Role of the Bicoid-related homeodomain factor Pitx1 in specifying hindlimb morphogenesis and pituitary development

Daniel P. Szeto,^{1,3} Concepción Rodríguez-Esteban,^{2,3} Aimee K. Ryan,¹ Shawn M. O'Connell,¹ Forrest Liu,¹ Chrissa Kioussi,¹ Anatoli S. Gleiberman,¹ Juan Carlos Izpisua-Belmonte,^{2,4} and Michael G. Rosenfeld^{1,4}

¹Howard Hughes Medical Institute, University of California, San Diego, School and Department of Medicine, La Jolla, California 92093-0648 USA; ²The Salk Institute, Gene Expression Laboratory, La Jolla, California 92037 USA

Pitx1 is a Bicoid-related homeodomain factor that exhibits preferential expression in the hindlimb, as well as expression in the developing anterior pituitary gland and first branchial arch. Here, we report that *Pitx1* gene-deleted mice exhibit striking abnormalities in morphogenesis and growth of the hindlimb, resulting in a limb that exhibits structural changes in tibia and fibula as well as patterning alterations in patella and proximal tarsus, to more closely resemble the corresponding forelimb structures. Deletion of the *Pitx1* locus results in decreased distal expression of the hindlimb-specific marker, the T-box factor, *Tbx4*. On the basis of similar expression patterns in chick, targeted misexpression of chick *Pitx1* in the developing wing bud causes the resulting limb to assume altered digit number and morphogenesis, with *Tbx4* induction. We hypothesize that Pitx1 serves to critically modulate morphogenesis, growth, and potential patterning of a specific hindlimb region, serving as a component of the morphological and growth distinctions in forelimb and hindlimb identity. *Pitx1* gene-deleted mice also exhibit reciprocal abnormalities of two ventral and one dorsal anterior pituitary cell types, presumably on the basis of its synergistic functions with other transcription factors, and defects in the derivatives of the first branchial arch, including cleft palate, suggesting a proliferative defect in these organs analogous to that observed in the hindlimb.

[Key Words: *Pitx1*; morphogenesis; hindlimb development; pituitary]

Received November 24, 1998; revised version accepted January 8, 1999.

The role of homeodomain factors in early and late development is genetically well established (Gehring et al. 1994; Scott 1997), with factors of the *Hox* gene cluster exhibiting specific domains of expression not only along the anterior-posterior axis but also in the developing appendages (Krumlauf 1994; Maconochie et al. 1996). For example, the most posterior members of the vertebrate, *Hox a* and *Hox d* gene clusters are expressed in a fashion that presage the subdivision of the limb along the anterior, posterior, and proximodistal axes (Dollé et al. 1989; Izpisua-Belmonte et al. 1991; Yokouchi et al. 1991; Nelson et al. 1996). In this paper we investigate the function of a member of a second family of homeodomain factors that exert critical regulatory roles during development, the *bicoid*-related family of homeobox genes, which in-

cludes *gooseoid* (*gsc*), *Otx1*, and *Otx2*. *Gooseoid* in vertebrates, *Orthodenticle* in *Drosophila*, and the vertebrate homologs *Otx1* and *Otx2* are critical in determination of head structures (Cho et al. 1991; Simeone et al. 1992, 1993). Deletion of *Otx1* results in loss of all head structures (Acampora et al. 1998), whereas forebrain and midbrain regions are deleted in *Otx2*^{-/-} mice (Acampora et al. 1995, 1996, 1998; Ang et al. 1996; Rhinn et al. 1998). Recently, a search for factors interacting with the pituitary-specific transcription factor Pit-1 (Szeto et al. 1996), or for interactions with a *cis*-acting element in the *POMC* promoter (Lamonerie et al. 1996), led to the cloning of a novel member of this *bicoid*-related gene family, *P-Otx/Ptx1*. The human homolog, *Backfoot*, was found in a screen for novel homeodomain factors (Shang et al. 1997). This factor, now referred to as Pitx1, has been shown to be expressed in the pituitary gland (Lamonerie et al. 1996; Szeto et al. 1996), in the first branchial arch, and its derivatives, and in the lateral mesenchyme and developing hindlimb, but only at very low levels in forelimb (Szeto et al. 1996; Lanctôt et al. 1997; Shang et al.

³These authors contributed equally to this work.

⁴Corresponding authors.

E-MAIL mrosenfeld@ucsd.edu; FAX (619) 534-8180.

E-MAIL belmonte@salk.edu; FAX (619) 455-1349.

1997). Although considerable insight has been obtained in the molecular basis underlying the establishment of the different limb axes (Johnson and Tabin 1997; Martin 1998; Schwabe et al. 1998), the intriguing question of the molecular mechanisms that underlie distinctions between forelimb and hindlimb are less well studied.

Pitx1 is one of the few known transcription factors that exhibit a striking hindlimb/forelimb difference in their expression. Its preferential expression in the hindlimb suggests that this transcription factor may exert a critical role in distinguishing hindlimb from forelimb identity. Two other genes, members of the *T-box* family (*Tbx*) of transcriptional activators, exhibit differential expression in limbs. *Tbx4* is expressed primarily in the developing hindlimb, whereas *Tbx5* is initially selectively expressed in the forelimb, although *Tbx5* later exhibits some expression in the hindlimb (Chapman et al. 1996; Gibson-Brown et al. 1996; Li et al. 1997).

Pitx1 is also expressed in the pituitary throughout its development (Lamoneier et al. 1996; Szeto et al. 1996), being uniformly expressed in oral ectoderm during the period of exclusion of *Sonic hedgehog* (*Shh*) from the invaginating Rathke's pouch (Treier et al. 1998), as well as dorsal-ventral *Fgf8* gradient (Erickson et al. 1998; Takuma et al. 1998; Treier et al. 1998). *Pitx1* expression continues during the subsequent ventral-dorsal emergence of distinct cell types including gonadotropes, expressing luteinizing hormone β , and follicle-stimulating hormone β (LH β , FSH β); thyrotropes, expressing thyroid-stimulating hormone β , (TSH β); somatotropes, expressing growth hormone (GH); lactotropes, expressing prolactin; and corticotropes, producing adrenocorticotrophic hormone (ACTH). Early in pituitary development, *Pitx1* is coexpressed with a second, highly related gene, *Pitx2*/*RIEG* (Semina et al. 1996; Gage and Camper 1997), which was initially identified by positional cloning of the gene responsible for the Rieger Syndrome in humans. This autosomal dominant disease is characterized by anterior chamber ocular abnormalities, dental hypoplasia, mild craniofacial dysmorphism, and occasionally decreased levels of growth hormone. *Pitx2* is asymmetrically expressed in lateral plate mesoderm and appears to exert critical roles in left-right situs (Logan et al. 1998a; Meno et al. 1998; Piedra et al. 1998; Ryan et al. 1998; St. Amand et al. 1998; Yoshioka et al. 1998).

In this paper we report evidence, on the basis of gene deletion in mice, that *Pitx1* exerts critical roles in the hindlimb, pituitary, and first branchial arch development. The most striking phenomena in the *Pitx1* gene-deleted mouse are alterations of specific skeletal structures within a specific region of the hindlimb, which assume many morphological and growth features of the corresponding bones in the forelimb, suggesting they are dependent on *Pitx1* expression in hindlimb mesenchymal populations. Misexpression of *Pitx1* in the chicken wing bud further supports the role of *Pitx1* in limb growth and morphogenesis. Further, *Pitx1*, probably on the basis of its synergistic actions with other transcription factors, is important for proliferation and differentiated function of specific pituitary cell phenotypes, as

well as for closure of the palate and mandibular development.

Results

Deletion of the Pitx1 genomic locus

Pitx1 and the highly-related gene *Pitx2*/*RIEG* referred to as *Pitx2*, which is linked to a human genetic disease (Semina et al. 1996) and to determination of left-right situs (Logan et al. 1998a; Meno et al. 1998; Piedra et al. 1998; Ryan et al. 1998; St. Amand et al. 1998; Yoshioka et al. 1998) are expressed from mouse embryonic day 7 (E7) onward, in distinct, yet highly overlapping patterns (e.g., Fig. 1A) (Szeto et al. 1996; Gage and Camper 1997; Lanctôt et al. 1997; Shang et al. 1997). Both *Pitx1* and *Pitx2* are robustly expressed in early development in specific mesenchymal populations and in the ectodermal primordium of the pituitary gland and derivatives of the first branchial arch (Fig. 1A). Most strikingly, *Pitx1* is selectively expressed in the mesenchyme of the developing hindlimb bud (Fig. 1B), where it is initially detected in the lateral plate mesenchyme at the level at which the hindlimb will emerge (E9–E10) (Szeto et al. 1996; Shang et al. 1997; Lanctôt et al. 1997). *Pitx1* transcripts are detected by whole mount in situ hybridization by E10.5–E11, and *Pitx1* remains robustly and widely expressed in the hindlimb mesenchyme through E12.5–E13.5. At later stages, *Pitx1* transcripts are absent in the centers of chondrogenesis, becoming confined to the perichondral regions and soft tissues of the hindlimb. Loss of *Pitx1* transcripts occurs in a proximal to distal fashion in the developing limb (Fig. 1B; data not shown). *Pitx1* is expressed in a very restricted fashion and only at later stages in the forelimb (Fig. 1B,D).

In contrast, *Pitx2* is expressed in the population of mesenchymal cells that migrate from the somite into both limb buds and eventually will differentiate into the limb musculature (Fig. 1B). *Pitx1* transcripts are transiently present in the pelvis (data not shown) and ultimately expressed in the most distal of the overlapping domains at the end of the long bones that express *parathyroid hormone related peptide* (*PTHrP*), *parathyroid hormone receptor* (*PTHr*) and *Indian hedgehog* (*Ihh*) (Lanske et al. 1996; Vortkamp et al. 1996). *Pitx1* mesenchymal expression overlaps with that of a member of the *Tbx* of transcriptional activators, *Tbx4*, and is later localized in the long bones (Fig. 1B). *Tbx4* provides a marker exhibiting hindlimb, but not forelimb, expression (Gibson-Brown et al. 1996), until late in development. In contrast, a second member of the family, *Tbx5*, is initially selectively expressed in forelimb, but later is also detected in the hindlimb (Chapman et al. 1996; Gibson-Brown et al. 1996; Li et al. 1997). Both *Pitx1* and *Pitx2* are also transiently expressed late in development in a few restricted regions of forelimb, and subsequently decline to practically undetectable levels in the mature limbs (Fig. 1B; data not shown). *Pitx1* appears to be selectively expressed in the olfactory pit, submandibular gland, ventral body wall mesenchyme, and *Pitx1* and

Figure 1. *Pitx1* and *Pitx2* expression patterns and targeted disruption of the *Pitx1* locus. (A) Expression of *Pitx1* and *Pitx2* analyzed by in situ hybridization. The distinct and overlapping expression patterns of *Pitx1* and *Pitx2* Rathke's pouch (RP) (E10.5) and branchial arch structures (E13.5) (T) tooth; (To) tongue; (M) mandible; salivary gland (SG); (E17.5) and pituitary gland (P). (B) Expression of *Pitx1*, *Pitx2*, and *Tbx4*. *Pitx1* is highly expressed in early limb bud, decreasing distally by E12.5; there is a differential expression of *Pitx1*, in the sheath and growth plate of the long bones, whereas *Pitx2* is expressed in muscle (arrow). Note the similar, limited expression of *Pitx1* and *Pitx2* in forelimb. *Pitx1* is robustly expressed in the developing hindlimb (arrows, top), throughout development with serial restriction from proximal, and then distal regions (second panel), finally in a pattern in perichondral regions, at the end of long bones, whereas *Pitx2* is selectively expressed in muscle (arrow) as well as transiently in developing pelvis (not shown), Hind (hindlimb) and Fore (forelimb). *Pitx1* expression overlaps with the *Tbx4* expression in limb development, and is shown in the perichondral region and growth plate of the femur (P0, bottom right). (C) Targeted deletion of the *Pitx1* genomic locus. Schematic representation of the *Pitx1* locus (top), the targeting vector (middle), and the *Pitx1* targeted allele (bottom). White and black boxes represent exons and introns, respectively, and restriction enzymes: *Hind*III (H); *Eco*RI (E). Analysis of transfected ES cells by genomic Southern blot analysis with the 5'-external probe A identifies a 11-kb *Hind*III fragment in the mutant allele and a 15-kb *Hind*III fragment in the wild-type allele. A 3'-external probe B was used to identify a 2.5-kb *Hind*III fragment in the mutant allele, while hybridizing to the 11-kb *Hind*III fragment in the wild-type mouse (not shown). Homozygous and heterozygous mice were identified by Southern blot analysis with probes A and B. (D) The normal *Pitx1* expression patterns analyzed by whole-mount *lacZ* staining at E10–E12.5. (R) Rathke's pouch; (NM) nasal mesenchyme; (BA) branchial arch; (U) umbilical cord; (Hind) hindlimb.

Pitx2 exhibit distinct expression patterns in the gastrointestinal tract and urogenital sinus (Fig. 1A; data not shown).

To examine the potential roles of *Pitx1* in development of the hindlimb and other tissues in which it is developmentally expressed, a targeting vector was designed to delete virtually the entire coding sequence region, except for the amino-terminal sequence 80 amino acids to which *lacZ* was fused in-frame (Fig. 1C). This targeting construct was used to obtain homologous recombinants in ES cells, which were injected into blastocysts to generate chimeric mice. Gene-deleted murine lines were generated by appropriate breeding, and homologous recombination was confirmed by genomic Southern blot analysis with both 5'- and 3'-specific probes (Fig. 1C), as well as lack of *Pitx1* transcripts in the first branchial arch, Rathke's pouch, and hindlimb (data

not shown). In these mice, *Pitx1*-directed *lacZ* expression is identical to the characteristic distribution of endogenous *Pitx1* transcripts (e.g., Fig. 1D). *Pitx1*^{-/-} mice, which die immediately or shortly after birth, and are born at levels statistically slightly below expected Mendelian ratios (~20 per 100 births). This could be explained by the finding that a small subset of null mice exhibit embryonic lethality after E11.5.

Pitx1 role in hindlimb morphogenesis

Although most distinctions between the mammalian hindlimb and forelimb are morphological, including joint articulations, shape, and size of radius/ulna versus tibia/fibula, there are apparently patterning differences that are evolutionarily conserved in mammals that include the patella in the hindlimb, and a particular proxi-

mal element of the forelimb carpus, referred to as the pisiform element, which is not present in the hindlimb tarsus (Romer 1986). Thus, the pisiform is a characteristic forelimb-specific structure arising from a specific condensation center. The hindlimb of *Pitx1*^{-/-} mice is significantly shorter than in wild-type littermates (Fig. 2A). Examination of the skeletal structure reveals that the long bones of the hindlimb, including the femur, tibia, and fibula are altered in length, width, and overall structure (Figs. 2B and 3A). The size of the pelvis, a structure in which *Pitx1* is also transiently expressed, is also remarkably reduced (Fig. 2B). However, the overall structure of pelvis and femur in *Pitx1*^{-/-} mice appears to retain the morphological features of the wild-type counterparts. In contrast, the tibia and fibula of the distal hindlimb in *Pitx1*^{-/-} mice both exhibit striking alterations in morphology, relative size, and shape. This includes alterations in the cnemial crest of the tibia (Romer 1986), altered angles of articulation of fibula both proximally and distally, and a striking alteration in relative size and diameters of tibia and fibula. Thus, the tibia and fibula of the *Pitx1*^{-/-} hindlimb are now morphologically more similar, although not identical, to the radius and ulna of the forelimb, exhibiting equivalent cross sectional diameters (Fig. 3A). Further, the patella is now absent (Fig. 2B) and there is a loss of the Zucker nodes (Fig. 3C), both characteristic features of the hindlimb (Fig. 3C).

There is also a striking alteration in the tarsal structure of the ankle (Fig. 3B), with the appearance of an additional proximal tarsal element that appears analogous to the pisiform, which is an evolutionarily con-

Figure 2. Morphological alterations of *Pitx1*^{+/+} and *Pitx1*^{-/-} mice at different stages of development. (A) *Pitx1*^{+/+} and *Pitx1*^{-/-} mice at E17.5; and E15.5 skeletal structures. Note the foreshortening of the mandible (solid arrowhead) and altered hindlimb (open arrowhead). (B) Details of skeletal structures of hindlimb and forelimb of *Pitx1*^{+/+} and *Pitx1*^{-/-} mice at P0. The forelimb (Fore) is unaltered, Pelvis (P) is abnormal with particular loss of the femur (F). The hindlimb (Hind) is altered in overall length and size. The patella (P) is absent (open arrowhead).

Figure 3. Analysis of the structural components of hindlimb development of *Pitx1*^{+/+} and *Pitx1*^{-/-} at P0. (A) Skeletal structures of the tibia (T) and fibula (F) in P0 *Pitx1*^{+/+} and *Pitx1*^{-/-} mice. There is alteration in size of the femur, whereas the growth and relative size of the tibia and fibula (solid arrowheads) now more closely resembles the morphological resemblance of the relationship between radius (R) and ulna (U) of the forelimb. (B) Tarsal development of the *Pitx1*^{+/+} and *Pitx1*^{-/-} mice. Note the normal development of the Zucker nodes (open arrowhead) and calcaneus (C) in *Pitx1*^{+/+} mouse. The hindlimb (Hind) proximal tarsus of the *Pitx1*^{-/-} mouse now contains an element that resembles the pisiform (P) seen in forelimb (Fore) carpus development of *Pitx1*^{+/+} littermate, with a very small calcaneus. (C) The development of the hindlimb distal components of *Pitx1*^{+/+} and *Pitx1*^{-/-} mice at P0. Digit morphology is not reproducibly altered. Zucker nodes (open arrowhead) are absent in the hindlimb (Hind) of the *Pitx1*^{-/-} mouse.

served characteristic element in the mammalian forelimb carpus (Romer 1986). However, the adjacent proximal tarsus does not assume a carpal-like morphology. In parallel, there is a marked alteration in morphology and size of the calcaneus, no longer characteristic of the wild-type hindlimb structure. The distal tarsus of the *Pitx1*^{-/-} mouse is not clearly distinct from the tarsus of the wild-type littermates. Whereas the size of digits is somewhat smaller, there do not appear to be clear structural alterations; consistent with the similarity of the forelimb and hindlimb digits in the wild-type mouse. Together, these alterations in morphology and bone patterning properties of the distal hindlimb of the *Pitx1*^{-/-} mouse, cause it to have bone structures, including the

tibia/fibula, patella, and the appearance of a pisiform element-like structure in the proximal tarsus, that are quite distinct from that of the wild-type hindlimb. The mutant hindlimb has assumed several morphological features that highly resemble those of the corresponding structures in the forelimb.

The regions of the cartilaginous growth plates, in which neither *Pitx1* nor *Pitx2* are expressed, appear to be normally maintained. In the *Pitx1*^{-/-} mice, expression of the hindlimb-specific marker gene, *Tbx4*, is reduced compared with normal, and is more strikingly reduced in the hindlimb region in which the morphological alterations are most dramatic, as shown both by in situ hybridization of sectioned embryos and whole-mount staining (Fig. 4A,B). No alterations in the *Tbx5* expression pattern are observed (Fig. 4A). Because *Pitx1* expression patterns correspond to structures adjacent to the growth plates, we evaluated expression *Ihh*, *PTHrP*, and *PTHrP*, and found no alterations in their expression (data not shown). No effects are observed on genes that are normally expressed at similar levels in hindlimb and forelimb including *Wnt5a*, *Fgf8*, *Bmp4*, *gsc*, *Hoxd10*, *d11*, or *d13* expression (data not shown).

To determine the effects of *Pitx1* gene deletion on the population of mesenchyme that normally expresses *Pitx1*, we examined *lacZ* expression in these mice. Surprisingly, *lacZ* expression is slightly diminished in the proximal developing mesenchyme (E11.5) compared with the levels in *Pitx1*^{+/+} mouse, and is reproducibly diminished in anterior, distal mesenchyme, as determined by the linear portion of limb in which *lacZ* staining can be detected (Fig. 4C,D). We therefore suggest that a population of *Pitx1*-expressing mesenchyme promotes

a *Pitx1*-dependent hindlimb-specific morphogenesis program, modulating growth and exerting specific effects on the tibia, fibula, patella, and tarsal morphology of ankle.

Effects of *cPitx1* misexpression

On the basis of the apparent requirement for *Pitx1* to achieve certain hindlimb-specific characteristics, it became of particular interest to investigate whether expressing *Pitx1* in forelimb would modify its developmental program. A chick *Pitx1* (*cPitx1*) cDNA was isolated, encoding a protein 80% identical to murine *Pitx1*, as has been recently reported (Logan et al. 1998b; Lanctôt et al. 1997). The *cPitx1* gene is expressed in a very similar pattern to the murine *Pitx1*. Initially, *cPitx1* transcripts are present almost exclusively in the limb bud that will develop into the leg but not in the limb bud that will give rise to the wing (Fig. 5A). As in the mouse, *cPitx1* is first detected in the lateral plate mesoderm before the limb bud emerges and as limb outgrowth proceeds, *cPitx1* is expressed throughout the entire limb mesenchyme (Fig. 5A). With progressive development of the limb bud, *cPitx1* becomes differentially expressed. Between stages 23 and 30, *cPitx1* transcripts are gradually excluded from the most proximal aspect of the developing limb bud and the adjacent flank. By stage 25, *cPitx1* transcripts begin to disappear from the distal region of the developing leg in which the digits are beginning to form, although low levels are observed in the interdigit region (Fig. 5A). Chick *Pitx1* transcripts start to be weakly detected in the developing wing at stage 26 outside of the prechondrogenic regions. Chick *Pitx1* transcripts are never detected in the limb ectoderm nor in the apical ectodermal ridge.

Figure 4. In situ hybridization and whole-mount analysis of limbs in *Pitx1*^{+/+} and *Pitx1*^{-/-} mice. (A) In situ hybridization analysis of markers known to be expressed during limb development. *Tbx4* expression is diminished distally, and no alteration in *Tbx5* or *Hoxd12* expression is noted in the hindlimb [*Tbx5* (H)] or forelimb [*Tbx5* (F)]. The expression of *Hoxd12*, *PTHrP*, *PTHrP*, and *Ihh* are also similar in ^{+/+} and ^{-/-} hindlimb at E12.5 and P0 (data not shown). (B) The expression patterns of *Pitx1* and *Tbx4* in the hindlimb by whole-mount in situ hybridization at E10.5. The *Tbx4* expression overlaps with that of *Pitx1* in the hindlimb of *Pitx1*^{+/+} mice and is reduced in the hindlimb of *Pitx1*^{-/-} mice. (C) Whole-mount *lacZ* staining analysis. *lacZ* (marker of targeted gene) expression is present in the mandibular structures (open arrow) and is subtly but reproducibly restricted in an anterior/distal portion of the hindlimb (Hind) of *Pitx1*^{-/-} mice (solid arrow in the bracket). (D) In situ hybridization analysis of *Pitx1* and *lacZ* expression in hindlimb at E11.5, showing anterior/distal restriction of *Pitx1*/*lacZ* expression, consistent with findings in the whole-mount *LacZ* staining (C).

Figure 5. (A) Expression of *cPitx1* in the developing chick hindlimb. Whole-mount and radioactive in situ hybridizations at different stages of hindlimb development. *cPitx1* transcripts start to be detected in the presumptive leg bud cells. At later stages, transcripts are detected throughout the hindlimb bud cells until stage 24 where they start to disappear from the condensing cartilage (panels 4–8). The bottom right panel shows the transient expression of *cPitx1* in the pelvis. (B) As seen by the ectopic patches (top) or broader expression (bottom) of *Tbx4*, retroviral infection of *cPitx1* in the presumptive wing cells can, later on, induce the expression of the hindlimb-specific gene, *Tbx4*. (C) Misexpression of *cPitx1* perturbs the outgrowth and patterning of the chick wing. Shown are whole-mount and skeletal preparations of control, and not injected, chick wing and leg. The chick wing has three digits of variable length, is covered by feathers, and displays a characteristic downward flexure at the wrist level. Instead, the chick leg is straight in its most distal part, not covered by feathers but by small scales, and has three digits of similar length and an additional

small fourth digit located toward the back of the foot. Ectopic expression of *cPitx1* in the wing transforms the curvature of the wing into an almost straight position, increases the size of digit 2 (open arrowhead) and induces the formation of a fourth digit (solid arrowhead). All of these changes in growth and patterning, together with the disappearance of feathers from the distal side of the wing, induce the infected wing to resemble a leg. Some of the infected embryos showed alterations in the size and morphology of the radius and ulna (solid arrow). (D,E) Results of a second experiment, to illustrate the range of phenotypic variations.

This expression pattern as well as cell-grafting experiments led Logan et al. (1998b) to hypothesize that *Pitx1* could be involved in specifying chick leg identity. If *cPitx1* is involved in encoding limb identity, its expression should be stable when leg tissue is grafted into wing tissue. When small pieces of leg mesoderm grafts are implanted beneath the apical ectodermal ridge of host wing buds, the original *cPitx1* expression is retained (Logan et al. 1998b).

On the basis of the hypothesis that the wing also contains the population of mesenchymal cells that would, if they expressed *Pitx1*, modulate a leg-like pattern, the retrovirus encoding full-length *Pitx1* was injected into the forelimb bud. Misexpression of *Pitx1* in the developing forelimb causes several morphological changes in the wing that are noticeably more striking at the distal aspect of the limb (Fig. 5E). One of the unique features of the chick wing is the posterior flexure of the most distal segment, the autopod, with respect to the middle segment, the zeugopod. This flexure is not observed in the leg in which the distal elements are placed in a straight orientation. Fifty-seven percent of the *Pitx1*-infected wings show a loss in the downward flexure of the wrist joint, thus giving them a leg-like appearance. When the skeletal pattern was examined, we observed a striking change in the relative size of the infected wing digits. The three wild-type wing digits have a variable length, with digit IV being the longest and II the shortest (Fig. 5C–E). In the leg, on the contrary, digits II, III, and IV are

very similar in length. In addition, the leg has an extra smaller digit positioned toward the back of the foot. In 61% of the *Pitx1* infected wings, we observed an increase in the size of digit II (Fig. 5C–E). In addition, an extra short digit appeared at the anterior side of the infected wings in 58% of the cases (Fig. 5C–E). This uniformity in digit length, as well as the appearance of an extra digit, has some resemblance to the digital patterning of the leg. The fact that only minimal abnormalities in the zeugopodal segment were observed after *Pitx1* misexpression, is likely to reflect a loss in developmental plasticity by the time the *Pitx1* virus is active in the regions fated to become the adult chick radius and ulna. The most distal cells of the limb bud, which will give rise to the digits, are, however, heavily infected by stage 20, a stage in which they are still plastic and competent to change cell fate (data not shown). This could explain the fact that the wing alterations are mainly restricted to the distal part of the limb. Finally, another effect of *Pitx1* misexpression was on the integument. The distal part of the chick wing, contrary to the leg, is normally covered by feathers. In 38% of the injected wings, we observed a reduction in the number of distal feathers, suggesting that *Pitx1* could act as a suppressor of feather formation during the developing leg integument.

In situ hybridization for various markers expressed during normal limb development in both leg and wing buds (including *Shh*, *Bmp2*, *Bmp4*, *Hoxd11*, or *Hoxd13*) showed no changes in their expression pattern. The limb

alterations were preceded by ectopic patches of expression of the hindlimb-specific *Tbx4* gene (15% of the injected limbs) (Fig. 5B). However, no change is observed for the forelimb-specific *Tbx5* gene (data not shown). Taken together, these results suggest that overexpression of *Pitx1* induces cell proliferation of a certain population of wing mesenchyme cells, complementing the lack of proliferation and morphological alterations observed after ablation of *Pitx1* in the mouse.

Role of *Pitx1* in pituitary and branchial arch development

Consistent with the pattern of *Pitx1* gene expression, the *Pitx1*^{-/-} mice also exhibit developmental defects in the anterior pituitary gland. Throughout the entire period in which cell phenotypes are established during pituitary organogenesis, *Pitx1* is continuously expressed. All known early events, including invagination of the Rathke's pouch, exclusion of *Shh* from invaginating epithelium, activation of *Fgf8*, *P-Lim*, *Msx-1*, *Lhx3*, α GSU, *Bmp2*, the ventral marker *Isl-1* (Treier et al. 1998) and *Prop-1* on E12.5 and *Pit-1* on E13.5, (Sornson et al. 1996) are normally maintained (Fig. 6A; data not shown). Analysis of expression of the trophic hormones that are characteristic of the differentiated pituitary cell types at E15.5 through postpartum day 0 (P0) indicates a selective decrease in the most ventral cell type populations. Examination of thyroid-stimulating hormone β (TSH β), luteinizing hormone β (LH β), follicle stimulating hormone β (FSH β), and the common glycoprotein α subunit (α GSU) expression suggests that both the number of gonadotropes and thyrotropes, as well as the level of LH β and TSH β transcripts and protein within the individual cells, are diminished (Fig. 6B,C; data not shown). Interestingly, the level of TSH β transcripts is most severely reduced in the rostral tip thyrotrope population, which does not require Pit-1 for TSH β gene activation (Lin et al. 1994). Growth hormone expression in somatotropes appears unchanged (Fig. 6B,C), whereas the number and expression levels of the *POMC* gene in the intermediate lobe melanotropes appears normal between E15.5 and P0. There is a consistent increase in the levels of both number of, and ACTH transcripts and peptide levels in the anterior pituitary corticotropes (Fig. 6B,C).

Development of the palate and derivatives of the first branchial arch are invariably severely affected in *Pitx1*^{-/-} mice (Fig. 7), probably accounting for the early postnatal death of the homozygous null mice. In addition to cleft palate (Fig. 7A), the distal mandible and the tongue are significantly foreshortened, the ventral sublingual mesenchyme is hypoplastic, and the submandibular gland does not form (Fig. 7B). However, the spatial relationships between most of the components of lower jaw and mouth are apparently normally maintained. The expression of a number of markers expressed early in the first branchial arch, including *Msx1*, *Msx2*, *gsc*, *Shh*, *Bmp2/4*, *Wnt5a*, and *Pitx2*, are unaltered in *Pitx1* gene-deleted mice (Fig. 7B; data not shown). The craniofacial defects in *Pitx1*^{-/-} mice, are particularly intriguing in light of

Figure 6. Effects of *Pitx1* deletion on development of the anterior pituitary gland. (A) Effects of *Pitx1* gene deletion on *P-Lim*, *Prop-1*, α GSU, and *Isl-1* expression in the pituitary gland. No differences were observed in the expression of these factors in *Pitx1*^{-/-} embryos as compared with that of the wild-type at E12.5. (B,C) *Pitx1* gene deletion alters expression of ventral pituitary-specific cell types at P0 in mice analyzed by in situ hybridization (B) or by immunohistochemical analysis at E17.5 (C). The expression of TSH β , FSH β , and LH β are markedly decreased, with some reduction of α GSU. Note particularly the loss of TSH β in the rostral tip (arrow). *Pitx2* and growth hormone (*GH*) gene expression are unchanged. *POMC* transcripts and ACTH immuno activity in anterior lobe are consistently increased. Because of saturation of the film with the *POMC* probe, the region is artificially black in a portion of the intermediate lobe (asterisk, B). Note that ACTH staining is similar on wild-type and ^{-/-} glands in the intermediate lobe (I), while clearly increased in the anterior lobe (A) (C).

the observation that the human *Pitx1* gene maps to 5q31 (Crawford et al. 1997), which the investigators suggest could indicate that mutant *Pitx1* alleles might be responsible for a subset of patients with Treacher–Collins syndrome (Rogers 1964; Fazen et al. 1967; McDonald and Gorski 1993).

Discussion

On the basis of these data, the *bicoid*-related gene, *Pitx1*, appears to be a critical transcriptional component of limb development, as well as exerting roles in development of a derivative of the midline stomadeum, the an-

Figure 7. Effects of *Pitx1* gene deletion on mandible, palate, and submandibular gland development at E17.5 and P0. (A) Marked reduction in size of mandible analyzed by skeletal staining (top) and failure of palatal closure (see brackets) analyzed by scanning electron microscopy in the *Pitx1*^{-/-} mouse (bottom). (B) Mandibular expression of *Pitx1* and *lacZ* as a marker in *Pitx1*^{+/+} and *Pitx1*^{-/-} mice, respectively at E11.5. Markers of mandibular development analyzed at E11.5 and E12.5 by in situ hybridization included *gsc*, *Shh*, and *Wnt5a*. Submandibular gland (SG) development in *Pitx1*^{+/+} and *Pitx1*^{-/-} mice at E17.5 marked by the *lacZ* probe. (To) tongue; (M) mandible.

terior pituitary, and on derivatives of the first branchial arch. Our data suggest a model in which expression of *Pitx1* in hindlimb mesenchyme is required for correct hindlimb morphogenesis and growth, particularly in the region encompassing the tibia, fibula, patella, and proximal tarsus. Thus, in the absence of *Pitx1*, tibia and fibula are morphologically more similar to the forelimb radius and ulna, and evolutionarily conserved hindlimb patterning features are altered. These include loss of the hindlimb-specific patella and Zucker's nodes, and appearance of a potential pisiform-like element, an evolutionary-conserved characteristic of the forelimb carpus, in the proximal tarsus, with altered growth and morphology of the calcaneus, but without clear alterations in other elements of the proximal tarsus. Because both of these morphological and potential patterning features are well-recognized aspects of the distinction between

forelimb and hindlimb, we suggest that the presence of *Pitx1* is required for a transcriptional program required for the characteristic growth and morphological alterations that are a component of the distinctions between hindlimb and forelimb. The molecular basis of this morphological change in the *Pitx1*^{-/-} mouse hindlimb could reflect either altered patterning, and/or altered formation of, or response to, an anterior-posterior growth gradient in the limb bud, resulting in normalization of the size of the tibia and fibula, loss of the patella and Zucker's nodes, and altered proximal tarsus. Consistent with this model, targeted misexpression of *Pitx1* in the chicken wing bud causes distinct proliferative alterations of the digits, as well as altered morphological features, suggesting that the appropriate population of mesenchyme is present in both limbs to mediate *Pitx1*-dependent morphological and proliferative alterations. The presence of *Pitx1* therefore appears to be required for a transcriptional program required for the characteristic growth and morphological alterations that are a component of the distinctions between hindlimb and forelimb. It is therefore tempting to speculate that *Pitx1* exerts either a patterning function or/and dictates the formation of the response to an anterior-posterior gradient-mediating proliferation in the limb bud.

Another family of genes that have been suggested as being involved in determining identity are the *Tbx* family (Chapman et al. 1996; Gibson-Brown et al. 1998; Isaac et al. 1998; Logan et al. 1998b; Ohuchi et al. 1998), with *Tbx4* specifically expressed in the hindlimb. Expression of *Tbx4* in the *Pitx1*^{-/-} hindlimb is clearly diminished, especially in its distal aspect. Expression of *Tbx4* is also induced in the chick forelimb after *Pitx1* misexpression. These results suggest that *Pitx1* is epistatic to at least a portion of the *Tbx4* program and required for the remainder of the gene inductions for segmental morphogenesis and patterning in a hindlimb-specific fashion. In the absence of *Pitx1*, the forelimb-specific gene *Tbx5* is not induced in the hindlimb: This may account for a portion of the distinctions that remain between the hindlimb of the *Pitx1*^{-/-} mouse and the wild-type forelimb.

The actions of *Pitx1* in pituitary development may provide several clues to the molecular basis of its actions in limb development. In pituitary, any potential early roles of *Pitx1* may be redundant with those of *Pitx2*/*RIEG* (Crawford et al. 1997), as there is no defect in early pituitary organogenesis; however, there is a consistent, late, pituitary developmental phenotype, which involves decreased proliferation and levels of distal target gene expression in two ventral pituitary cell types—gonadotropes and thyrotropes—and an element of a distal cell type, the corticotrope, expressing ACTH, in which *Pitx1* protein is expressed (Lamonerie et al. 1996). On the basis of identification of synergistic interactions between *Pitx1* and other transcription factors (Szeto et al. 1996; Tremblay et al. 1998), it is likely that a major aspect of the phenotype reflects the synergistic role of *Pitx1* in target gene induction. Furthermore, as there are likely to be proliferative roles of the *Fgf8*, *Shh*, *Bmp*, and *Wnt* signaling factors in these cell types (Treier et al. 1998),

Pitx1 may alter sensitivity to critical trophic factors in a cell-autonomous fashion, and may exert similar effects in the limb. A similar explanation is likely to account for *Pitx1* effects in closure of the palatal bone structure. Thus, in the affected targets, *Pitx1* could alter expression of, or response to, trophic factors thereby exerting its effects on growth, as well as morphology. In this regard, the mechanism of *Pitx1* actions may be analogous in the various affected target organs.

On the basis of our *in vivo* data, we speculate that the growth, and morphogenetic roles of *Pitx1* in hindlimb actually contribute a critical component of the differential hindlimb and forelimb developmental programs that generate limb identity.

Materials and methods

Generation of *Pitx1*-gene-deleted mice

A mouse *Pitx1* genomic clone was isolated from a J1 129/Sv mouse genomic library with the complete *Pitx1* cDNA probe. The 5'-flanking region comprising a 4.0-kb *EcoRI*-*HindIII* fragment and a 3.0-kb *PmlI* 3'-flanking fragment were subcloned into the corresponding cloning sites of the *lacZ*/neomycin containing vector, in which expression of *lacZ* is driven by the *Pitx1* promoter and a neomycin gene is driven by the mouse phosphoglycerate kinase (PGK) promoter (Bermingham et al. 1996). The targeting vector was completed by subcloning into PGK-thymidine kinase plasmid. The entire homeodomain and virtually all of the 3'-coding region is replaced by the *lacZ*/neomycin gene. The R1 cell line was cultured in DMEM high glucose medium containing 15% FCS and supplemented with leukemia inhibitory factor (LIF). Targeting vector DNA was linearized (12 µg) and electroporated into 2×10^7 ES cells in 0.8 ml of electroporation buffer at 250 V and 500 µF with a Genepulser. Cells were grown for 7–9 days in 150 µg/ml G418 and 2 mM Gancyclovir and 500 double drug-selected clones were grown for an additional 3 days. Cell lines that had undergone homologous recombination were identified with the 5' external (1.0 kb) probe that hybridizes to a 15-kb *HindIII* wild-type *Pitx1* locus fragment and a 11-kb *HindIII* fragment in the *Pitx1*^{-/-} allele. A 3' internal (0.5 kb) probe, which recognizes a 2.5-kb *HindIII* fragment in both wild-type and *Pitx1*^{-/-} allele, was used to identify homologous recombination in the 3'-flanking region. Three ES cell lines, that met the requirement for homologous recombination at the *Pitx1* locus, were microinjected into C57BL/6 blastocysts that were then transferred to pseudopregnant females. Chimeric male mice were backcrossed to C57BL/6 females and germ-line transmission was scored by the presence of the agouti coat color. Heterozygotes and homozygotes were identified by Southern blot analysis (Bermingham et al. 1996). Three lines were generated and analyzed.

In situ hybridization, whole-mount hybridization, *lacZ* staining, immunohistochemistry, and differential bone/cartilage staining

Isolation, fixation, and hybridization with ³⁵S-labeled antisense RNA probes and exposure were performed as described previously (Ryan et al. 1998; Simmons et al. 1989). Chick embryos were staged according to Hamburger and Hamilton (1951). Whole-mount *in situ* hybridization of chick embryos and sectioning was performed as described (Ryan et al. 1998). After

fixation, whole-mount *in situ* hybridization was performed on mouse embryos, which were dehydrated with methanol and treated with hydrogen peroxide and proteinase K. Transcripts were then detected with AP-conjugated DIG antibodies and stained with NBT/BCIP. Whole-mount *lacZ* staining was performed in the presence of 1 mg/ml X-gal substrate. Immunohistochemistry was done on 5/7-mm-thick paraffin sections stained by indirect immunoperoxidase method. Peroxidase activity was visualized with DAB/metal enhancer (Pierce, Rockford, IL). Sections were counterstained with methyl green and mounted in Permount (Fisher). Antibodies were obtained and used diluted as follows: ACTH (Chemicon, Temecula, CA) 1:1000; GH and TSHβ (DAKO, Carpinteria, CA) 1:1000; αGSU (National Hormone and Pituitary Program) 1:1000. Anti-rabbit horseradish peroxidase-conjugated antibodies were from Chemicon and used at a 1:500 dilution. For bone and cartilage staining, embryos were isolated by cesarean section and the abdomens of embryos were immediately cut open prior to being placed into 95% ethanol for 24 hr. After embryos were skinned and eviscerated, they were fixed in 95% ethanol for 72 hr, and placed in 0.3% Alcian Blue/0.1% Alizarin Red S staining solution for at least 72 hr. After staining, each embryo was washed in tap water to remove excess dye, and then placed in 0.75% potassium hydroxide solution for maceration. After 24 hr, the embryos were cleared by successive washes in 20% and 50% glycerin solution.

Retroviral infection

The replication competent retroviral vectors RCASBP(A)-containing cDNAs encoding full-length *cPitx1* were generated as described (Ryan et al. 1998). Chicken embryos (MacIntyre Poultry, San Diego) were infected by injecting virus into the presumptive wing region at stages 10–12 (Hamburger and Hamilton staging table). After appropriate periods of incubation, chick embryos were fixed in 4% paraformaldehyde overnight, dehydrated in methanol, evaluated under a dissecting microscope, and stored at -20°C prior to *in situ* hybridization.

Acknowledgments

We thank Mario Capecchi, Mitch Kronenberg, and Andy McMahon for generously providing reagents, and Andy McMahon, Cliff Tabin, and Mathias Treier for discussions and sharing of results prior to publication. We thank Peggy Meyer for her expertise and generous assistance in preparation of illustrations and Marie Fisher for assistance in manuscript preparation. M.G.R. is an Investigator of the Howard Hughes Medical Institute. This work was supported by National Institutes of Health Grants to M.G.R. and J.C.I.B., and the Leila Y. Mathers Charitable Foundation Grant to J.C.I.B.

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked 'advertisement' in accordance with 18 USC section 1734 solely to indicate this fact.

References

- Acampora, D., S. Mazan, Y. Lallemand, V. Avantaggiato, M. Maury, A. Simeone, and P. Brûlet. 1995. Forebrain and mid-brain regions are deleted in *Otx2*^{-/-} mutants due to a defective anterior neuroectoderm specification during gastrulation. *Development* **121**: 3279–3290.
- Acampora, D., S. Mazan, V. Avantaggiato, P. Barone, F. Tuorto,

- Y. Lallemand, P. Brûlet, and A. Simeone. 1996. Epilepsy and brain abnormalities in mice lacking the Otx1 gene. *Nat. Genet.* **14**: 218–222.
- Acampora, D., S. Mazan, F. Tuorto, V. Avantsgiato, J.J. Tremblay, D. Lazzaro, A. di Carlo, A. Mariano, P.E. Macchia, G. Corte, V. Macchia, J. Drouin, P. Brulet, and A. Simeone. 1998. Transient dwarfism and hypogonadism in mice lacking Otx-1 reveal prepubescent stage-specific control of pituitary levels of Gh, FSH and LH. *Development* **125**: 1229–1239.
- Ang, S.L., O. Jin, M. Rhinn, N. Daigle, L. Stevenson, and J. Rossant. 1996. A targeted mouse Otx2 mutation leads to severe defects in gastrulation and formation of axial mesoderm and to deletion of rostral brain. *Development* **122**: 243–252.
- Bermingham, J.R., Jr., S.S. Scherer, S. O'Connell, E. Arroyo, K.A. Kalla, F.L. Powell, and M.G. Rosenfeld. 1996. Tst-1/Oct-6/SCIP regulates a unique step in peripheral myelination and is required for normal respiration. *Genes & Dev.* **10**: 1751–1762.
- Chapman, D.L., N. Garvey, S. Hancock, M. Alexiou, S.I. Agulnik, J.J. Gibson-Brown, J. Cebra-Thomas, R.J. Bollag, L.M. Silver, and V.E. Papaioannou. 1996. Expression of the T-box family genes, Tbx1-Tbx5, during early mouse development. *Dev. Dynam.* **206**: 379–390.
- Cho, K.W., B. Blumberg, H. Steinbeisser, and E.M. De Robertis. 1991. Molecular nature of Spemann's organizer: The role of the *Xenopus* homeobox gene goosecoid. *Cell* **67**: 1111–1120.
- Crawford, M.J., C. Lanctôt, J.J. Tremblay, N. Jenkins, D. Gilbert, N. Copeland, B. Beatty, and J. Drouin. 1997. Human and murine PTX1/Ptx1 gene maps to the region for Treacher Collins syndrome. *Mamm. Genome* **8**: 841–845.
- Dolle, P., J.C. Izpisua-Belmonte, H. Falkenstein, A. Renucci, and D. Duboule. 1989. Coordinate expression of the murine Hox-5 complex homeobox-containing genes during limb pattern formation. *Nature* **342**: 767–772.
- Erickson, J., S. Norlin, T.M. Jessel, and T. Edlund. 1998. Integrated FGF and BMP signaling controls the progression of progenitor cell differentiation and the emergence of pattern in the embryonic anterior pituitary. *Development* **125**: 1005–1015.
- Fazen, L.E., J. Elmore, and H.L. Nadler. 1967. Mandibulo-facial dysostosis (Treacher-Collins syndrome). *Am. J. Dis. Child.* **113**: 405–410.
- Gage, P.J. and S.A. Camper. 1997. Pituitary homeobox 2, a novel member of the bicoid-related family of homeobox genes, is a potential regulator of anterior structure formation. *Hum. Mol. Genet.* **6**: 457–464.
- Gehring, W.J., Y.Q. Qian, M. Billeter, K. Furukubo-Tokunaga, A.F. Schier, D. Resendez-Perez, M. Affolter, G. Otting, and K. Wuthrich. 1994. Homeodomain-DNA recognition. *Cell* **78**: 211–223.
- Gibson-Brown, J.J., S.I. Agulnik, D.L. Chapman, M. Alexiou, N. Garvey, L.M. Silver, and V.E. Papaioannou. 1996. Evidence of a role for T-box genes in the evolution of limb morphogenesis and the specification of forelimb/hindlimb identity. *Mech. Dev.* **56**: 93–101.
- Gibson-Brown, J.J., S.I. Agulnik, L.M. Silver, L. Niswander, and V.E. Papaioannou. 1998. Involvement in T-box genes Tbx2-Tbx5 in vertebrate limb specification and development. *Development* **125**: 2499–2509.
- Hamburger, V. and H. Hamilton. 1951. A series of normal stages in the development of the chick embryos. *J. Morphol.* **88**: 49–92.
- Isaac, A., C. Rodriguex-Estaban, A. Ryan, M. Altan, T. Tsukui, K. Patel, C. Tickle, J.C. Izpisua-Belmonte. 1998. Tbx genes and limb identity in chick embryo development. *Development* **125**: 1867–1875.
- Izpisua-Belmonte, J.C., C. Tickle, P. Dolle, L. Wolpert, and D. Duboule. 1991. Expression of the homeobox Hox-4 genes and the specification of position in chick wing development. *Nature* **50**: 585–589.
- Johnson, R.L. and C.J. Tabin. 1997. Molecular models for vertebrate limb development. *Cell* **90**: 979–990.
- Krumlauf, R. 1994. Hox genes in vertebrate development. *Cell* **78**: 191–201.
- Lamonerie, T., J.J. Tremblay, C. Lanctôt, M. Therrien, Y. Gauthier, and J. Drouin. 1996. Ptx1, a bicoid-related homeobox transcription factor involved in transcription of the proopiomelanocortin gene. *Genes & Dev.* **10**: 1284–1295.
- Lanctôt, C., B. Lamolet, and J. Drouin. 1997. The bicoid-related homeoprotein Ptx1 defines the most anterior domain of the embryo and differentiates posterior from anterior lateral mesoderm. *Development* **124**: 2807–2817.
- Lanske, B., A.C. Karaplis, K. Lee, A. Luz, A. Vortkamp, A. Pirro, M. Karperien, L.H.K. Defize, C. Ho, R.C. Mulligan, A.B. Abou-Samra, H. Jüppner, G.V. Segre, and H.M. Kronenberg. 1996. PTH/PTHrP receptor in early development and Indian hedgehog-regulated bone growth. *Science* **273**: 663–666.
- Li, H., C. Tierney, L. Wen, J.Y. Wu, and Y. Rao. 1997. A single morphogenetic field gives rise to two retina primordia under the influence of the prechordal plate. *Development* **124**: 603–615.
- Lin, S.C., S. Li, D.W. Drolet, and M.G. Rosenfeld. 1994. Pituitary ontogeny of the Snell dwarf mouse reveals Pit-1-independent and Pit-1-dependent origins of the thyrotrope. *Development* **120**: 515–522.
- Logan, M., S.M. Pagan-Westphal, D.M. Smith, L. Paganessi, and C.J. Tabin. 1998a. The transcription factor of Ptx2 mediates situs-specific morphogenesis in response to left-right asymmetric signaling. *Cell* **94**: 307–317.
- Logan, M., H. Simon, and C. Tabin. 1998b. Differential regulation of T-box and homeobox transcription factors suggests roles in controlling chick limb-type identity. *Development* **125**: 2825–2835.
- Maconochie, M.S., S. Nonchev, A. Morrison, and R. Krumlauf. 1996. Paralogous Hox genes: function and regulation. *Annu. Rev. Genet.* **30**: 529–556.
- Martin, G. 1998. The roles of Fgf in the early development of vertebrate limbs. *Genes & Dev.* **12**: 1571–1586.
- McDonald, M.T. and J.L. Gorski. 1993. Nager acrofacial dysostosis. *J. Med. Genet.* **30**(9): 779–782.
- Meno, C., A. Shimono, Y. Saijoh, K. Yashiro, K. Mochida, S. Ohishi, S. Noji, H. Kondoh, and H. Hamada. 1998. lefty-1 is required for left-right determination as a regulator of lefty-2 and nodal. *Cell* **94**: 287–297.
- Nelson, C.E., B.A. Morgan, A.C. Burke, E. Laufer, E. DiMambro, L.C. Murtaugh, E. Gonzales, L. Tessarollo, L.F. Parada, and C. Tabin. 1996. Analysis of Hox gene expression in the chick limb bud. *Development* **122**: 1449–1466.
- Ohuchi, H., J. Takenchi, H. Yoshioka, Y. Ishimaru, K. Ogura, N. Takahashi, T. Ogura, and S. Noji. 1998. Correlation of wing-leg identity in ectopic FGF-induced chimeric limbs with the differential expression of chick Tbx5 and Tbx4. *Development* **125**: 51–60.
- Piedra, M.E., J.M. Icardo, M. Albajar, J.C. Rodriguez-Rey, and M.A. Ros. 1998. Pitx2 participates in the late phase of the pathway controlling left-right asymmetry. *Cell* **94**: 319–324.
- Rhinn, M., A. Dierich, W. Shawlot, R.R. Behringer, M. Le Meur, and S.L. Ang. 1998. Sequential roles for Otx2 in visceral endoderm and neuroectoderm for forebrain and midbrain induction and specification. *Development* **125**: 845–856.

- Rogers, B. 1964. Barry-Treacher Collins syndrome: A review of 200 cases. *Br. J. Plast. Surg.* **17**: 109–136.
- Romer, A.S. 1986. *The vertebrate body*. (ed. T.S. Parsons), Saunders, Philadelphia, PA.
- Ryan, A.K., B. Blumberg, C. Rodriguez-Esteban, S. Yonei-Tamura, K. Tamura, T. Tsukui, J. de la Pena, W. Sabbagh, J. Greenwald, S. Choe, D.P. Norris, E.J. Robertson, R.M. Evans, M.G. Rosenfeld, and J.C. Izpisua Belmonte. 1998. Ptx2 determines asymmetries in vertebrate. *Nature* **394**: 545–551.
- St. Armand, T.R., J. Ra., Y. Zhong, Y. Hu, S.I. Baber, M. Qiu, and Y. Chen. 1998. Cloning and expression pattern of chicken Pitx2: A new component in the SHH signaling pathway controlling embryonic heart looping. *Biochem. Biophys. Res. Commun.* **247**: 100–105.
- Scott, M.P. 1997. Hox genes, arms and the man. *Nat. Genet.* **15**: 117–118.
- Schwabe, J.W., C. Rodriguez-Esteban, and J.C. Izpisua Belmonte. 1998. Limbs are moving: Where are they going? *Trends Genet.* **14**: 229–235.
- Semina, E.V., R. Reiter, N.J. Leysens, W.L. Alward, K.W. Small, N.A. Datson, J. Siegel-Bartelt, D. Bierke-Nelson, P. Bitoun, B.U. Zabel, J.C. Carey, and J.C. Murray. 1996. Cloning and characterization of a novel bicoid-related homeobox transcription factor gene, RIEG, involved in Rieger syndrome. *Nat. Genet.* **14**: 392–399.
- Shang, J., Y. Luo, and D.A. Clayton. 1997. Backfoot is a novel homeobox gene expressed in the mesenchyme of the development hind limb. *Dev. Dynam.* **209**: 242–253.
- Simeone, A., D. Acampora, M. Gulisano, A. Stornaiuolo, and E. Boncinelli. 1992. Nested expression domains of four homeobox genes in developing rostral brain. *Nature* **358**: 687–690.
- Simeone, A., D. Acampora, A. Mallamaci, A. Stornaiuolo, M.R. D'Apice, V. Nigro, and E. Boncinelli. 1993. A vertebrate gene related to orthodenticle contains a homeodomain of the bicoid class and demarcates anterior neuroectoderm in the gastrulating mouse embryo. *EMBO J.* **12**: 2735–2747.
- Simmons, D.M. 1989. A complete protocol for in situ hybridization of messenger RNAs in brain and other tissues with radiolabeled single stranded RNA probes. *J. Histotechnol.* **12**: 169–181.
- Sornson, M.W., W. Wu, J.S. Dasen, S. Flynn, D.J. Norman, S.M. O'Connell, I. Gukovsky, C. Carrière, A.K. Ryan, A.P. Miller, L. Zuo, A.S. Gleiberman, B. Andersen, W.G. Beamer, and M.G. Rosenfeld. 1996. Pituitary lineage determination by the Prop-1 homeodomain factor defective in Ames dwarfism. *Nature* **384**: 327–333.
- Szeto, D.P., A.K. Ryan, S.M. O'Connell, and M.G. Rosenfeld. 1996. P-OTX: A Pit-1-interacting homeodomain factor expressed during anterior pituitary gland development. *Proc. Natl. Acad. Sci.* **93**: 7706–7710.
- Takuma, N., H.Z. Sheng, Y. Furuta, J.M. Ward, K. Sharma, B.L. Hogan, S.L. Pfaff, H. Westphal, S. Kimura, and K.A. Mahon. 1998. Formation of Rathke's pouch requires dual induction from the diencephalon. *Development* **125**: 4835–4840.
- Treier, M., A.S. Gleiberman, S.M. O'Connell, D.P. Szeto, J.A. McMahon, A.P. McMahon, and M.G. Rosenfeld. 1998. Multistep signaling requirements for pituitary organogenesis in vivo. *Genes & Dev.* **12**: 1691–1704.
- Tremblay, J.J., C. Lanctôt, and J. Drouin. 1998. The pan-pituitary activator of transcription, Ptx1 (pituitary homeobox 1), acts in synergy with SF-1 and Pit 1 and is an upstream regulator of the Lim-homeodomain gene Lim3/Lhx3. *Mol. Endocrinol.* **112**: 428–441.
- Vortkamp, A., K. Lee, B. Lanske, G.V. Segre, H.M. Kronenberg, and C.J. Tabin. 1996. Regulation of rate of cartilage differentiation by Indian hedgehog and PTH-related protein. *Science* **273**: 613–622.
- Yokouchi, Y., H. Sasaki, and A. Kuroiwa. 1991. Homeobox gene expression correlated with the bifurcation process of limb cartilage development. *Nature* **353**: 443–445.
- Yoshioka, H., C. Meno, K. Koshiba, M. Sugihara, H. Itoh, Y. Ishimaru, T. Inoue, H. Ohuchi, E.V. Semina, J.C. Murray, H. Hamada, and S. Noji. 1998. Pitx2, a bicoid-type homeobox gene, is involved in a lefty-signaling pathway in determination of left-right asymmetry. *Cell* **94**: 299–305.

Role of the Bicoid-related homeodomain factor Pitx1 in specifying hindlimb morphogenesis and pituitary development

Daniel P. Szeto, Concepción Rodríguez-Esteban, Aimee K. Ryan, et al.

Genes Dev. 1999, **13**:

References

This article cites 50 articles, 22 of which can be accessed free at:
<http://genesdev.cshlp.org/content/13/4/484.full.html#ref-list-1>

License

Email Alerting Service

Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or [click here](#).