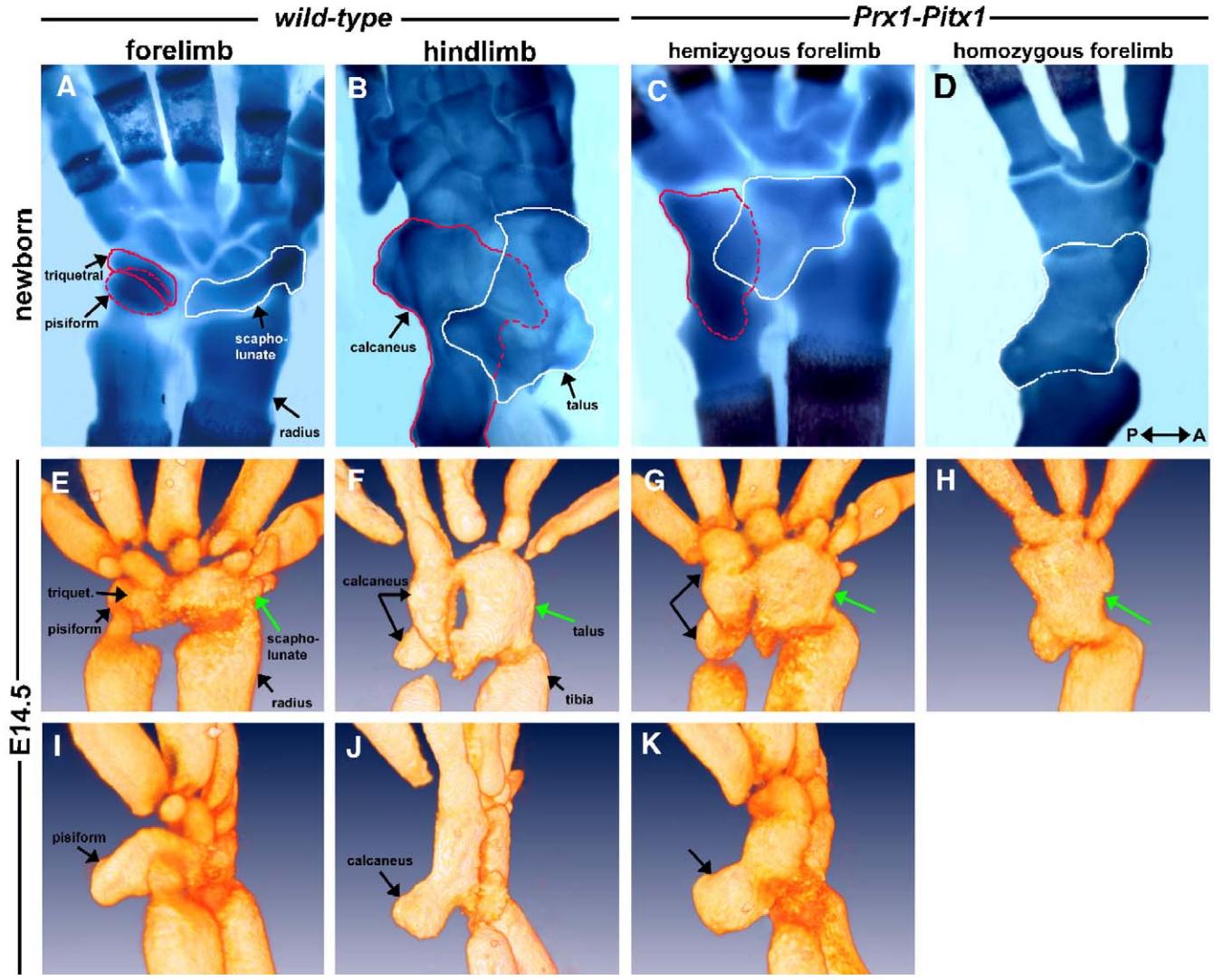


Fig. 2. Bones of the wrist of *Prx1–Pitx1* transgenics are transformed to reflect characteristics of the ankle. Panels A–D are skeletal preparations of newborn pups. Panels E–K are volume renderings of OPT images of *Col2-gfp* and *Prx1–Pitx1;Col2-gfp* embryos at E14.5. Dotted lines indicate the bone is subjacent to the bone with the solid outline. "P-A" refers to posterior-anterior orientation. Panels A–H are dorsal views of the left autopod. (A) Wild-type forelimb showing the position and relationship between the triquetral and pisiform (outlined in red), the scapholunate (outlined in white) bones of the wrist, and the radius. (B) Wild-type hindlimb showing the calcaneus (outlined in red) and talus (outlined in white) of the ankle. (C) Hemizygote *Prx1–Pitx1* forelimb with a single bone at the posterior side of the wrist (outlined in red) resembling the calcaneus, and an enlarged scapholunate (outlined in white), resembling the talus of the hindlimb. (D) Homozygote *Prx1–Pitx1* forelimb with a single wrist bone (outlined in white) resembling the talus of the hindlimb. (E) Wild-type *Col2-gfp* forelimb with triquetral, pisiform, and scapholunate bones and the radius indicated. (F) Wild-type *Col2-gfp* hindlimb with calcaneus, talus, and tibia indicated. (G) Hemizygous *Prx1–Pitx1;Col2-gfp* forelimb with apparent fusion of triquetral and pisiform bones (black arrows) to form a calcaneus-like bone. The scapholunate (green arrow) reflects the shape of the talus of the hindlimb (see panel F). (H) Homozygous *Prx1–Pitx1;Col2-gfp* forelimb with a single bone of the wrist similar to the talus of the hindlimb (see F). (I-K) 90° rotation of wild-type forelimb (I), hindlimb (J), and hemizygous *Prx1–Pitx1* forelimb (K) to demonstrate the transformation of the shape of the "pisiform" which forms part of the fused posterior skeletal element of the wrist of transgenics to reflect the shape of the calcaneus of the hindlimb.

Bones of the wrist are transformed in Prx1–Pitx1 mice to resemble bones characteristic of the ankle

Analysis of carpal bones of embryos at E14.5 using OPT and in newborns using Alcian blue/Alizarin red stains demonstrates that bones of the wrist of *Prx1–Pitx1* transgenic mice are partially transformed to resemble the morphology of equivalent bones in the ankle (Figs. 2A–K). In wild-types, the scapholunate bone of the wrist articulates with the radius (Figs. 2A, outlined in white, and E, indicated by green arrow). In *Prx1–Pitx1* transgenics (Figs. 2C and D, outlined in white, and in Figs. 2G and H, indicated by green arrow) this element is similar to the size and shape of the talus in the ankle, which articulates with the tibia (Figs. 2B outlined in white, and F indicated by green arrow). An additional transformation in transgenics is the apparent fusion of the triquetral and pisiform (Figs. 2C, outlined in red, and G, indicated by black arrows). These are separate bones in the wild-type wrist (Figs. 2A, outlined in red, and E, indicated by black arrows). In the transgenics, bones are fused to form a structure similar in shape

to the calcaneus of the ankle (Figs. 2B, outlined in red, and F, indicated by black arrows). Rotating the 3D models of the limbs 90° shows that the ventral projection of the pisiform is transformed to form the shape of the calcaneus (Figs. 2I–K).

Forelimb muscle and tendon morphology in Prx1–Pitx1 mice is transformed to resemble the hindlimb morphology

Muscle and tendon morphogenesis is integrated with skeletal development to give rise to the unique structures of each limb type. Muscles move the skeleton via their tendon connections to bone; hence, these tissues are structurally and functionally integrated. It is therefore advantageous to visualise both muscle and tendon together. Using a fluorescent reporter line, *Scx-GFP* (R. Schweitzer, unpublished) to detect tendon, and immunohistochemistry (using a contrasting fluorophore) to detect muscles, we used OPT to examine both tendon and muscle simultaneously (Figs. 3A–I).

This approach has significant advantages compared to conventional 2D imaging techniques that provide no information about underlying muscles or the relationships between muscles with bones or tendons (Fig. 3H). A further advantage of

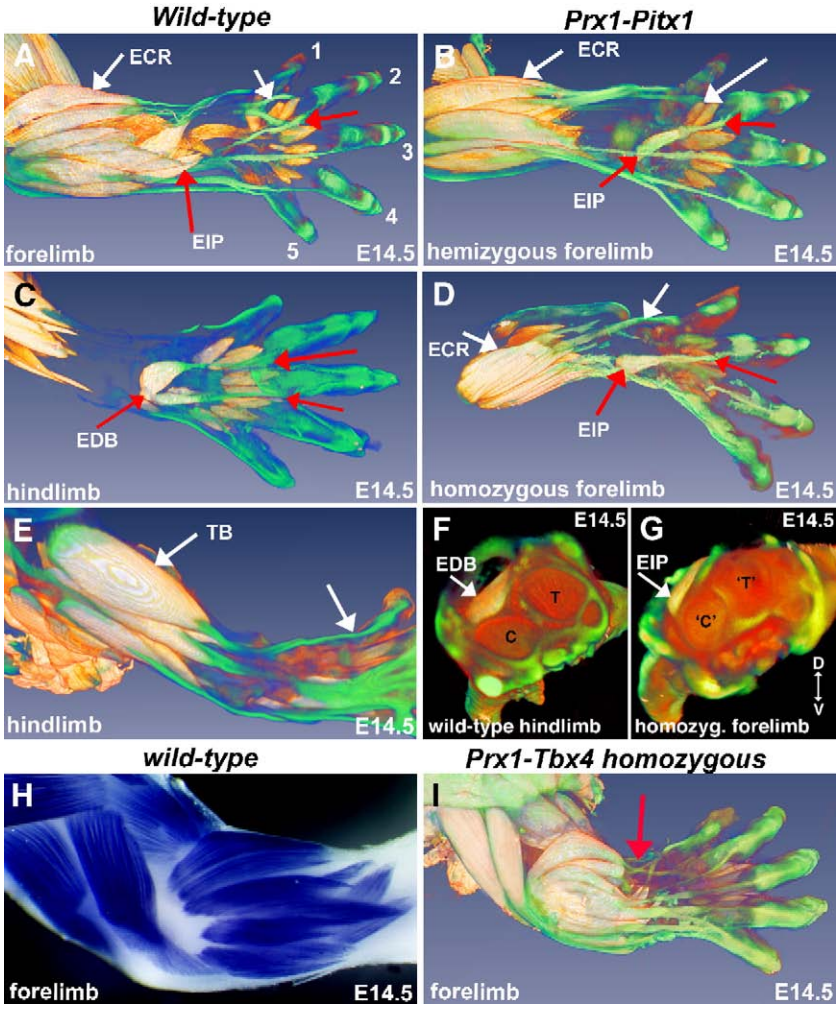


Fig. 3. Muscles and tendons in the *Prx1–Pitx1* transgenic forelimb are transformed to reflect characteristics of the hindlimb. Panels A–K and M are volume renderings of OPT images of *Scx-gfp*, *Prx1–Pitx;Scx-gfp*, and *Prx1–Tbx4;Scx-gfp* transgenic limbs at E14.5. Panel L is a whole-mount stain of a forelimb using alkaline-phosphatase-conjugated anti-skeletal muscle myosin (*My32*). ECR = extensor carpi radialis; EIP = extensor indicis proprius; EDB = extensor digitorum brevis; TBL = triceps brachii longus; FCU = flexor carpi ulnaris. (A) Wild-type forelimb showing ECR (white arrows) as two muscle bodies with tendons branching to insert into digits 2 and 3. The EIP muscle (red arrow) originates from the mid-shaft of the ulna, and its distal tendon inserts into digit 2. (B) Hemizygous *Prx1–Pitx1* forelimb showing ECR is less distinct as two separate muscle bodies, with a single distal tendon inserting into digit 2 (white arrows), similar to the TB of the hindlimb (see panel E). The EIP muscle is translocated to the wrist (red arrow) but retains its distal tendon insertion into digit 2, as in the EDB of the hindlimb (see C). (C) Wild-type hindlimb showing two muscle bodies of the EDB with distal tendon insertions into digits 2 and 3 (red arrows). (D) Homozygous *Prx1–Pitx1* forelimb showing the ECR muscle as a single body (white arrows) with one distal tendon inserting into digit 1, as in the TB muscle of the hindlimb and EIP inserting into digit 2 (red arrow) (see E). (E) Wild-type hindlimb showing TB muscle with one distal insertion into digit 1 (white arrows). (F and G) "Virtual" sagittal sections through wild-type hindlimb (F) and hemizygous *Prx1–Pitx* forelimb (G) demonstrating the association of EDB muscle with the calcaneus (C) in the ankle, and the association of the translocated EIP muscle with the "calcaneus" ('C') in the wrist. T and 'T' refer to the talus present in the ankle, and "talus"-like bone present in the wrist of transgenics. "D-V" refers to dorsal–ventral orientation. (H) Wild-type forelimb stained using *My32*. (I) Homozygous *Prx1–Tbx4* forelimb demonstrating no transformation of muscle and tendon patterning, but showing shortened muscles of the zeugopod (red arrow).

OPT is that it allows the generation of "virtual" sections through any plane of the specimen (Figs. 3F and G). Individual muscles and their associated tendons from their origins to their insertions on bone can be visualised in three-dimensional space, providing a novel way of describing specific changes in the morphology and arrangement of these tissues following the misexpression of transgenes in the limb.

Many aspects of soft tissue morphology are altered following ectopic expression of *Pitx1* in the forelimb. We focus on two fundamental alterations in the forelimb musculature that represent two distinct aspects of the transformation of limb-type morphology: (1) transformation of a forelimb muscle to resemble a hindlimb muscle, (2) translocation of a forelimb muscle to the equivalent position and attachment sites of a hindlimb muscle (Table 1).

Transformation of the forelimb extensor carpi radialis muscle (ECR) to a hindlimb tibialis (TB) muscle

The *extensor carpi radialis* (ECR) muscle of the forelimb is comprised of two muscle bodies with associated tendons that branch to insert into digits 2 and 3 (Fig. 3A, indicated by white arrows). In hemizygote and homozygote forelimbs, the ECR is comprised of a single muscle bundle and has a single distal tendon inserting into digit 2 in hemizygotes and digit 1 in homozygotes (Figs. 3B and D, respectively, indicated by white arrows). This transformed muscle and associated tendon insertion pattern is equivalent to the *tibialis* (TB) muscle of the hindlimb (Fig. 3E, indicated by white arrows), which is comprised of a single muscle body, with one distal tendon inserting into digit 1. Therefore, following ectopic expression of *Pitx1* in transgenic embryos the forelimb ECR muscle appears to be transformed to a hindlimb TB muscle in both shape and tendon insertion pattern.

Table 1
Summary of patterning alterations in muscle and tendon in the *Prx1–Pitx1* forelimb

	Muscle	Wild-type forelimb	Wild-type hindlimb	*Prx1–Pitx1* forelimb
Transformation	Extensor carpi radialis	2 muscle bodies, distal tendon branches to insert into digits 2 and 3		1 muscle body, one distal tendon inserting into digit 1 or 2
	Tibialis		1 muscle body, single distal tendon inserts into digit 1	
Translocation	Extensor indicis proprius	Originates from mid-shaft of ulna		Originates at ankle
	Extensor digitorum brevis		Originates at ankle	

Translocation of the extensor indicis proprius (EIP) muscle to the wrist

In the wild-type forelimb, the *extensor indicis proprius* (EIP) muscle originates from the mid-shaft of the ulna and has an associated distal tendon that inserts into digit 2 (Fig. 3A, indicated by red arrows). In *Prx1–Pitx1* transgenic embryos, the EIP muscle is not present in its normal location in the forearm and instead is translocated distally to the wrist region (Figs. 3B and D, indicated by red arrows). This 'ectopic' muscle also has a distal tendon that inserts into digit 2, as in the wild-type forelimb. The position of this 'ectopic' muscle in the *Prx1–Pitx1* transgenic is equivalent to the position of the *extensor digitorum brevis* (EDB) muscle in the wild-type hindlimb, which originates from an attachment to the calcaneus, and also inserts into digit 2 (Fig. 3C, indicated by red arrows). Analysis of the same data set by virtual section indicates that this muscle has an origin of attachment to the calcaneus-like bone present in the wrist of transgenics. This is equivalent to the attachment of the EDB in the wild-type hindlimb (Figs. 3F and G, indicated by white arrows). This indicates that under the influence of *Pitx1*, the EIP muscle translocates from its normal location to a position of a hindlimb muscle with equivalent tendon attachment site.

Pitx1 misexpression results in ectopic expression of Hoxc10 and Tbx4 in the forelimb

To understand how *Pitx1* controls limb-type tissue morphogenesis, we analysed the expression of potential downstream targets. Genes belonging to the *Hoxc* cluster have a limb-type-restricted expression pattern in the developing embryo and have been previously implicated in patterning limb structures (Nelson et al., 1996; Peterson et al., 1994). *Hoxc10* is normally expressed in the hindlimb (Figs. 4D and G) and not in the forelimb (Fig. 4A) (Nelson et al., 1996). Following misexpression of *Pitx1*, *Hoxc10* expression is ectopically induced in the forelimbs of hemizygous and homozygous embryos at E10.5 (data not shown) and E12.5 (Figs. 4B, E, and H). Analysis of sections shows *Hoxc10* expression is localised to the mesenchyme in both wild-type hindlimbs and transgenic forelimbs at E12.5 (Figs. 4G and H). In both the transgenic limb and the wild-type hindlimb (data not shown), this expression domain overlaps largely with regions of the limb expressing markers of the myoblasts (*MyoD*, Fig. 4C) and tendon progenitors (*Scx*, Fig. 4F). Other genes that are also expressed in the hindlimb bud, e.g., *Hoxc9* and *Hoxc11* (Nelson et al., 1996; Peterson et al., 1994), are not ectopically expressed in limb buds of *Prx1–Pitx1* transgenic embryos however (data not shown). In contrast, *Hoxc10* expression is not induced in the forelimb of *Prx1–Tbx4* transgenic embryos (Fig. 4I).

Tbx4 is ectopically expressed in the forelimbs of *Prx1–Pitx1* embryos at E10.5 (Figs. 4J and K). Ectopic expression of *Tbx4* is still present at E12.5, although levels are reduced (data not shown). Expression of *Tbx5*, however, is unchanged

from normal levels in the transgenic forelimbs at E10.5 (Figs. 4L and M) and at E12.5 (data not shown). *Hoxc5*, which is expressed in an anterior domain of the forelimb, is also expressed normally in the forelimb buds of *Prx1–Pitx1* embryos (data not shown). In *Prx1–Tbx4* embryos, *Tbx5* expression is also normal (data not shown). These findings demonstrate that whereas hindlimb-restricted markers are ectopically induced following ectopic expression of *Pitx1* in the forelimb, expression of forelimb-restricted markers are unaffected.

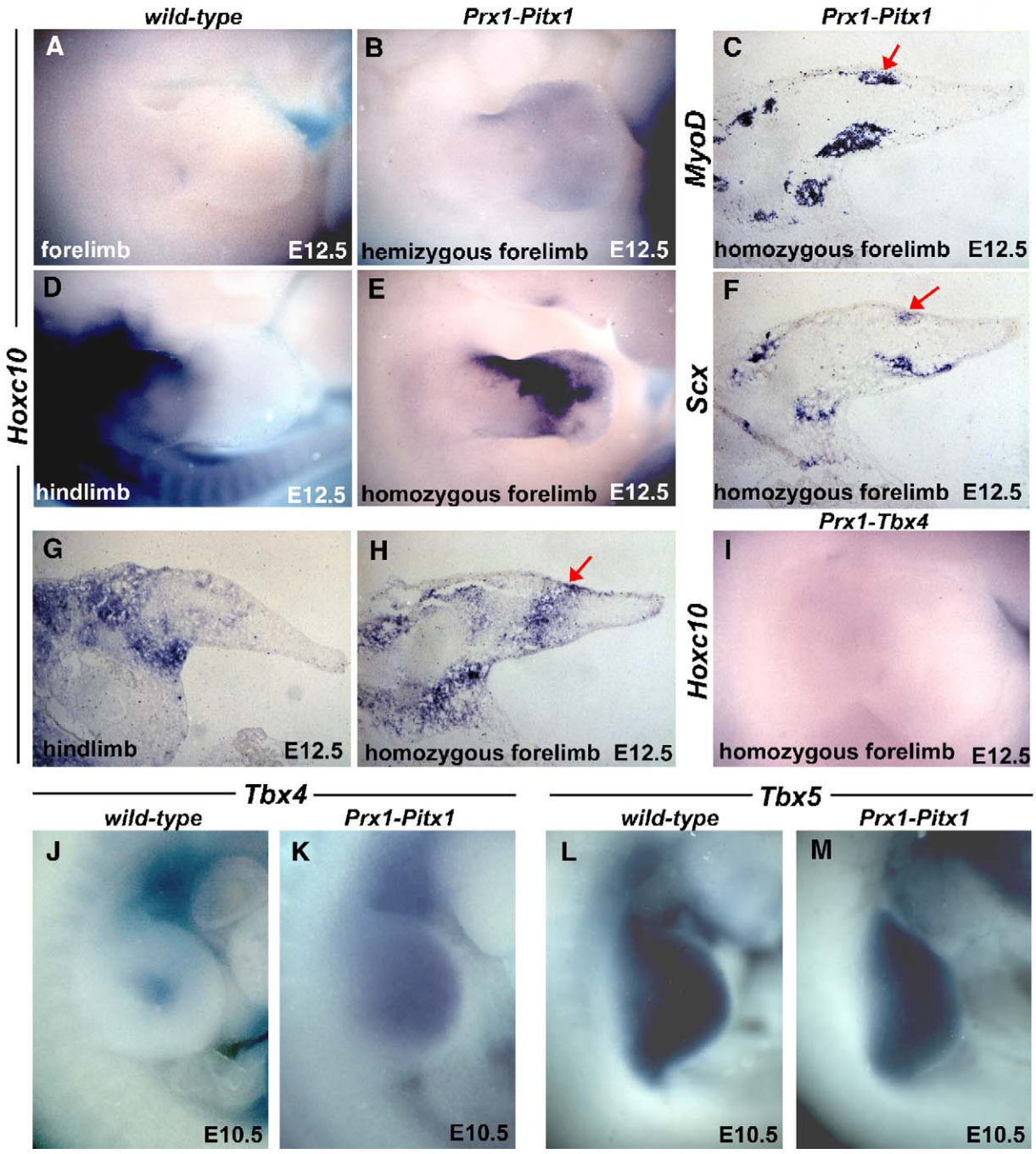


Fig. 4. Misexpression of *Pitx1* results in ectopic expression of *Hoxc10* and *Tbx4* in the forelimb. Panels A, B, D, E, and I are whole-mount RNA *in situ* hybridisatisations of E12.5 embryos. Panels C, F, G, and H are serial section RNA *in situ* hybridisations of E12.5 embryos. Panels J–M are whole-mount RNA *in situ* hybridisations of E10.5 embryos. (A) Wild-type forelimb showing no expression of *Hoxc10.* (B) Hemizygous *Prx1–Pitx1* forelimb showing weak ectopic expression of *Hoxc10* throughout the limb. (C and F) Homozygous *Prx1–Pitx1* forelimb demonstrating the expression pattern of *MyoD* (C) and *Scx* (F), which overlap with regions of ectopic expression of *Hoxc10* in the forelimb of transgenics (see H). (D and G) Wild-type hindlimb demonstrating expression of *Hoxc10*. (E and H) Homozygous *Prx1–Pitx1* forelimb showing ectopic expression of *Hoxc10*. (I) Homozygote *Prx1–Tbx4* forelimb showing no ectopic induction of *Hoxc10* in the limb. (J) Wild-type forelimb bud showing normal expression of *Tbx4*. (K) Homozygous *Prx1–Pitx1* forelimb demonstrating ectopic induction of *Tbx4* in the limb. (L) Wild-type forelimb bud showing normal expression of *Tbx5*. (M) Homozygous *Prx1–Pitx1* forelimb showing comparable expression of *Tbx5* with wild-type.

Pitx1 misexpression in the forelimb is associated with changes in expression of Shh, Gli3, and Ptc1

Interestingly, ectopic expression of *Pitx1* in homozygote *Prx1–Pitx1* forelimbs leads to a loss of digits and a loss of one bone of the zeugopod that is unrelated to a transformation of limb-type identity. In the transgenic hindlimb, where *Pitx1* is over-expressed, anterior–posterior patterning of structures is normal. To address the molecular mechanism underlying loss of digits and the zeugopodal bone, and the identity of the remaining structures, we examined the expression of components of the *Shh* signaling pathway that are involved in anterior–posterior patterning. There appear to be differences in the way the zone of polarising activity (ZPA), a signaling center in the posterior of the limb critical for correct anterior–posterior patterning, is established in forelimbs and hindlimbs (Charite et al., 2000; Hornstein et al., 2005; Stratford et al., 1997). However, it is unknown if *Pitx1* has a role in influencing this process in the hindlimb.

Shh is normally expressed in the ZPA in the posterior of the limb (Figs. 5A and C). *Shh* protein diffuses from the ZPA, and the response of cells in the limb to a gradient of *Shh* determines anterior–posterior patterning (Ahn and Joyner, 2004). In *Prx1–Pitx1* homozygote forelimbs, the levels of *Shh* transcripts are markedly reduced in the limb (at 28 somites, when *Shh* should be initially induced), and later at E10.5 (Figs. 5B and D, indicated by arrows). Associated with the reduced expression of *Shh*, *Ptc1*, part of the receptor complex for *Shh*, which is also positively regulated by *Shh*, is expressed at lower levels in the transgenic forelimb (Figs. 5E and F, indicated by arrow). At early stages of limb formation (19 somites), *Gli3* is normally expressed broadly throughout the limb bud (Fig. 5G), but later becomes anteriorly restricted due to negative regulation by *Shh* (Fig. 5I). In *Prx1–Pitx1* transgenics, *Gli3* expression appears consistent with wild-type expression pattern at 19 somites (Fig. 5H) but is expanded posteriorly in the forelimbs later at E10.5 (Fig. 5J).

dHand (also referred to as *Hand2*) is required to establish the zone of cells in the posterior bud that express *Shh* (Charite et al., 2000; te Welscher et al., 2002). At stages prior to *Shh* induction in the limb (19 somites), *dHand* expression is expressed at lower levels in *Prx1–Pitx1* homozygotes compared to wild-type (Figs. 5K and L, indicated by arrow). At later stages *dHand* itself is positively regulated by *Shh* (Charite et al., 2000; te Welscher et al., 2002). At E10.5, *dHand* expression is reduced in transgenics, consistent with the reduction in *Shh* signaling (Figs. 5M and N, indicated by arrow).

At early stages of limb initiation, *Tbx3* is expressed in an overlapping domain with *dHand* and may act as a licensing factor for *Shh* expression in the ZPA (Rallis et al., 2003). In the wild-type limb, *Tbx3* is expressed at the anterior and posterior margins of the limb bud (Figs. 5O and Q). *Tbx3* expression is downregulated in the posterior margin of the limb bud in *Prx1–Pitx1* homozygous forelimbs at 25 somites (Figure P, indicated by arrow). Later, at E10.5, there is no posterior expression, whereas the anterior domain appears unaffected (Fig. 5R, indicated by arrow).

An additional component of the hierarchy of signals that have been implicated in the regulation of *Shh* expression in the forelimb is *Hoxb8* which, when expressed throughout the limb bud, leads to ectopic expression of *Shh* in the anterior forelimb bud, but not in the hindlimb bud (Charite et al., 1994). *Hoxb8* is normally expressed in the posterior of the forelimb, and this domain is unaffected in *Prx1–Pitx1* homozygotes (Figs. 5S and T). These results suggest that ectopic expression of *Pitx1* in the forelimb is acting in parallel or downstream of any influences of *Hoxb8* on *Shh* expression.

In summary, the loss of expression factors in the posterior domain of the limb including *Shh*, *dHand*, *Ptc1*, and the posterior domain of *Tbx3* suggests a loss of posterior digits and the ulna in homozygotes. The remaining digits and the single zeugopod, which have formed in conditions of reduced *Shh* signaling, have an anterior identity.

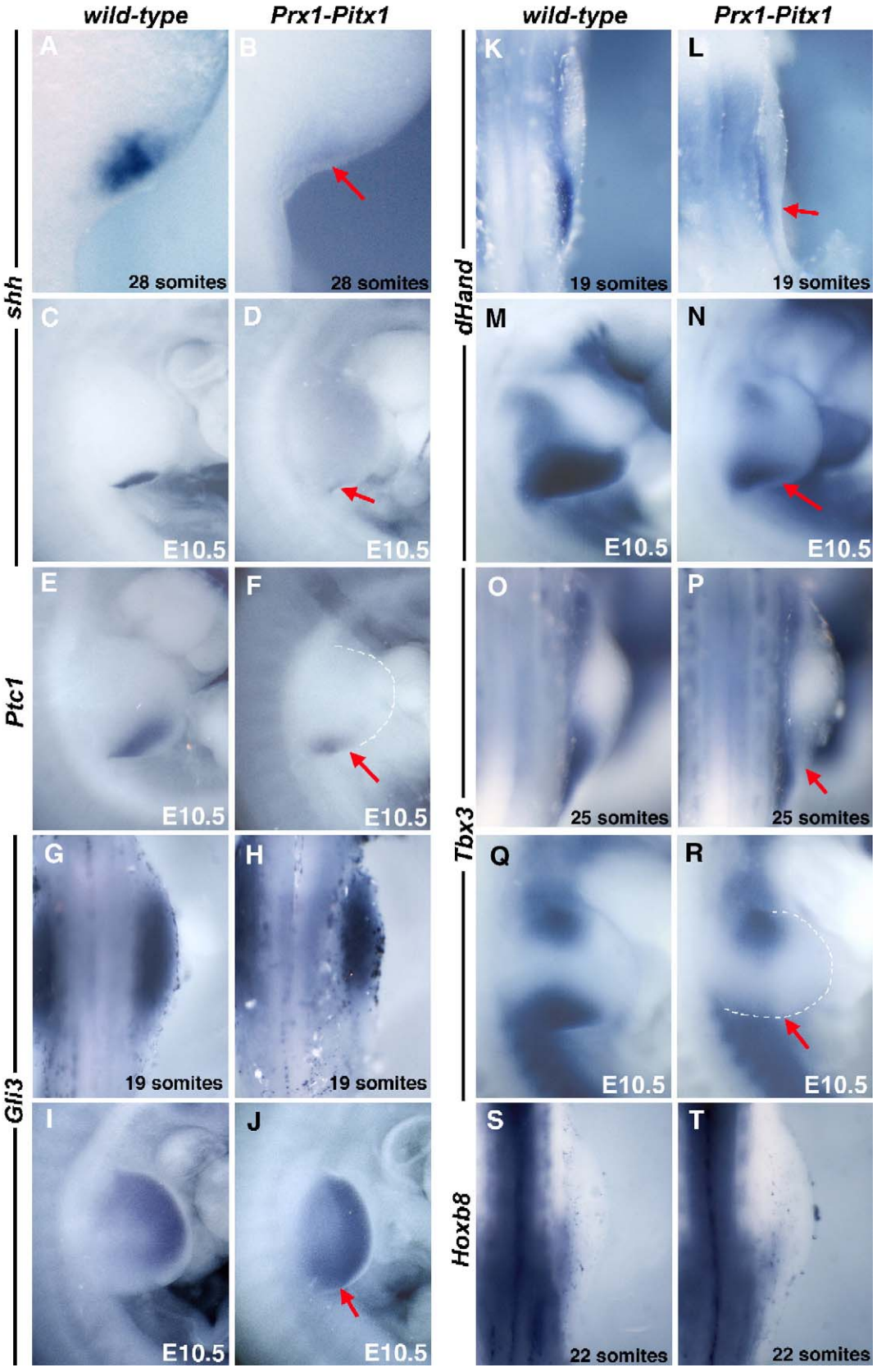
Discussion

Pitx1 has a role in determining hindlimb identity

This study demonstrates that under the influence of a single hindlimb-restricted gene, *Pitx1*, cells in the forelimb can be directed to form structures characteristic of the hindlimb. We have demonstrated this using a novel 3D imaging technique that has allowed us for the first time to analyse the organisation and relationships between muscles, tendons, and bones in the limb at stages of development when these tissues are maturing into their final shape. The level of resolution of the OPT technique is particularly well suited to imaging structures larger than is appropriate or feasible using confocal microscopy. Previous studies have relied on 2D imaging, and single-tissue staining methods; however, our 3D approach has tremendous potential as a powerful tool for future research on understanding the formation of multiple tissues in the limb. More generally, this technique can also be applied towards understanding development of other organ systems.

Fig. 5. Misexpression of *Pitx1* in the forelimb is associated with changes in expression of *Shh*, *Ptc1*, *Gli3*, *dHand*, and *Tbx3*, but not *Hoxb8*. Panels are whole-mount RNA *in situ* hybridisations of wild-type and homozygous *Prx1–Pitx1* transgenic forelimb buds. Panels A and B are of 28 somite stage embryos. Panels C, D, E, F, I, J, M, N, Q, and R are of E10.5 embryos. Panels G, H, K, and L are of 19 somite stage embryos. Panels O and P are of 25 somite stage embryos. Panels S and T are of 22 somite stage embryos. (A and C) Wild-type limb buds showing normal expression of *Shh*. (B and D) Transgenic limb buds showing reduced *Shh* expression (arrowed). (E) Wild-type limb bud showing normal expression of *Ptc1*. (D) Transgenic limb bud showing reduced *Ptc1* expression (arrowed). (G and I) Wild-type limb buds demonstrating normal expression of *Gli3*. (H) Transgenic limb bud showing normal broad expression of Gli3 at early stages. (J) Transgenic limb bud at a later stage still showing broad expression of *Gli3* throughout the limb (arrowed). (K and M) Wild-type limb buds showing normal expression of *dHand*. (L and N) Transgenic limb buds showing reduced posterior expression of *dHand* (arrowed). (O and Q) Wild-type limb buds showing normal expression of *Tbx3*. (P and R) Transgenic limb buds showing reduced posterior expression of *Tbx3* (arrowed), and normal anterior expression. (S) Wild-type limb bud showing expression of *Hoxb8*. (T) Transgenic limb bud showing comparable expression of *Hoxb8* to wild-type.

In our transgenic mouse model, ectopic expression of *Pitx1* in the forelimb is capable of partially transforming multiple limb elements to resemble equivalent structures of the hindlimb. This is consistent with studies in the chick, which have shown that misexpression of *Pitx1* in the wing-bud can transform some elements of the wing to a leg-like identity (Logan and Tabin,



1999). It is also consistent with the deletion of *Pitx1* in the mouse that results in a loss of hindlimb characteristics (Lanctot et al., 1999; Marcil et al., 2003; Szeto et al., 1999) and the observation that rescue of forelimb outgrowth in the mouse using both *Pitx1* and *Tbx4*, rather than *Tbx4* alone, results in limbs with a partially transformed forelimb to hindlimb phenotype (Minguillon et al., 2005). Significantly, in this study, we demonstrate that *Pitx1* has multiple roles in directing the patterning of different tissue types of the limb and that this is complete by E14.5. These roles include influencing the morphogenesis of the cartilaginous precursors of the skeleton, the organisation of myoblasts into muscle bundles, as well as the migration and attachment of tendon cells to positions between bone and muscles. An example of this process is the fusion of the triquetral, and pisiform bones of the wrist to form a calcaneus-like bone, and the translocation of the *extensor indicis proprius* muscle to form an attachment to this transformed bone, similar to the attachment of the *extensor digitorum brevis* muscle of the foot.

By using 3D imaging, we have identified examples of transformation of bone, muscle, and tendon to a hindlimb-like morphology. We demonstrate a translocation of muscles from their normal position to locations similar to equivalent muscles in the hindlimb. We also observed disruptions to the normal patterning of elements in the limb that arose from a primary defect in the skeleton. In the absence of the correct attachments sites on bones muscles either fail to insert or form insertions at abnormal locations.

By taking a multiple-tissue imaging approach, it is possible to observe the alteration of morphology in one tissue as well as associated, but different tissues. For example, when the *extensor carpi radialis* muscle in the transgenic forelimb is transformed to assume the morphology of a hindlimb *tibialis* muscle, the tendon attachments for this muscle are also transformed to reflect the insertion pattern of the tendons of the *tibialis*. During their development, reciprocal interactions between muscles and tendons are required to direct their correct position and attachment in the limb (Kardon, 1998). *Pitx1* may cell-autonomously direct the transformation of certain tissues, such as muscles, which then influence the transformation of associated tissues, such as tendons, in a non-cell-autonomous manner. Alternatively, because *Pitx1* is normally expressed throughout the limb mesenchyme, which includes the tendon progenitors, it may act cell autonomously in both muscles and tendons to affect their position and morphology. The *Prx1* enhancer leads to ectopic expression of *Pitx1* in all cells of the forelimb mesenchyme, including muscle and tendon progenitors; therefore, we cannot distinguish whether the transgene is acting cell autonomously or non-cell autonomously.

Misexpression of Pitx1 in the forelimb induces expression of Hoxc10, a potential downstream target involved in hindlimb patterning

Genes of the *Hoxc* cluster have a rostral–caudal bias to their expression domains along the axis of the embryo. *Hoxc4* and *Hoxc5* are expressed rostrally, in the forelimb region, whereas *Hoxc9*, *Hoxc10*, and *Hoxc11* are expressed caudally, in the hindlimb region (Nelson et al., 1996; Peterson et al., 1992, 1994; Simon and Tabin, 1993). The restricted expression pattern of *Hox* genes between forelimbs and hindlimbs has been implicated in limb-type specification (Favier and Dolle, 1997; Peterson et al., 1992), although the *Hoxc* cluster knockout has no reported limb phenotype (Suemori and Noguchi, 2000). Misexpression of *Pitx1* in the forelimb can induce the expression of the hindlimb marker *Hoxc10*. A similar result is observed when *Pitx1* is misexpressed in the chick wing bud (Logan and Tabin, 1999). The regulatory relationship between *Pitx1* and *Hoxc10* remains unclear, however. The results from ectopic expression of *Pitx1* in the forelimb demonstrate that *Pitx1* is capable of positively regulating the expression of *Hoxc10*, suggesting that this regulatory relationship may normally exist in the hindlimb. However, the expression of *Hoxc10* is reported to be normal in the *Pitx1* knockout mouse indicating that other factors can contribute to its expression (Marcil et al., 2003). The retention of normal expression patterns of *Tbx5* and *Hoxc5* indicates that the normal program of forelimb-restricted expression is unaffected by ectopic expression of *Pitx1*. Therefore, there is no evidence that *Pitx1,* or the genes that are ectopically induced in response to exposure to this factor, *Tbx4* and *Hoxc10*, have any role in repressing the forelimb transcriptional program. The retention of factors normally present in forelimb may explain why some structures of transgenic limbs retain some forelimb traits.

Role for Tbx4 in limb initiation and outgrowth

Our results demonstrate that *Tbx4* does not have a role in determining limb-type identity, consistent with a study in which *Tbx5* expression in the forelimb is replaced with *Tbx4* (Logan and Tabin, 1999; Minguillon et al., 2005). *Tbx4* may be a downstream target of *Pitx1* in the chick hindlimb, as misexpression of *Pitx1* in the wing bud induces ectopic *Tbx4* expression (Logan and Tabin, 1999; Szeto et al., 1999), and deletion of *Pitx1* in the mouse results in downregulation of *Tbx4* in the hindlimb (Lanctot et al., 1999; Marcil et al., 2003; Szeto et al., 1999). *Tbx4* is upregulated in the forelimb of *Prx1–Pitx1* transgenics, supporting a model in which *Tbx4* is a target of *Pitx1*. Although results from the deletion of *Tbx5* and replacement with *Tbx4* suggests that these genes do not have a role in specifying limb-type identity, *Tbx5* and *Tbx4* are excellent markers of whether progenitor cells will give rise to forelimb of hindlimb structures, respectively.

Our results demonstrate that misexpression of *Pitx1* during limb bud stages is capable of diverting the forelimb program to a hindlimb program, at least partially. In these contexts our observations of ectopic *Tbx4* and *Hoxc10* serve as a readout that cells in the forelimb have been diverted to a hindlimb transcriptional program. While ectopic *Tbx4* would not be predicted to have an instructive role in transforming forelimb elements, *Hoxc10* remains a candidate. In addition, ectopic expression of *Tbx4* may not occur as a result of it being a direct target of *Pitx1* but rather as a

consequence of the transformation of a complex of transcriptional networks to a hindlimb program.

Relationship between Pitx1 and expression of Shh

Ectopic expression of *Pitx1* in the forelimb results in downregulation of posterior markers including *Shh* and *Ptc* and a corresponding loss of posterior digits of the forelimb, whereas over-expression in the hindlimb results in a normal limb. There is accumulating evidence that the mechanisms establishing the ZPA are different between forelimbs and hindlimbs (Charite et al., 1994, 2000; Hornstein et al., 2005; Stratford et al., 1997). Although our results do not implicate *Pitx1* as a factor in establishing the ZPA in the hindlimb, they support evidence that the factors acting upstream to positively regulate *Shh* expression in the ZPA differ between forelimbs and hindlimbs. 5′ Hox genes are required for *Shh* expression in the limb (Zakany et al., 2004). A potential explanation for the disruption of *Shh* expression could be that ectopic *Pitx1* alters the expression of these Hox genes.

In conclusion, the results of this study support a model in which *Pitx1* determines hindlimb identity. By using novel 3D imaging techniques offering an unprecedented level of detail of structure, organisation, and relationship between tissues of the limb, we show that misexpression of this gene in the forelimb can transform individual muscles, tendons, and bones to reflect characteristics of the hindlimb. Furthermore, we show that *Pitx1* is capable of positively regulating other hindlimb-specific genes including *Hoxc10* and *Tbx4*. The restricted expression of *Hox* genes between forelimbs and hindlimbs has been implicated in limb-type specification (Favier and Dolle, 1997; Peterson et al., 1992), and our results place *Pitx1* as a potential upstream regulator of *Hox* genes in the hindlimb. Misexpression of *Tbx4* in the forelimb does not transform the limb, reinforcing the evidence that its function is not to determine identity of the hindlimb.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2006.06.055.

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