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

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**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 induction, outgrowth and maintenance (Martin, 1998). An early marker of limb field specification is provided by the expression of the posteriorizing signal molecule sonic hedgehog (Shh) (Chen et al., 1997). This early restricted expression of Shh in the zygopod and stylopod domains of the limb (Capdevila et al., 1996) and/or Wnt signalling molecules (Kawakami et al., 2001). In particular, the proximal limb bud 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 zygopod 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 (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...
Pitx1\textsuperscript{+/–} 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\textsuperscript{+/–} 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\textsuperscript{+/–} 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\textsuperscript{+/–} 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\textsuperscript{+/–} mice with Pitx2\textsuperscript{2+/–} mice. Surprisingly, we did not get the expected Mendelian ratio of 25% double heterozygotes (Pitx1\textsuperscript{+/–},Pitx2\textsuperscript{2+/–}) 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\textsuperscript{+/–} mice. Double mutant embryos (Pitx1\textsuperscript{1+/–},Pitx2\textsuperscript{2+/–}) were obtained by crossing Pitx1\textsuperscript{1+/–} mice with Pitx1\textsuperscript{1+/–},Pitx2\textsuperscript{2+/–} mice. We only ever got one Pitx1\textsuperscript{1+/–},Pitx2\textsuperscript{2+/–} embryo by intercrossing double heterozygotes and a few Pitx1\textsuperscript{1+/–},Pitx2\textsuperscript{2+/–} embryos were obtained by crossing double heterozygotes of each Pitx2 allele.

Mutant (Pitx1\textsuperscript{1+/–},Pitx2\textsuperscript{2neo/neo}) mice with the most extreme phenotype showed a much more extensive phenotype than single mutant mice (Fig. 2). Whereas Pitx1\textsuperscript{1+/–} 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\textsuperscript{1+/–} 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 Pitx2\textsuperscript{2neo/neo} alleles (data not shown) or the Pitx2\textsuperscript{2+/–} 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\textsuperscript{1+/–} 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\textsuperscript{1+/–} and Pitx1\textsuperscript{1+/–},Pitx2\textsuperscript{2+/–} 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\textsuperscript{1+/–} or double mutant embryos. The reduction in Pitx1\textsuperscript{1+/–} 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\textsuperscript{1+/–} embryos (approx. somites 25.5 to 28.0) and to a length of about 2 somites in Pitx1\textsuperscript{1+/–},Pitx2\textsuperscript{2+/–} 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-
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 Pitx2 (Fig. 4D). 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

![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.](image-url)
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 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 Mx2. (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 Pitx2+/- 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.

**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,

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**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.
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 Hox10 and Hox11 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 Pitx2–/–, 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 proximantoanteri or 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 Cappechi, 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., 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 identity. 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.

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