Hindlimb patterning and mandible development require the \textit{Ptx1} gene

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SUMMARY

The restricted expression of the \textit{Ptx1} (\textit{Ptx1}) gene in the posterior half of the lateral plate mesoderm has suggested that it may play a role in specification of posterior structures, in particular, specification of hindlimb identity. \textit{Ptx1} is also expressed in the most anterior ectoderm, the stomodeum, and in the first branchial arch. \textit{Ptx1} expression overlaps with that of \textit{Ptx2} in stomodeum and in posterior left lateral plate mesoderm. We now show that targeted inactivation of the mouse \textit{Ptx1} gene severely impairs hindlimb development: the ilium and knee cartilage are absent and the long bones are underdeveloped. Greater reduction of the right femur size in \textit{Ptx1} null mice suggests partial compensation by \textit{Ptx2} on the left side. The similarly sized tibia and fibula of mutant hindlimbs may be taken to resemble forelimb bones: however, the mutant limb buds appear to have retained their molecular identity as assessed by forelimb expression of \textit{Tbx5} and by hindlimb expression of \textit{Tbx4}, even though \textit{Tbx4} expression is decreased in \textit{Ptx1} null mice. The hindlimb defects appear to be, at least partly, due to abnormal chondrogenesis. Since the most affected structures derive from the dorsal side of hindlimb buds, the data suggest that \textit{Ptx1} is responsible for patterning of these dorsal structures and that as such it may control development of hindlimb-specific features. \textit{Ptx1} inactivation also leads to loss of bones derived from the proximal part of the mandibular mesenchyme. The dual role of \textit{Ptx1} revealed by the gene knockout may reflect features of the mammalian jaw and hindlimbs that were acquired at a similar time during tetrapod evolution.

Key words: Limb specification, Craniofacial development, Homeobox, Bicoid-related, Mouse

INTRODUCTION

\textit{Ptx1} is the prototypical member of a \textit{paired} family of homeobox transcription factors which have DNA binding specificity similar to that of \textit{Drosophila bicoid} (Drouin et al., 1998b; Lamonerie et al., 1996; Lanctôt et al., 1997; Szeto et al., 1996); its transcriptional properties have been defined for a number of late downstream target genes in the pituitary gland (Lamonerie et al., 1996; Tremblay et al., 1998; Tremblay and Drouin, 1999). Although the related gene products, \textit{Ptx2} and \textit{Ptx3}, have similar transcriptional properties, their expression patterns and developmental roles are different (Drouin et al., 1998a,b; Smidt et al., 1997). Most striking is the left-side-specific expression of \textit{Ptx2} in lateral plate mesoderm and its implication in asymmetric development of internal organs such as heart and stomach (Logan et al., 1998; Piedra et al., 1998; Ryan et al., 1998; Yoshioka et al., 1998). While \textit{Ptx1} and \textit{Ptx2} expression overlap partially, for example in stomodeum and posterior left side lateral plate mesoderm (Lanctôt et al., 1997; Semina et al., 1996), \textit{Ptx3} has very different expression, for example, in midbrain dopaminergic neurons (Smidt et al., 1997) and lens (Semina et al., 1998).

Little is known about the molecular mechanisms or genes involved in specification of limb identity. Few genes have limb-specific expression: the early expression of \textit{Ptx1} throughout the lateral plate mesoderm of the posterior half of the embryo results in \textit{Ptx1} expression in hindlimb but not in forelimb mesenchyme (Lanctôt et al., 1997). This hindlimb-specific expression has suggested that \textit{Ptx1} may be involved in determination of hindlimbs. Two \textit{brachyury}-related genes, \textit{Tbx4} and \textit{Tbx5}, also have limb-specific expression; indeed, although not exclusive to limbs, \textit{Tbx5} is only expressed in forelimb while \textit{Tbx4} is restricted to hindlimbs (Chapman et al., 1996; Gibson-Brown et al., 1996). Human mutations in the \textit{Tbx5} gene were shown to be responsible for the Holt-Oram syndrome which affects forelimb and heart (Basson et al., 1997; Li et al., 1997). At present, the relationship between \textit{Ptx1} and \textit{Tbx4} in specification of hindlimb features is not clear although \textit{Ptx1} expression appears to precede \textit{Tbx4} (Chapman et al., 1996; Lanctôt et al., 1997). The relationship between these genes and general mechanism for limb patterning (reviewed by Johnson and Tabin, 1997) also needs to be clarified; the latter are thought to play equivalent roles in both fore- and hindlimbs. For example, the \textit{Lmx1b} gene is required for patterning of dorsal structures of both limbs (Chen et al., 1998). To investigate the role of \textit{Ptx1} in limb development, we
have generated mice deficient in Ptx1 activity. Hindlimb and mandible development are severely affected by this mutation.

**MATERIALS AND METHODS**

**Gene targeting**

The murine *Ptx1* gene was cloned from a Sv129 genomic library (kind gift from Drs M. Aubry and J. P. Julien). To construct the targeting vector, a 7.09 kb EcoRI fragment was subcloned in pKS and a 1.8 kb *XbaI-KpnI* fragment encompassing the second homeodomain-containing exon was replaced with a pGKneo-pA cassette (kind gift from Dr D. Lohnes). A pGK-TK-pA cassette was introduced at an *AatII* site at the 3’ end of the gene targeting fragment. Mutant ES cells were obtained using previously described protocols (Ramirez-Solis et al., 1993). The targeting vector was linearized with *ClaI* and electroporated in 1x10^7 R1 ES cells (kind gift from Dr A. Nagy). Homologous recombination occurred at the *Ptx1* locus in 5 out of the 480 transfectants that were picked. One of these efficiently contributed to the germ line when introduced in Balb/c mouse transfectants that were picked. One of these efficiently contributed to the germ line when introduced in Balb/c mouse blastocysts. All animals analyzed for this work were hybrid Sv129 x Balb/c of the first three generations of crossing with Balb/c mice.

**RESULTS AND DISCUSSION**

In order to investigate the role of *Ptx1* during embryogenesis, we generated a null mutation by deletion of the homeodomain-coding exon 2 of the mouse *Ptx1* gene (Fig. 1A). Mice that are heterozygous for the mutated allele are phenotypically normal and fertile. Mice that are homozygous for the *Ptx1* deletion die at birth. They show the expected Mendelian ratios at birth (Fig. 1B) and RNA extracted from day E13.5 *Ptx1*+/− mandibles no longer contained Ptx1 mRNA as revealed by northern blot (Fig. 1C). Thus, deletion of the central *Ptx1* exon impaired transcription and/or mRNA accumulation, effectively creating a null mutation.

*Ptx1*−/− mice appear to develop normally until E12.5. In particular, the allantois and stomodeum are not affected, presumably due to compensation by Ptx2 in these tissues (Lanctôt et al., 1997; Mucchielli et al., 1996; Semina et al., 1996; Szeto et al., 1996). Starting at E14.5, *Ptx1*−/− mice are readily recognized by shortening of the jaw (micrognathia).
Skeletal preparations of newborns also revealed marked hindlimb defects (Fig. 2A). The Ptx1<sup>+</sup> phenotype is 100% penetrant and showed few variations between animals (except those noted below). Thus, Ptx1 deficiency affects both the first arch and posterior mesoderm domains of Ptx1 expression (Lancôt et al., 1997).

**Apparent partial transformation of hindlimbs into forelimb-like skeleton**

The axial skeleton is not affected in the mutant embryos, including the sacral region where knockout animals have an abnormal pelvic girdle (Fig. 2B). The ischium and pubic bone are slightly smaller but appear normal; however, the ilium is completely absent (Fig. 2C). In some animals, there are small cartilaginous remnants of the ilium (Fig. 2B, arrow). Strikingly, the pelvic bones of Ptx1<sup>+</sup> mice are attached to the first sacral vertebra (S1) through the acetabulum (ac) rather than through the tip of the ilium (Fig. 2C). In two embryos, the acetabulum was asymmetrically positioned beside S2 (left side) and S3 (right side) level (not shown). Arrow shows cartilaginous remnants of the ilium. (C) Dissected pelvic bones show the absence of ilium (il) and a slight reduction in the size of the ischium (is) and pubic bone (pb) in Ptx1<sup>+</sup> embryos. (D) Forelimbs are normal in Ptx1<sup>+</sup> embryos. (E) Severe truncation of hindlimbs in Ptx1<sup>+/−</sup> embryos showing reduced diameter of tibia and enlarged fibula. (F) Comparison of femurs from wild-type and knockout skeletons showing greater effect on right than left femur. This was observed in 80% of skeletons. In some cases, the right femur was also kinked (not shown). (G) Comparison of knee joints with forelimb (elbow) joint. (H) Forelimb digits are not affected in Ptx1<sup>+/−</sup> animals. Digits are numbered and radius (R), ulna (U), pisiform (P1) and prepollex (PP) bones are indicated. (I) Hindlimb digits are not affected in knockout animals. Tibia (T), fibula (F) and calcaneus (Ca) are indicated. (J) Lateral view of hindlimbs showing protruding calcaneus (Ca) in both wild-type and knockout skeleton.

Ptx1 deficiency by left side-specific presence of Ptx2 which is specifically expressed in left lateral plate mesoderm during early development where it acts as an effector of left-right asymmetry (Logan et al., 1998; Piedra et al., 1998; Ryan et al., 1998; Yoshioka et al., 1998).

The knee joints of Ptx1<sup>+/−</sup> mice lack distinct patella and fabella, and cartilage of the distal femur and tibia heads is greatly reduced (Fig. 2G). The tibias are shorter and narrower, such that their size is now comparable to that of the fibula (Fig. 2E,G). These similarly sized tibia and fibula are reminiscent of the radius and ulna; also, the simplified knee joint may resemble the elbow (Fig. 2G). These changes may reflect a transformation of hindlimb into forelimb-like structures. However, examination of the digits does not support this conclusion. Indeed, both size and ossification patterns of Ptx1<sup>+/−</sup> hindlimb digits 1 and 5 are similar to those of wild-type hindlimbs (Fig. 2I) rather than to those of forelimb digits (Fig. 2H). Also, the tarsal bones can be recognized as such and are distinct from carpal bones even though their development is affected. A calcaneus is also present in hindlimbs of knockout mice (Fig. 2I).

**Limb identity**

In order to assess the possibility that the changes in hindlimb structure in Ptx1<sup>+/−</sup> mice might reflect an anterior limb transformation, we examined Tbx5 expression, a T box gene shown to be exclusively expressed in forelimbs (Gibson-Brown...
et al., 1996). Tbx5 expression remains forelimb-specific in Ptx1-/- embryos and is never detected in hindlimbs (Fig. 3A). Further, the level of Tbx5 expression is not significantly different in Ptx1 null mice either at E10.5 or in forelimb at E13.5 (Fig. 3A). The expression of the hindlimb-specific Tbx4 gene was also assessed by whole-mount in situ hybridization: Tbx4 mRNA levels appeared lower in Ptx1-/- hindlimbs compared to wild-type sibs (Fig. 3B) but Tbx4 expression is not abolished. Hence, Ptx1 may contribute to the control of Tbx4 expression but it is not absolutely required for it.

Bone development

The severe reduction in femur and tibia size suggested that their development might be impaired rather than re-specified. Histological analysis showed that calcification of Ptx1-/- tibia is greatly reduced, with narrower cortical bone and less trabecular bone (Fig. 4A). At the distal end of Ptx1-/- tibia, hypertrophied chondrocytes are enlarged, their layer is expanded, they produce less extracellular matrix and almost no mineralization (Fig. 4C). At the proximal end, undifferentiated chondrocytes are almost absent as are articular cartilage, secondary ossification centers and pre-hypertrophied chondrocytes (Fig. 4B). The unusual calcification of the tibia head might be related to abnormal chondrocyte differentiation/proliferation at the growth plate (Fig. 4B). Endochondral bone is also greatly diminished in femurs (not shown). Taken together, the unaltered expression of the most specific forelimb marker, Tbx5, and the hindlimb chondrogenic defect argue against a true transformation of hindlimb into forelimb. However, these defects would not be incompatible with a model in which Ptx1 is responsible (possibly in association with Tbx4) for development of hindlimb-specific features starting from a ground state or generic tetrapod limb program.

All the hindlimb structures affected by the Ptx1 mutation are on the dorsal side, i.e. the ilium, knee and tibia. Further, the severity of the defects is greatest proximally; indeed, the ilium...
is absent, the femur is greatly reduced in size and the tibia is smaller. This correlates well with the greater expression of *Ptx1* in the proximal and dorsal parts of the hindlimb buds, both in mice (Lanctôt et al., 1997) and in chick (Logan et al., 1998). Inactivation of the *Lmx1b* gene also affected the same hindlimb bones, although less severely, but contrary to *Ptx1*, *Lmx1b* plays a similar role in forelimb patterning (Chen et al., 1998).

The abnormal chondrogenesis in hindlimb may suggest a role for *Ptx1* in proliferation, differentiation and/or signaling in the dorsal hindlimb bud mesenchyme.

Impaired development of proximal mandible

The knockout embryos have severe micrognathia (Fig. 5A), cleft palate (Fig. 5B) and a bifurcate tongue (Fig. 5C). By E14.5, these defects are clearly visible (Fig. 5G-I). As seen in Fig. 5I, palatal shelves have failed to elevate and to fuse in the midline. The resulting cleft palate may be caused by a deficiency in maxillary epithelium, a site of *Ptx1* expression, or be secondary to the tongue defect. Tooth development is not significantly affected in *Ptx1* −/− mice: neither upper nor lower (Fig. 5I) molars or incisors (Fig. 5H) are affected. In the mandible, the proximal part is the most affected (Fig. 5D). Indeed, both incisor and molar cavities appear normal despite the shortening of the mandibular bone. The knockout mandible has novel bone deposition around Meckel’s cartilage all the way up to the malleus (Fig. 5D,E). In places, the Meckel is replaced by this extended mandibular bone. We could not find evidence of the gonial bone which may be absent unless it has become fused to the mandible. Also, the tympanic bone is smaller: it does not reach Meckel’s cartilage (Fig. 5F) and the middle ear cartilages (malleus and incus which are unaffected). All these defects affect neural crest derivatives of the

Fig. 5. First branchial arch derivatives are absent or severely reduced in *Ptx1* −/− embryos. (A) The heads of knockout embryos show severe micrognathia. (B) Ventral view of the palate (p) reveals cleft palate (cp) in *Ptx1* −/− embryos. (C) Dissected mandible (mand) and tongue (to) show bifurcate tongue in *Ptx1* −/− embryos as well as smaller size of the jaw. (D) Alizarin red/Alcian blue preparation of wild-type and *Ptx1* −/− mandibles. A short segment of Meckel’s cartilage (Me) is attached to the *Ptx1* +/+ mandible whereas the malleus (ma) is directly attached to mandibular bone in *Ptx1* −/− mandible. (E) Dissected middle ear cartilage showing intact malleus (ma) and incus (ic). Note that tympanic bone (T) does not reach Meckel’s cartilage in the *Ptx1* −/− ear (not shown) and that the gonial bone (go) is either absent or fused to the mandibular bone (md) that surrounds/replaces Meckel’s cartilage (Me) in *Ptx1* −/− ear. (F) Ventral views of cranium show cleft palate and smaller tympanic bone (T) in *Ptx1* −/− embryos. (G) Sagittal section through E14.5 *Ptx1* +/+ head showing tongue (to), primary palate (pp), secondary palate (sp), choana (ch) and upper incisor (in). (H) Sagittal section through E14.5 *Ptx1* −/− head showing shorter tongue (to), absence of secondary palate and larger choana (ch). md, mandibular bone. (I) Transverse section through E14.5 *Ptx1* −/− head showing bifurcate tongue (to), open palatal shelves (ps), and normal lower molar (mo). ns, nasal septum; vo, vomeronasal organ.
mandibular arch, i.e. the mandible, tympanic and gonial bones. The position of these structures correlates well with the presence of Ptx1-expressing mesenchyme in the middle of the first branchial arch at E10.5 (Lancotì et al., 1997).

In both hindlimbs and mandible, Ptx1 is required for bone development, and we have shown specific defects in hindlimb chondrogenesis. Thus, Ptx1 may be implicated in proliferation and/or differentiation of specific mesenchymes and consequently, the loss of Ptx1 function appears to have resulted in deletion of structures rather than in a change of fate. This interpretation would be consistent with a role for Ptx1 in dorso-ventral patterning of hindlimbs in which Ptx1 may direct development of specific dorsal hindlimb structures such as the patella.

Ptx1 mutation affects mandibular structures whose function has changed during transition from a reptilian to a mammalian jaw joint. These correspond to the tympanic and gonial bones that derived from the angular bone of primitive tetrapods, and to the proximal part of the mandibular bone which may be considered an extension of the ancestral dentary bone (Ballard, 1964). The hindlimb defects may also represent the loss of a function acquired during limb evolution. Some fossil snakes as well as some pythons have vestigial hindlimbs that resemble those of the Ptx1−/− mice in that they have tibia and fibula of similar size, simple joint structure, and a small ilium (Caldwell and Lee, 1997; Coates, 1994; Lee, 1997). The association of hindlimb and mandibular deficiencies in Ptx1 null mice may reflect the parallel evolution of these structures.

REFERENCES


