Development 130, 45-55

doi:10.1242/dev.00192

Pitx1 and *Pitx2* are required for development of hindlimb buds

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Accepted 11 October 2002

SUMMARY

Two closely related homeobox transcription factors, Pitx1 and Pitx2, have been implicated in patterning of lateral plate mesoderm derivatives: Pitx1 for specification of hindlimb identity and Pitx2 for determination of laterality. We show that, together, Pitx1 and Pitx2 are required for formation of hindlimb buds and, when present in limited doses, for development of proximal (femur) and anterior (tibia and digit 1) hindlimb structures. Although Pitx1 is expressed throughout developing hindlimb buds, Pitx2 is not expressed in limb bud mesenchyme itself, but is co-expressed with Pitx1 in the presumptive hindlimb field before bud growth. Thus, *Pitx1* and *Pitx2* genes are required for sustained hindlimb bud growth and formation of hindlimbs.

Key words: Pitx1, Pitx2, Limb, Patterning, Mouse

INTRODUCTION

Limb patterning and growth appear to be intimately interrelated as the same signals have been implicated in both processes. In particular, members of the fibroblast growth factor (Fgf) family are associated with early events of limb induction, outgrowth and maintenance (Martin, 1998). An early marker of limb field specification is provided by the restriction of *Fgf10* expression within the lateral plate mesoderm (lpm) that corresponds to this field (Ohuchi et al., 1997). Signals for restriction of *Fgf10* expression to this field may involve *Fgf8* in the corresponding intermediate mesoderm (Crossley et al., 1996) and/or Wnt signalling molecules (Kawakami et al., 2001). This early restricted expression of *Fgf10* appears responsible for induction of *Fgf8* in the overlying surface ectoderm that is destined to become the apical ectodermal ridge (AER) of the growing limb. This initial action of *Fgf10* appears essential for limb outgrowth as *Fgf10*-deficient mice failed to develop limbs (Min et al., 1998; Sekine et al., 1999). In turn, the AER and growth factors it produces, including *Fgf8*, play an essential role for limb bud outgrowth (Fallon et al., 1994; Sun et al., 2002; Lewandoski et al., 2000; Crossley et al., 1996; Niswander et al., 1993). One of these roles is for the maintenance of *Fgf10* expression in the growing limb mesenchyme (Crossley et al., 1996; Ohuchi et al., 1997). In addition, the AER and *Fgf8* are important for induction of anteroposterior (AP) polarity. Indeed, *Fgf8* is required for induction of the zone of polarizing activity (ZPA), a structure that expresses the posteriorizing signal molecule sonic hedgehog (*Shh*) (Lewandoski et al., 2000). In turn, *Shh* feedback onto the AER induces expression of another growth factor, *Fgf4* (Zuniga et al., 1999).

En1 and *Lmx1b* are both transcription factors of the homeodomain family and they appear to play roles in marking the identity of ventral or dorsal limb domains, respectively (Chen and Johnson, 1999). Indeed, the knockout of these genes lead to the loss of ventral (*En1*) (Logan et al., 1997; Loomis et al., 1998) or dorsal (*Lmx1b*) structures in mice (Chen et al., 1998; Dreyer et al., 1998). Similarly, the identity of proximodistal (PD) domains in the limb appears to be defined very early in the growing limb bud. In particular, the proximal limb domain where stylopod (femur or humerus) will form is marked by expression of other homeobox-containing transcription factors, Meis1 and Meis2, and gain-of-function experiments in chick embryos have suggested that this restricted expression is required for specification of both zeugopod and stylopod domains of the limb (Capdevila et al., 1999; Mercader et al., 2000).

The scheme described above for limb induction, patterning and growth is thought to be a generic one acting both at forelimbs (FL) and hindlimbs (HL). However, the appearance of distinct HL during evolution has probably required a new set of signals and transcription factors to mark the identity of HL by comparison to FL. The extent to which FL represent a default pathway for limb formation remains a subject of debate, although FL-specific transcription factors such *Tbx5* have been identified (Chapman et al., 1996; Gibson-Brown et al., 1996; Gibson-Brown et al., 1998; Logan et al., 1998b) and are involved in FL formation (Basson et al., 1997; Li et al., 1997; Rodriguez-Esteban et al., 1999; Takeuchi et al., 1999). The implication of transcription factors for specification of HL identity is clearer. Indeed, the homeobox containing transcription factor Pitx1 has been shown to become specifically restricted to HL mesenchyme following its early

expression throughout posterior lpm (Lanctôt et al., 1997). The role of *Pitx1* in HL identity was clearly supported by gene inactivation experiments in mice that resulted in HLs showing features of FL in particular at the level of zeugopod and knee joint (Lanctôt et al., 1999b; Szeto et al., 1999). The interpretation of these studies were further supported by gain-of-function experiments using retrovirus-mediated *Pitx1* expression in FL buds of chick embryos: the resulting wings developed with partial features of legs both at the level of skeleton and muscle (Logan and Tabin, 1999). Another transcription factor, a member of the T-box family *Tbx4*, was also implicated in specification of HL identity but its expression appears to be downstream and, in part, under control of *Pitx1* (Lanctôt et al., 1999b; Szeto et al., 1999; Logan and Tabin, 1999).

A surprising observation made on *Pitx1*$^{-/-}$ embryos was a relatively frequent left-right (LR) asymmetry in the severity of the phenotype (Lanctôt et al., 1999b). Indeed, femur length was found to be more often reduced on the right compared with left HLs. As the *Pitx1*-related homeobox factor *Pitx2* was shown to be an effector for LR asymmetry in the lpm (Logan et al., 1998a; Piedra et al., 1998; Ryan et al., 1998; Yoshioka et al., 1998), we have suggested that redundancy between the Pitx genes may explain the LR asymmetry in the phenotype of *Pitx1*$^{-/-}$ embryos. This redundancy is somewhat counter-intuitive because under normal conditions both limbs are symmetrical and are not subject to LR patterning. In part to verify this hypothesis, we generated mice that are double mutants for *Pitx1* and *Pitx2*. The analysis of these mice not only confirmed an apparent redundancy between the two factors but unexpectedly highlighted a co-operative role of both Pitx genes in formation of HL buds.

MATERIALS AND METHODS

Mice

The *Pitx1* and *Pitx2* mutant alleles (Fig. 1A) and their phenotypes were described previously (Gage et al., 1999; Lanctôt et al., 1999b). Genotyping of embryos or pups was carried out by PCR as described (Gage et al., 1999; Lanctôt et al., 1999b) using DNA isolated from the tail or umbilical cord/amniotic membrane of the newborn. Separate PCR reactions were carried out for *Pitx1* and *Pitx2* genotyping. Except for *Pitx1*$^{-/-}$ mice in the 129sv background, all other mice used in this study were in mixed genetic background. Noon of the day on which a vaginal plug was detected was considered as ~E0.5. Embryos were staged more precisely by counting the number of somites posterior to the forelimb bud and scoring the first one counted as somite 13 (Lewandoski et al., 2000).

Skeletal preparation and staining

E17.5 or E16.5 embryos were stained with Alcian Blue and Alizarin Red, and younger embryos (E13.5) were only stained with Alcian Blue as described (McLeod, 1980).

Whole-mount embryo staining

Whole-mount in situ hybridization and immunohistochemistry was done as described in protocols from Dr Janet Rossant's laboratory. These two protocols used can be found at http://www.mshri.on.ca/develop/rossant/protocols.html

Immunohistochemistry

Section immunohistochemistry was performed as described (Lanctôt et al., 1999a) using previously characterized Pitx1 and Pitx2 primary antibodies (Tremblay et al., 1998; Hjalt et al., 2000). MyoD antibody was purchased from Pharmingen. Biotinylated anti-rabbit (Vector Labs, 1/150), was used as secondary antibody and revealed using streptavidin-HRP (NEL750, NEN, 1/1000) and DAB. Slides were counter-stained with Methyl Green.

RESULTS

Inactivation of the mouse *Pitx1* (Fig. 1A) resulted in loss of some HL-specific features and their replacement by features reminiscent of FL (Lanctôt et al., 1999b; Szeto et al., 1999). More specifically, the diameters of tibia and fibula were similar, unlike the normal bones but much like radius and ulna of FL, and secondary cartilage of the knee joints did not form in *Pitx1*$^{-/-}$ mice, resembling instead the FL articulation. In addition, the fibula contacted directly the femur in *Pitx1*$^{-/-}$ mice as opposed to contacting the tibia in wild-type or heterozygous littermates. Femur length was also reduced in

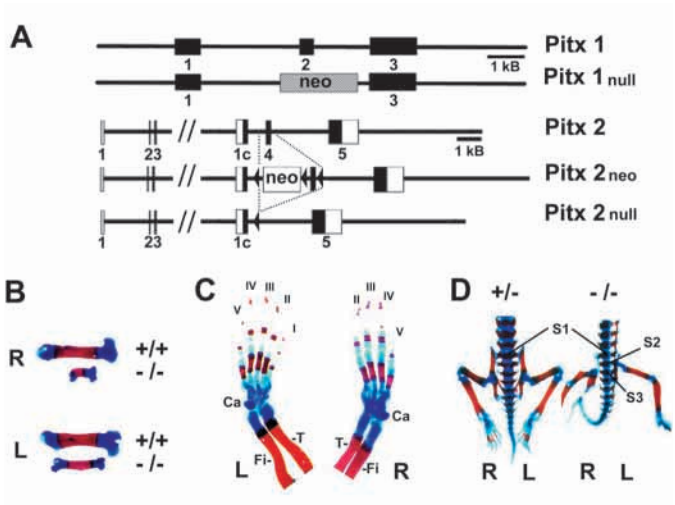

Fig. 1. Pitx gene alleles and left-right (LR) asymmetry during hindlimb development. (A) Schematic representation of the mouse *Pitx1* and *Pitx2* genes and of mutant alleles used in the present study. Numbered boxes represent exons, and in each case, the null alleles were produced by deletion of the homeodomain-encoding exon. In the *Pitx2neo* allele, arrowheads indicate the position of loxP sites used by CRE recombinase to yield the null allele. (B) Size reduction in femur length observed in *Pitx1*$^{-/-}$ embryos. Dissected femurs from right and left side of the same skeleton stained at E17.5 for bone (red) and cartilage (blue) are shown for wild-type and knockout ($^{-/-}$) embryos. (C) All *Pitx1*$^{-/-}$ embryos examined in the pure 129sv genetic background showed a loss of hindlimb (HL) digit 1 (I) on the right side only, whereas in mixed genetic background (129sv / Balb/c) all five digits were present on both sides. (D) Ventral view of skeletal preparations showing vertebrae and HL of E17.5 mice either heterozygous ($^{+/-}$) or homozygous ($^{-/-}$) for the *Pitx1* knockout allele. The first sacral vertebra is indicated (S1); pelvic bones are normally ($^{+/-}$) attached to S1 by the distal end of the ilium. In the majority of *Pitx1*$^{-/-}$ embryos, this attachment is through the acetabulum because the ilium does not form in *Pitx1*$^{-/-}$ embryos (Lanctôt et al., 1999b). However, in few rare cases, femur and pelvic bone attachment to the vertebrae is displaced posteriorly, usually to S2, and in some cases (like the one shown here), displacement is even greater (S3) on the right than left side.

Pitx1$^{-/-}$ HLs. Surprisingly, this reduction was often greater (in about two-thirds of mice) on the right compared with the left side (Fig. 1B). In a mixed genetic background (129sv/Balb/c), *Pitx1*$^{-/-}$ mice occasionally exhibited loss of digit one of the right but not left HL. However, loss of the right HL digit one occurred in all homozygous mice when the knockout allele was present in a pure 129sv background (Fig. 1C). Also occasionally, the attachment of pelvic bones to vertebrae was displaced posteriorly in *Pitx1*$^{-/-}$ mice. Normally, the anterior tip of the ilium is attached to first sacral vertebra, S1. In knockout embryos that lack ilium, this attachment is through the acetabulum, and in some embryos it is displaced posteriorly to S2 or S3. In a few cases, this posterior displacement was asymmetrical, with greater displacement on the right than left side (Fig. 1D). Taken together, these observations indicate greater penetrance of the Pitx1 null phenotype on the right than left side. In view of the predominant expression of *Pitx2*, a factor closely related to *Pitx1*, in left-side lpm, we put forward the hypothesis of a partial redundancy between these two *Pitx* genes.

In order to ascertain the putative redundancy between *Pitx1* and *Pitx2* genes, *Pitx1*$^{+/-}$ mice were crossed with mice carrying either a hypomorphic (neo) or null allele of the *Pitx2* gene (Fig. 1A) (Gage et al., 1999). To obtain double mutant mice, we crossed *Pitx1*$^{+/-}$ mice with *Pitx2*$^{+/-}$ mice. Surprisingly, we did not get the expected Mendelien ratio of 25% double heterozygotes (*Pitx1*$^{+/-}$,*Pitx2*$^{+/-}$) but only 2%. We cannot explain the poor viability of these mice. This was not observed with the *Pitx2neo* allele, which gave close to the expected yield (20%) when crossed with *Pitx1*$^{+/-}$ mice. Double mutant embryos (*Pitx1*$^{-/-}$,*Pitx2*$^{+/-}$) were obtained by crossing *Pitx1*$^{+/-}$ mice with *Pitx1*$^{+/-}$,*Pitx2*$^{+/-}$ mice. We only ever got one *Pitx1*$^{-/-}$,*Pitx2*$^{-/-}$ embryo by intercrossing double heterozygotes and a few *Pitx1*$^{-/-}$,*Pitx2neo/–* embryos were obtained by crossing double heterozygotes of each *Pitx2* allele.

Mutant (*Pitx1*$^{-/-}$,*Pitx2neo/neo*) mice with the most extreme phenotype showed a much more extensive phenotype than single mutant mice (Fig. 2). Whereas *Pitx1*$^{-/-}$ mice exhibit the patterning defects described above, *Pitx2* mutant embryos do not exhibit any obvious limb defect (Gage et al., 1999; Kitamura et al., 1997; Lin et al., 1999; Lu et al., 1999). By contrast, double mutant mice have lost three HL skeletal elements. Indeed, both right and left femur, tibia and digit one are missing in these embryos (Fig. 2). The pelvis is not more severely affected than in *Pitx1*$^{-/-}$ mice. The identification of the only remaining zeugopodal element as fibula is based on the contact between this bone and the calcaneus. Except for the loss of digit one, it is striking how the autopod is unaffected by the double gene mutation.

In agreement with the hypothesis of a gene dose-dependent phenotype, the loss of HL skeletal elements followed a reproducible pattern in series of embryos deficient for *Pitx1*, either carrying the *Pitx2neo/neo* alleles (data not shown) or the *Pitx2*$^{+/-}$ alleles (Fig. 3). The order of bone loss with progressive penetrance of the phenotype is as follows. Right digit 1 was the most sensitive to loss of Pitx function (Fig. 3B), as was observed in some *Pitx1*$^{-/-}$ mice (Fig. 1C). In more affected embryos, the right tibia partially or completely failed to develop (Fig. 3C) and then the right femur was lost (Fig. 3D). On the left side, dependence on Pitx function followed a similar sequence: digit 1 (Fig. 3D), tibia (Fig. 3E), followed by reduction (Fig. 3F) and loss of left femur (Fig. 2). All skeletal preparations examined (over 20 embryos) fit within this sequence of bone losses. The phenotype of these double mutant mice is in part reminiscent of embryos deficient for limb AER *Fgf8* expression. Indeed, these mice also failed to develop femur and digit 1 and the tibia is hypoplastic (Lewandoski et al., 2000).

Analysis of early limb bud development revealed smaller HL buds (in all of over 100 embryo pairs examined), both in *Pitx1*$^{-/-}$ and *Pitx1*$^{-/-}$,*Pitx2*$^{+/-}$ embryos compared with wild-type littermates (Fig. 4). In most cases (~60% of pair comparisons), limb bud size reduction was greater for double than single mutant embryos (Fig. 4A). Greater reduction was observed on right compared with left side in about 50% of either *Pitx1*$^{-/-}$ or double mutant embryos. The reduction in *Pitx1*$^{-/-}$ HL bud size is surprising as these embryos show patterning defects but no loss of skeletal elements, except for reduction in femur size. This observation could however be consistent with a joint role of *Pitx1* and *Pitx2* genes in early expansion of lpm in the HL field and of early limb bud mesenchyme. When measured relative to somites (Fig. 4A), the reduction in HL bud size observed in mutant embryos is striking because it results from a narrowing of the HL bud from a length of about 3.5/4 somites (approx. somites 24.5 to 28.5) to a length of 2.5 somites in *Pitx1*$^{-/-}$ embryos (approx. somites 25.5 to 28.0) and to a length of about 2 somites in *Pitx1*$^{-/-}$,*Pitx2*$^{+/-}$ embryos (approx. somites 26 to 27.5-28.0). In all cases, the limb bud is centered on somite 27. This narrowing along the AP axis was best revealed in embryos labeled by whole-mount in situ hybridization with a probe for *Tbx4*, a HL-

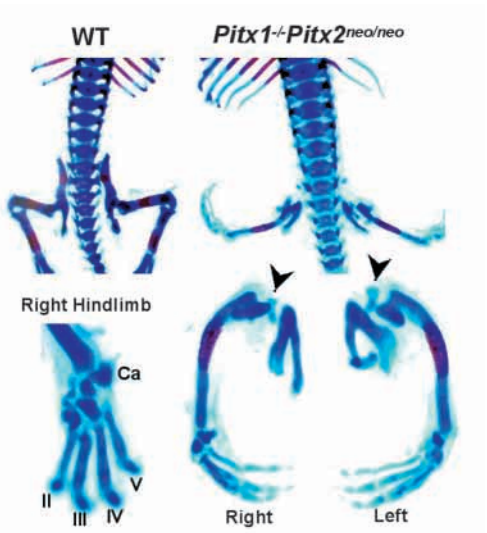

Fig. 2. Loss of proximal (femur) and anterior (tibia and first digit) bones in hindlimbs (HL) of mice mutant for both *Pitx1* and *Pitx2*. Skeletal preparations (Alizarin Red, bone; Alcian Blue, cartilage) of E16.5 wild-type (WT) and *Pitx1*$^{-/-}$,*Pitx2neo/neo* embryo showing the pelvic area (top right), the right and left dissected HL with one remaining zeugopod bone and four digits (I,III,IV,V; bottom right), as well as an enlargement of the right HL autopod (bottom left) showing the remaining zeugopod bone contacting the calcaneus (Ca). Based on this, it is concluded that the remaining bone is the fibula. Small cartilaginous remnants (arrowheads) between the pelvic bone and fibula could be the only remain of the femur. This skeleton represents the most extreme phenotype seen in this embryo series.

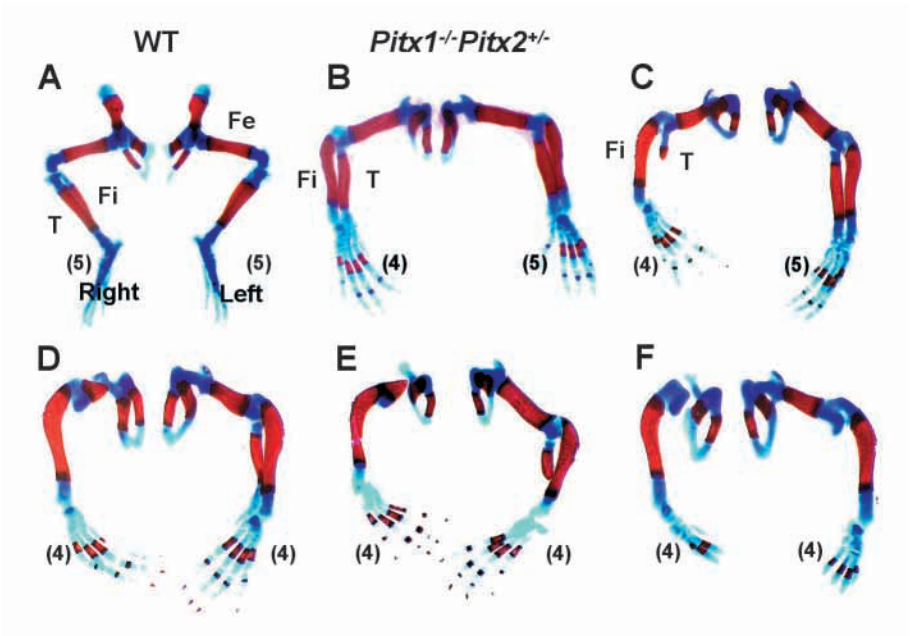

Fig. 3. Progressive penetrance of hindlimb (HL) phenotype observed in series of *Pitx1*$^{-/-}$,*Pitx2*$^{+/-}$ embryos. All embryos observed fitted the sequence of bone losses illustrated here. All dissected hindlimbs are positioned similar to the wild-type preparation (A). (B) The first digit of the right HL is missing. (C) The right tibia is severely affected. (D) The right tibia and femur did not form and the left digit 1 has disappeared. (E) The left tibia is partially lost. (F) There is only a remnant of the left femur. Fe, femur; T, tibia; Fi, fibula; digit numbers are shown in parentheses.

specific marker that has previously been shown to be decreased in *Pitx1*$^{-/-}$ embryos (Lanctôt et al., 1999b; Szeto et al., 1999) and which is similarly decreased in double mutant embryos (Fig. 4A). Thus, HL bud size reduction affects both outgrowth and width of the bud along the AP axis.

Bud outgrowth is thought to be controlled by growth factors produced by the AER. In particular, Fgf8 is the earliest growth factor to mark the AER and at E10.5, this *Fgf8* expression appears similar in single and double mutant embryos compared with wild-type (Fig. 4B). *Fgf10* expressed throughout the mesenchyme of the limb bud is also thought to play a role in growth control (Ohuchi et al., 1997). *Fgf10* expression did not appear to be affected in the single or double mutant embryos (Fig. 4C). Although expression of *Fgf8* and *Fgf10* are not grossly affected in mutant embryos, the loss of skeletal elements in double mutant embryos may reveal a failure to specify limb bud segments, for example, the proximal segment from which the stylopod (femur) develops. As this proximal segment is marked by expression of Meis genes, we investigated Meis gene expression in embryos mutant for *Pitx1* or for *Pitx1* and *Pitx2*. In both, *Meis2* expression was similar to that in wild-type embryos (Fig. 4D); similar results were obtained for *Meis1* (data not shown). These data suggest that failure to develop stylopod (femur) in *Pitx1*$^{-/-}$,*Pitx2*$^{+/-}$ embryos does not result from a failure to specify the proximal limb domain.

However, limb outgrowth could be curtailed if early expression of Fgf genes was delayed (Min et al., 1998; Sekine et al., 1999; Lewandoski et al., 2000; Moon and Capecchi, 2000). For this reason, we investigated early expression of *Fgf10*, *Fgf8* and other markers. As shown in Fig. 4E, early HL expression (25 somites) of *Fgf10* was not significantly altered in either *Pitx1*$^{-/-}$ or *Pitx1*$^{-/-}$,*Pitx2*$^{+/-}$ embryos. Examination of 5-10 embryos/genotype suggested a slight decrease of *Fgf10* expression, but this proved difficult to substantiate objectively. Similarly, early HL expression of *Bmp7* was not different in double compared with single mutant embryos (Fig. 4F) and AER expression of *Msx2* was also unaffected in mutant embryos (Fig. 4G). AER expression of *Fgf8* in HL starts at stage 27 somites in wild-type embryos. A similar onset was observed for *Pitx1*$^{-/-}$ embryos, although expression could be slightly reduced (Fig. 4H). AER expression of *Fgf8* was delayed in *Pitx1*$^{-/-}$,*Pitx2*$^{+/-}$ embryos with an onset at stage 30 somites (Fig. 4H). Hence, a delay and/or reduction in AER expression of *Fgf8* may account in part for the phenotype of double mutant embryos, as proposed to explain the differential effect in FL or HL of conditional *Fgf8* knockout (Lewandoski et al., 2000).

The reduction in HL bud size along the AP axis suggests that AP patterning of the limb bud might be altered. In order to assess this within the context of global AP patterning, the expression in HL of posterior Hox genes was ascertained by whole-mount in situ hybridization. At E11.5, the anterior border of *Hoxc11* expression was found to be on the rostral side of somite 27, which lies in the middle of the developing HL buds (Fig. 5A). In *Pitx1*$^{-/-}$ and *Pitx1*$^{-/-}$,*Pitx2*$^{+/-}$ embryos, the anterior border of *Hoxc11* expression was the same relative to somite 27 (Fig. 5A) but the narrowing of the HL bud in mutant embryos appeared to result in loss of anterior bud mesenchyme. In agreement with this, the strong band of *Hoxc11* expression observed in the posterior third of wild-type HL buds is similarly posterior in mutant limb buds but the band now accounts for about half of the bud mesenchyme, as if anterior bud mesenchyme was missing (Fig. 5A). Expression of *Hoxc9* and *Hoxc10* was not affected in these mutant embryos (data not shown).

In order to further investigate AP patterning within the buds, we assessed *Shh* and *Gli3* expression by whole-mount in situ hybridization. *Shh* labels the ZPA, which is known to play an important organizer function to define AP polarity in the limb bud and *Gli3* marks the anterior bud mesenchyme. In both wild-type and *Pitx1*$^{-/-}$ embryos, *Shh* expression was similar at the posterior margin of the limb bud (Fig. 5B). By contrast, *Shh* expression extended halfway up the limb bud in *Pitx1*$^{-/-}$,*Pitx2*$^{+/-}$ embryos (Fig. 5B). Thus, the ZPA of double mutant embryos appears to extend further anteriorly compared

with wild-type or *Pitx1*$^{-/-}$ embryos. By contrast, anterior bud expression of *Gli3* was similar in mutant and wild-type embryos (Fig. 5C), indicating that anterior signals are still present in *Pitx* mutant embryos. AER expression of *Fgf4* was also extended anteriorly in mutant embryos (Fig. 5D). Given the narrowing of the limb bud, the apparent extension of the ZPA may be secondary to the loss of mesenchyme and/or extension of posterior signal. This was further assessed using another marker of posterior limb mesenchyme, *Hand2* (*dHand*), that has previously been associated with AP patterning defects at the zeugopod and autopod levels (Charité et al., 1995; Fernandez-Teran et al., 2000). Indeed, overexpression of *Hand2* in the HL has resulted in loss of tibia, similar to our double mutant mice (Charité et al., 1995). Expression of *Hand2* was found to extend more into the anterior half of the HL bud in mutant embryos. A striking example of this is shown in Fig. 5E, where *Hand2* expression extends the entire width of the right limb bud at zeugopod level but still only covers the posterior side of the left HL. Thus, the effect of the loss of Pitx genes, in particular at the zeugopod level, might be in part ascribed to a more anterior expression of *Hand2* within the limb bud.

Clearly, the role of Pitx genes would be best revealed in double null mutant embryos. We only obtained one such embryo in almost two years of breeding and we got a few *Pitx1*$^{-/-}$,*Pitx2neo/–* embryos, which should express less Pitx2 than null heterozygotes. These latter embryos had more severely affected HL, in particular autopods (Fig. 6A-C). Indeed, both embryos shown in Fig. 6 have three remaining digits on the left side and only two on the right, as revealed either by Alcian Blue staining of cartilage (Fig. 6B) or by in situ hybridization for *Sox9*, which also marks cartilaginous condensations (Fig. 6C). The further loss of digits as *Pitx2* gene dose was decreased is suggestive of a dependence on *Pitx* genes for expansion of limb bud mesenchyme. This idea is further supported by the single *Pitx1*$^{-/-}$,*Pitx2*$^{-/-}$ embryo that we obtained (Fig. 6D). Indeed, at E12.5, this embryo had severely retarded HL development. Furthermore, the left HL bud exhibited some AER expression of *Fgf8* and it was bigger than the right HL bud. This LR asymmetry cannot be attributed to *Pitx2* and may suggest involvement of other regulators. It thus appears that induction of AER function was not prevented in absence of both Pitx genes, although growth of HL buds was severely curtailed. Total Pitx gene expression level appears to be the most important parameter for HL bud growth as *Pitx1*$^{+/-}$,*Pitx2*$^{-/-}$ embryos from the same litter (Fig. 6D) had relatively normal HL bud development, in agreement with the idea that *Pitx1* has the highest expression level and is the most important for HL bud formation.

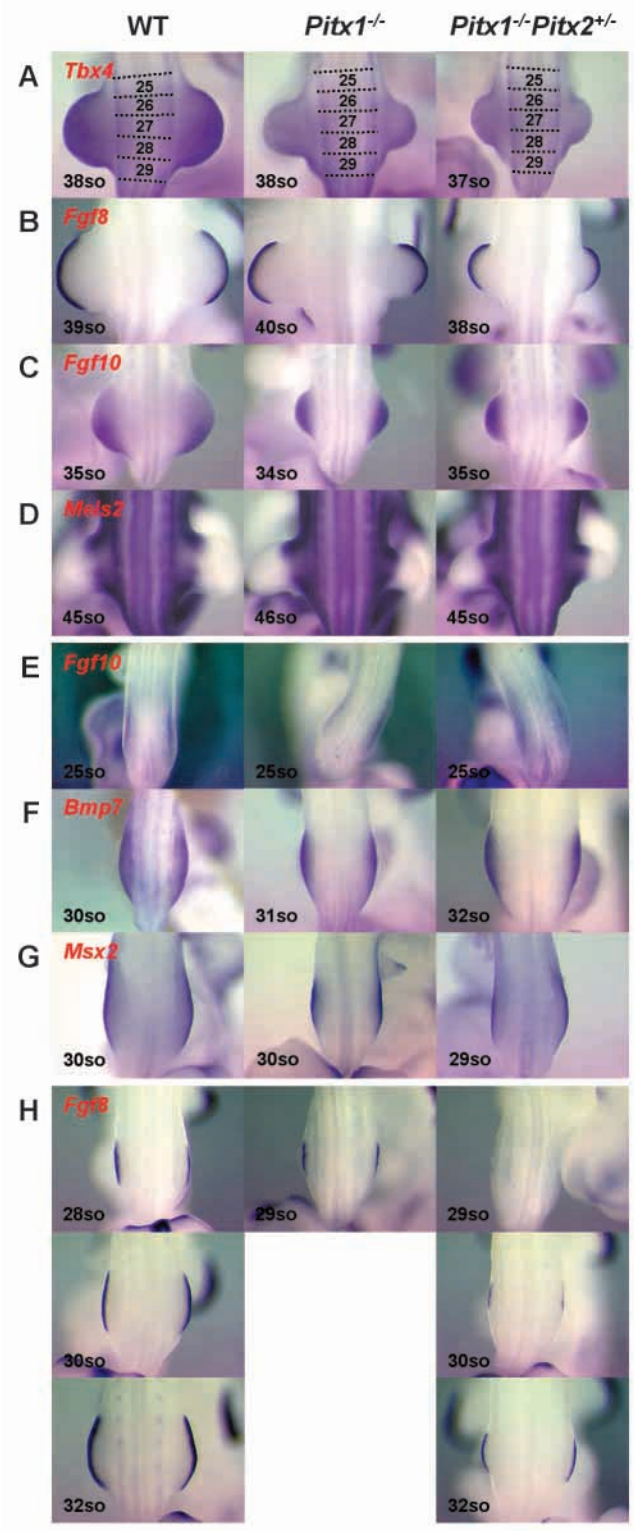

Fig. 4. Analysis of hindlimb (HL) bud formation in wild-type, *Pitx1*$^{-/-}$ and *Pitx1*$^{-/-}$,*Pitx2*$^{+/-}$ embryos. Dorsal views of embryos are shown with assessment of developmental stage provided by somite (so) count. (A) The HL-specific transcription factor *Tbx4* mRNA was revealed by whole-mount in situ hybridization and found to be downregulated in mutant embryos (~E10.5). This staining offered the best contrast to outline the position of somites along the AP axis and these are indicated by numbers for each embryo. Both mutant embryos show smaller right and left HL bud compared with WT, with greater reduction on the right side. (B) In situ hybridization for *Fgf8* revealing the AER. (C) In situ hybridization for *Fgf10* marking the HL bud mesenchyme (~E10.0). (D) In situ hybridization for *Meis2* mRNA revealing the proximal segment of the HL bud (~E11.5). Similar results were obtained with *Meis1* (data not shown). (E) In situ hybridization for *Fgf10* in HL field of 25-somite embryos (~E9). (F) In situ hybridization for *Bmp7* in HL field at onset of bud growth. (G) In situ hybridization for *Mxs2*. (H) In situ hybridization for *Fgf8* (~E9.5), revealing early expression of *Fgf8* and initiation of HL bud outgrowth. *Fgf8* expression is delayed in *Pitx1*$^{-/-}$,*Pitx2*$^{+/-}$ embryos from about 27- to 30-somite stages of development.

The genetic requirement for both *Pitx1* and *Pitx2* during growth and patterning of HL is surprising in view of the previously characterized expression of these genes. Whereas *Pitx1* was known to be expressed from early-on throughout the HL mesenchyme, *Pitx2* is not known to be expressed in this mesenchyme (Campione et al., 1999; Kitamura et al., 1997; Logan et al., 1998a; Mucchielli et al., 1996; Piedra et al., 1998; Ryan et al., 1998; Semina et al., 1997; Yoshioka et al., 1998). It was therefore surprising to observe such strong genetic requirement for both genes, and this led us to reinvestigate in detail the expression of both Pitx genes from early development throughout limb growth. Both whole-mount and sectioned embryos were analyzed for mRNA expression using in situ hybridization and for protein using immunohistochemistry. Whole-mount histochemical analysis of Pitx1 and Pitx2 in early E8.5-E9.0 embryos revealed that, in addition to their joint expression in the stomodeum, both factors are also co-expressed in the tail bud region presumed

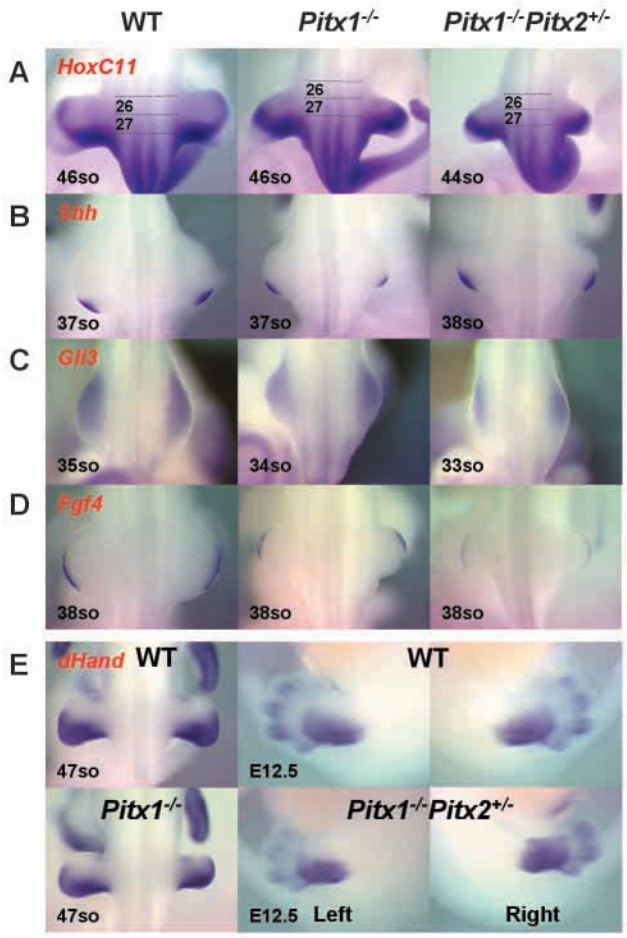

Fig. 5. Hindlimb (HL) anteroposterior markers reveal apparent loss of anterior bud mesenchyme. Dorsal views of whole-mount in situ hybridization embryos are shown. (A) The anterior border of *Hoxc11* mRNA is revealed at the junction between somites 26 and 27. In mutant embryos, the proportion of *Hoxc11*-negative anterior mesenchyme relative to *Hoxc11*-positive mesenchyme is reduced, consistent with the loss of anterior bud mesenchyme revealed in Fig. 5A. (B) The zone of polarizing activity (ZPA) is revealed by hybridization for sonic hedgehog (*Shh*). Whereas in wild-type and *Pitx*$^{-/-}$ embryos the ZPA occupies the posterior quadrant of the HL bud, this structure extends all the way up to half the HL buds in *Pitx1*$^{-/-}$,*Pitx2*$^{+/-}$ embryos. (C) Expression of *Gli3* in anterior hindlimb buds is present in embryos of the three genotypes. (D) Expression of *Fgf4* in AER. The extent of *Fgf4* expression appears anteriorized in mutant embryos compared with wild type, again in agreement with the loss of anterior mesenchyme. (E) Expression of the posterior limb bud mesenchyme marker, *Hand2* (*dHand* in figure) is also extended anteriorly. Two examples at different developmental stages are shown with LR differences in the anterior extension of *Hand2* expression. (Bottom row) Anterior bud expression of *Hand2* is shown in the right HL bud of an ~E11.5 *Pitx1*$^{-/-}$ embryo, and in the right HL of a E12.5 *Pitx1*$^{-/-}$,*Pitx2*$^{+/-}$ embryo.

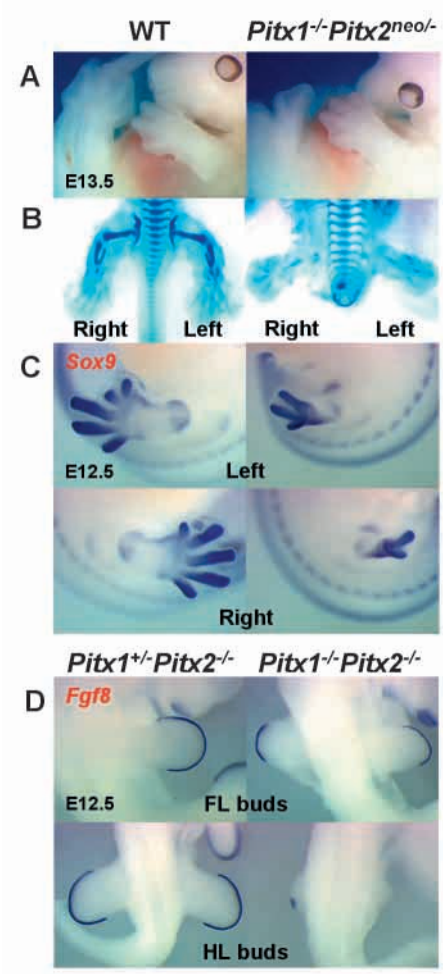

Fig. 6. Further loss of digits in *Pitx1*$^{-/-}$,*Pitx2neo/–* embryos. A few *Pitx1*$^{-/-}$, *Pitx2neo/–* embryos were obtained and found to miss more than one hindlimb (HL) digit. (A,B) Photograph (A) and skeletal preparations (B) of E13.5 wild-type and *Pitx1*$^{-/-}$,*Pitx2neo/–* embryos showing loss of one digit on the left side and of two digits on the right side. Note absence of forelimb (FL) defects. (C) Similar embryos in which cartilaginous condensation of the digits were revealed at E12.5 using whole-mount in situ hybridization for *Sox9*. The *Pitx1*$^{-/-}$,*Pitx2neo/–* embryo has three digits on left and two digits on right side. The left HL of the wild-type embryo was damaged during preparation. (D) Whole-mount in situ hybridization of AER *Fgf8* in the single *Pitx1*$^{-/-}$,*Pitx2*$^{-/-}$ embryo obtained. This embryo (E12.5) was underdeveloped and smaller than the *Pitx1*$^{+/-}$,*Pitx2*$^{-/-}$ embryo shown for comparison. Whereas FL bud development appeared normal in those embryos, very small HL buds were present in the double null embryo, with a small patch of *Fgf8* expressing tissue on the left side.

to become the HL field (Fig. 7). As previously reported (Lanctôt et al., 1997), Pitx1 expression was restricted to the lpm of the posterior end of the embryo (Fig. 7A-D). Pitx2 immunoreactivity was observed in left lpm as previously reported (Logan et al., 1998a; Piedra et al., 1998; Ryan et al., 1998; Yoshioka et al., 1998). However, this expression appeared to extend throughout the length of the embryo down to the tail bud and weak expression was also detected on the right side of the tail bud (Fig. 7A-D). This expression is much weaker than that of Pitx1. The unexpected observation of co-expression of Pitx1 and Pitx2 in the tail bud region destined to become HL may offer the explanation for the genetic interaction between the two Pitx genes. Later in development, Pitx1 expression is maintained throughout HL mesenchyme (Fig. 7E,F), whereas Pitx2 is not present in HL mesenchyme (Fig. 7E). The only limb bud expression of Pitx2 was observed in myoblasts (Fig. 7F) as indicated by the similarity with the pattern of MyoD (Fig. 7F) and Pax3 (data not shown) expression. It had previously been shown that *Pitx2* is expressed in chick myotomes and myoblasts (Logan et al., 1998a; Piedra et al., 1998). It is very unlikely that *Pitx2* expression in muscle cells may be an important determinant for the growth and patterning defects observed in double mutant mice as splotch mice, which do not form limb muscle, still form all skeletal elements (Henderson et al., 1999). Thus, co-expression of *Pitx1* and *Pitx2* is limited to the mesoderm of the very early HL field and both genes appear required for early expansion of limb bud mesenchyme.

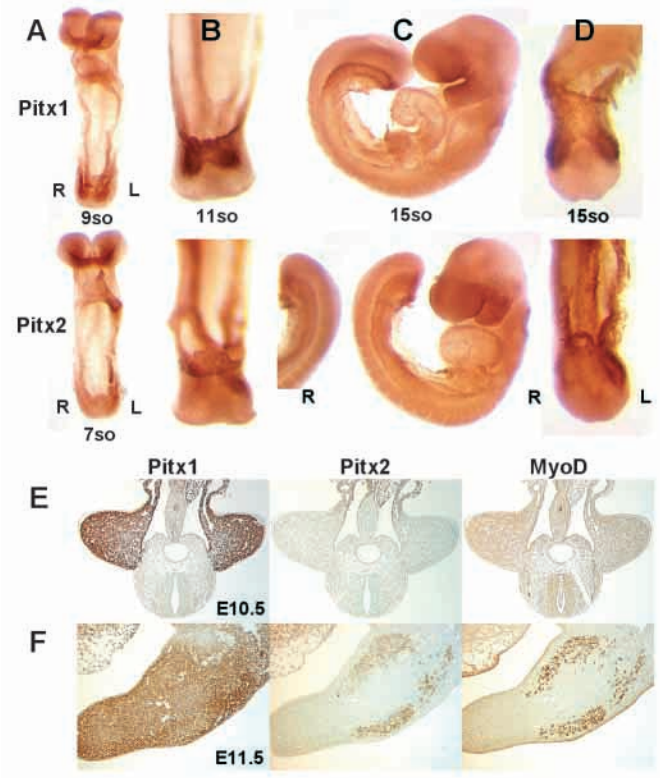

Fig. 7. Early expression of Pitx1 and Pitx2 proteins revealed by whole-mount and section immunohistochemistry. Pitx1 (top) protein is revealed in stomodeum (oral ectoderm) of nine- (A) and 15- (C) somite embryos. Expression in posterior lpm of nine- (A), 11- (B) and 15-somite (C, right side view; D, ventral view) embryos is shown to be bilateral. Pitx2 (bottom) protein is revealed in the head (bilateral) and in left lpm of seven- (A), 11- (B) and 15- (C) somite embryos. In tail bud area, note stronger expression on left side and weaker but significant expression on the right side.
(E) Immunohistochemical analysis of Pitx1, Pitx2 and MyoD protein expression in consecutive transverse sections of E10.5 embryos, revealing Pitx1 only in hindlimb (HL) buds. (F) Consecutive sections of E11.5 HL, revealing Pitx1 protein throughout the mesenchyme and Pitx2 protein in muscle cells that colocalize with MyoD-positive cells.

DISCUSSION

The present work indicates that Pitx genes play essential roles for patterning and growth of HL structures. At least one function of these genes appears to take place much before the onset of limb bud outgrowth and may determine the potential of the HL field. This was not expected from analysis of *Pitx1*-deficient embryos, because *Pitx1* was primarily associated with patterning defects during HL specification (Lanctôt et al., 1999b; Szeto et al., 1999). We have found that HL buds of *Pitx1*$^{-/-}$ and, even more so, *Pitx1*$^{-/-}$,*Pitx2*$^{+/-}$ embryos are significantly narrower (along the AP axis) and shorter (along the P/D axis) than those of littermate controls (Figs 4, 5). Although these smaller limb buds are similarly positioned along the AP axis (centered around somite 27), the loss of bud mesenchyme appeared greater on the anterior side upon inactivation of the *Pitx1* gene, and further loss was observed in double mutant embryos (Figs 4, 6). This stepwise loss of limb bud mesenchyme leads to a different ratio of anterior to posterior mesenchyme. Indeed, using *Hoxc11*, *Shh* or *Hand2* as markers of posterior bud mesenchyme, it is clear that the proportion of the limb bud expressing those posterior marker genes becomes greater in *Pitx1*$^{-/-}$ and *Pitx1*$^{-/-}$,*Pitx2*$^{+/-}$ mice (Fig. 5). The greater loss of skeletal elements observed in the few *Pitx1*$^{-/-}$,*Pitx2neo/-* embryos that we obtained (Fig. 6A-C) might have resulted from even greater losses of early limb bud mesenchyme than that shown in Fig. 4. This is supported by the very small HL buds observed on the single *Pitx1*$^{-/-}$,*Pitx2*$^{-/-}$ embryo obtained (Fig. 6D). This embryo may be similar to *Fgf10*$^{-/-}$ embryos which have almost no limb buds (Min et al., 1998; Sekine et al., 1999). Be that as it may, the loss of skeletal structures (Figs 2, 3) observed in the single and double mutant embryos appeared most likely to result from an essential and dose-dependent role of *Pitx* genes in early mesoderm (Fig. 7), much before the initiation of limb bud outgrowth. As Pitx2 is not expressed in the growing HL bud (Fig. 7), we are left to speculate that the early co-expression of Pitx1 and Pitx2 in mesoderm either determines the growth potential of this tissue in the HL-forming region and/or that the two Pitx genes are essential for patterning the limb field (Fig. 8).

Hindlimb specification and patterning role of Pitx1

Pitx1 was identified as the most upstream gene in a cascade that also includes *Tbx4* for specification of HL identity. This model derived from knockout of the *Pitx1* gene in mice (Lanctôt et al., 1999b; Szeto et al., 1999), and overexpression of *Pitx1* (Logan and Tabin, 1999) and of *Tbx4* (Takeuchi et al., 1999) in chick wing buds. The consequences of these manipulations were mostly observed at the level of zeugopod and at the boundary between zeugopod and stylopod. Indeed,

the autopod is not drastically affected by *Pitx1* inactivation in mice. Other HL-specific factors include *Hoxc10* and *Hoxc11* (Nelson et al., 1996; Peterson et al., 1994) and these were shown to be induced by ectopic expression of *Pitx1* in FL (Logan and Tabin, 1999), suggesting that they may be downstream of *Pitx1*. Our results do not agree with this interpretation as *Hoxc10* and *Hoxc11* expression is unaffected in *Pitx1*$^{-/-}$ or double mutants.

In view of the effect of *Pitx1* deficiency on early HL bud outgrowth (Figs 4, 5), it is worthwhile re-visiting the phenotype of *Pitx1*$^{-/-}$ mice in order to differentiate, if possible, Pitx1 functions that may be truly involved in specification as opposed to those that involve dose dependence and redundancy with Pitx2. Two aspects of the *Pitx1* knockout qualitatively affect HL skeletal structures, producing a resemblance to FL structures. These are the absence of secondary cartilage development leading to the formation of an articulation that is more elbow than knee like, and the contact of fibula with femur instead of tibia much like the contact between equivalent bones in FLs (Lanctôt et al., 1999b). These transformations are most likely to reflect a true HL specification role of Pitx1. By contrast, the reduction in femur length may be associated with defects in growth regulation rather than specification or patterning.

Pitx gene expression in posterior mesoderm and in HL bud mesenchyme

The demonstration of strong genetic interaction between the *Pitx1* and *Pitx2* genes poses the question of where and when might the two genes be co-expressed or, if not co-expressed, what might be the tissues that interact to account for the phenotype of the double mutants. The expression of Pitx1 from very early in posterior lpm and throughout the HL bud mesenchyme was already well established (Lanctôt et al., 1997). However, Pitx2 did not appear to be present in limb buds, except in myoblasts, and, when re-assessed using immunocytochemistry, we confirmed that *Pitx2* is not expressed in HL mesenchyme (Fig. 7E). However, Pitx1 and Pitx2 were detected with similar patterns of expression on both sides of the tail bud at the 7-15 somite stages of development (E8.5-E9.0), with Pitx2 showing LR asymmetry (Fig. 7A-D). Thus, this very early co-expression of Pitx factors probably accounts for their function in limb bud formation. The higher Pitx1 protein levels (compared with Pitx2) in this area would be consistent with the absence of marked HL phenotype in *Pitx2*$^{-/-}$ embryos (Gage et al., 1999; Kitamura et al., 1999; Lin et al., 1999; Lu et al., 1999) or in *Pitx*$^{+/-}$,*Pitx2*$^{-/-}$ embryos (Fig. 6D). In normal conditions, the function of *Pitx* genes in the HL field would thus be primarily served by *Pitx1* and it is only in its absence that the contribution of *Pitx2* to limb bud growth becomes evident. This interpretation would also be consistent with the fact that asymmetrical development of HL is only observed in the absence of *Pitx1*.

Mesoderm outgrowth and limb development

The earliest phenotype observed in Pitx-deficient embryos is the reduction in HL bud size both along the AP and PD axes (Fig. 4). The observation that this phenotype is sometimes asymmetrical is consistent with the partial penetrance of the *Pitx2* alleles in the *Pitx1*$^{-/-}$ background (Fig. 3). Hence, this phenotype is correlated with Pitx gene dose effects observed in the present study. In *Pitx1*$^{-/-}$ embryos, the variable reduction in femur length with its strong bias for the right side correlates well with the reduction of HL bud size (both right side biases observed in 50-60% embryos). The impairment of HL bud growth was almost complete in absence of both Pitx genes (Fig. 6D), despite relatively conserved AER and bud functions in *Pitx1*$^{-/-}$,*Pitx2*$^{+/-}$ embryos, as revealed using markers such as *Fgf8, Fgf10, Bmp7, Msx2, Fgf4, Hoxc11, Hand2, Shh, Gli3* and *Meis* (Figs 4-6). The similarity of HL phenotypes produced by inactivation of both Pitx genes (Fig. 6D) or of *Fgf10* (Min et al., 1998; Sekine et al., 1999) suggests that they may be mediated through similar mechanisms. Although both mutant mice initiate bud outgrowth, *Fgf10*$^{-/-}$ embryos did not exhibit AER function, whereas Pitx mutant embryos do. As *Fgf10* expression was not significantly affected in double mutant embryos (Fig. 4C,E), it may not be the production of *Fgf10* or of another signal [such as *Fgf8*, which was still induced in AER of the *Pitx1*$^{-/-}$,*Pitx2*$^{-/-}$ embryo (Fig. 6D)], that is dependent on Pitx genes. Rather, it may be the ability to respond to signals that is Pitx dependent. The simplest model for the role of *Pitx1* and *Pitx2* genes in HL bud formation may thus be that these genes are required for appropriate growth response of HL field mesenchyme to growth factors, such as Fgf10 (Fig. 8A). Alternatively, we cannot exclude the possibility that *Pitx* genes are required for *Fgf10* expression itself (Fig. 8B) because we could not assess its expression in a double null mutant.

How could Pitx genes be essential for formation of proximal (femur) and anterior (tibia and first digit) structures? Given their early co-expression, *Pitx* genes may be required for patterning the proximoanterior domain of the HL field. The Pitx genes would thus be essential for expression of an anterior-specific factor that remains to be identified. Indeed, a factor with the expected expression or function is not currently known. The Pitx genes themselves do not appear to be the anterior-specific signal, as their expression does not show AP differences at the HL level (Fig. 7), but they may nonetheless serve a permissive function. Alternatively, the progressive loss of anterior and proximal structures first on the right and then on the left side (Fig. 3) would be consistent with an impairment of bud mesenchyme growth dependent on Pitx gene dose.

The loss of anterior HL bud mesenchyme (Fig. 4A) is associated with loss of anterior skeletal elements, first digit and tibia (Figs 2, 3). These observations correlate well with excision experiments performed on chick wing buds in which removal of the anterior half bud resulted in loss of anterior structures, i.e. anterior digit and radius (FL equivalent of tibia), together with proximal part of humerus (Warren, 1934; Saunders, 1948). Thus, the primary defects associated with Pitx gene deficiency is the early loss of bud mesenchyme, which may result in loss of anterior skeletal elements. Because most signalling appears to be intact in double Pitx mutant embryos, including *Shh* and *Gli3*, their reduced HL buds may be subjected to disproportionate posteriorizing activity (Fig. 5) and this may also contribute to the loss of anterior skeletal elements.

It is interesting to compare *Pitx1* and *Pitx2* deficiency with conditional inactivation of AER *Fgf8*. In one study, HL knockout of *Fgf8* resulted in loss of femur and first digit, but not tibia (Lewandoski et al., 2000). In another study in which *Fgf8* knockout was targeted to FL, radius and first digit were lost in 100% of embryos and the humerus lost in 70% of

embryos (Moon and Capecchi, 2000). It was proposed that rescue of the zeugopod might be ascribed to AER expression of *Fgf4*, which is expressed later and more posteriorly than *Fgf8* (Lewandoski et al., 2000; Tickle and Munsterberg, 2001). This is consistent with the double knockout of limb *Fgf8* and *Fgf4*, which abrogated limb bud development (Sun et al., 2002); this latter work also supported a model of sequential growth of bud mesenchyme pre-specified for PD structures. In Pitx double mutant embryos, the delay in AER expression of *Fgf8* (Fig. 4H) may thus contribute to the reduced size of proximal structures. However, although AER expression of *Fgf8* was delayed from the 27- to 30-somite stage (Fig. 4H), it is noteworthy that other AER or bud markers are not significantly affected in mutant embryos. These include *Fgf10* (Fig. 4E), which is essential for *Fgf8* expression (Ohuchi et al.,

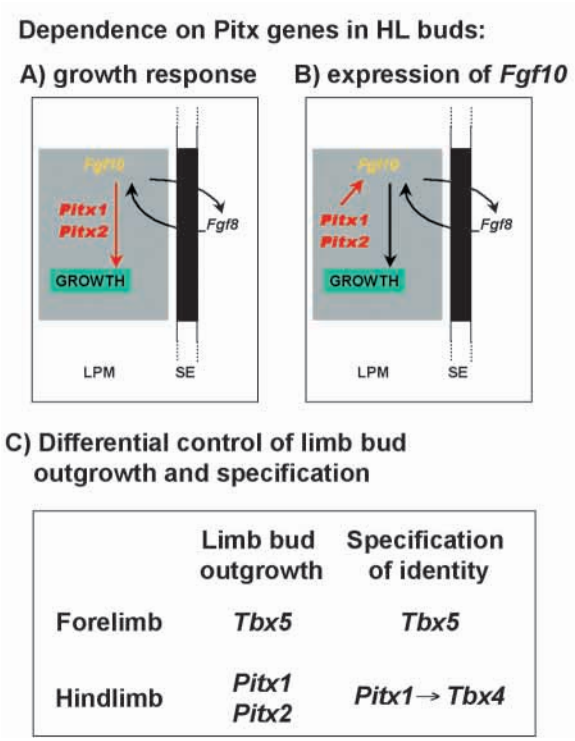

Fig. 8. Role of Pitx genes in limb bud development. (A) Model for role *Pitx1* and *Pitx2* genes in hindlimb bud formation. The early co-expression of Pitx genes in the mesoderm of the limb bud field appears to be required for growth of bud mesenchyme in response to signals such as Fgf10. At this time, we do not have specific evidence to implicate Fgf10 more than other signals, except for the early expression of Pitx genes. This model is consistent with relatively normal signaling in Pitx mutant embryos. (B) As double null Pitx mutants could not be studied extensively, it cannot be excluded that Pitx genes are required for expression of *Fgf10* and that they control the growth capacity of hindlimb bud mesenchyme in this way. (C) Differential control of limb bud outgrowth and specification by Pitx and Tbx genes. Previous work suggested that *Tbx5* is a determinant for specification of forelimb identity whereas *Pitx1* and the downstream *Tbx4* gene both contribute to specification of hindlimb identity. The present work shows that *Pitx1* and *Pitx2* genes are required for hindlimb bud outgrowth but this function does not appear to require mouse *Tbx4* (V. Papaioannou, personal communication). By contrast, *Tbx5* appears to fulfill in forelimb buds an outgrowth function similar to that fulfilled by Pitx genes in hindlimb buds (Agarwal et al., 2003) (M. Logan, personal communication).

1997), *Bmp7* (Fig. 4F), *Msx2* (Fig. 4G), *Gli3* (Fig. 5C) and *Fgf4* (Fig. 5D). In addition, the presence of AER *Fgf8* in HL bud of the *Pitx1*$^{-/-}$,*Pitx2*$^{-/-}$ embryo (Fig. 6D) argues against a primary role of Pitx genes in establishment of AER function. Taken together with intact *Fgf10* expression in *Pitx1*$^{-/-}$,*Pitx2*$^{+/-}$ embryos and with the restricted co-expression of Pitx genes in early limb field lpm, these data are consistent with a role of Pitx genes in determining the growth capacity of limb bud mesenchyme (Fig. 8A).

Limb malformations resulting from thalidomide exposure may resemble to some extent the loss of HL skeletal elements in Pitx-deficient mice. In children with thalidomide defects, upper limbs are affected more frequently than lower limbs, but the sequence of limb loss with severity is usually thumb (first digit), radius, humerus and ulna (Smithells and Newman, 1992). In legs, tibia and femur are most often affected. These deficiencies are similar to those observed for HL in Pitx mutant embryos, suggesting a possible relationship in mechanism.

What about forelimbs?

The present study suggests an important function for Pitx gene dose in the growth and patterning of HLs. However, none of the mutant embryos described in the present work has any phenotype in FL. We must therefore conclude that Pitx genes do not play any role in FL development and this is consistent with the absence of *Pitx1* or *Pitx2* expression in FL buds, except in myoblasts. This is a somewhat surprising conclusion but the later appearance of HL during evolution would not be incompatible with the recruitment of Pitx genes for growth and patterning of HLs, independently of mechanisms acting at FLs. It is unlikely that another Pitx gene may fulfill a similar function in FLs as the only other Pitx gene known, *Pitx3*, is not expressed in early FL buds (A. M. and J. D., unpublished observations). In this context, the control of HL bud growth by Pitx genes may be viewed as a recent function.

Recent work suggests that the *Tbx5* gene plays an essential role for outgrowth of forelimb buds that resembles that of Pitx genes in HL. Indeed, FL buds do not develop in *Tbx5*$^{-/-}$ mouse embryos (Agarwal et al., 2003) (M. Logan, personal communication). By contrast, the HL-specific *Tbx4* gene does not appear to play a similar limiting role for HL bud outgrowth because *Tbx4*$^{-/-}$ embryos develop HL buds (V. Papaioannou, personal communication). The role of *Tbx4* thus appears to be primarily in specification of HL identify. Taken together, these studies suggest different mechanisms for outgrowth and specification in HL and FL (Fig. 8C). In FL, the primary gene controlling both outgrowth and specification appears to be *Tbx5*, whereas in HL, these roles are taken by *Pitx1*, with the downstream *Tbx4* contributing together with *Pitx1* only for specification of HL identity.

We are grateful to Cliff Tabin and David Lohnes, as well as to members of our laboratories, for critical comments on the manuscript. We are thankful to Julie D'Amours for managing the mouse colony and to Alain Moreau for setting up our mouse database. We also thank Jennifer Farrah, Pascale Blaise, Marta Camilot and Thi Som Mai Le for genotyping. We are grateful to Jeff Murray and Tord Hjalt for the Pitx2 antiserum. The *Hoxc10* and *Hoxc11* probes were kindly provided by Mario Cappechi, *FgF8* by Gail Martin, *Fgf10* by Nobu Itoh, *Meis2* by Guy Sauvageau and *Hand2* by Jay Cross. We are grateful for the efficient help of Claire Crevier for preparation of histology sections and Lise Laroche for secretarial help. A. M. was

supported by a studentship from the National Sciences and Engineering Research Council of Canada (NSERC). This work was supported by the Canadian Institutes of Health Research.

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