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Interactions between dorsal-ventral patterning genes *lmx1b, engrailed-1* and *wnt-7a* in the vertebrate limb

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ABSTRACT The vertebrate limb has characteristic morphological features that distinguish dorsal and ventral regions. For example, humans and most other mammals have nails on the dorsal surface of their digits, while the ventral surface is covered by skin or footpads. Internally, there is a high degree of organization along the dorsal-ventral axis. Extensor muscles are generally located dorsally while flexor muscles are generally located ventrally. The skeleton has subtle differences that allow for attachment of these muscles and distinct pools of motor neurons innervate either dorsal or ventral muscles. How is this complex arrangement of tissues generated? Recent studies have identified a molecular cascade of three factors that govern early events in dorsal-ventral limb patterning. Two of these factors, *engrailed-1* and *wnt-7a* are expressed in the dorsal and ventral ectoderm respectively. The function of *engrailed-1* is to repress the expression of *wnt-7a* in the ventral limb bud ectoderm. The third factor, a LIM-homeodomain transcription factor, *lmx1b* is induced in dorsal mesenchyme by wnt-7a and it is both necessary and sufficient to specify dorsal limb pattern. In this report, we examine genetic interactions between *wnt-7a, engrailed-1,* and *lmx1b* by analyzing the phenotypes of mice that are double mutants for *lmx1b* and either *wnt-7a* or *engrailed-1*. These studies indicate that *lmx1b* is the only target of *wnt-7a* and *engrailed-1* that is of consequence for dorsal-ventral patterning. Moreover, this genetic analysis suggests that *lmx1b* plays additional roles in anterior-posterior patterning and growth that were not previously appreciated.

KEY WORDS: *limb, lmx1b, engrailed-1, wnt-7a, development*

Introduction

The developing vertebrate limb is amenable to genetic and embryological manipulation and has long been used as a model system to understand pattern formation (Johnson and Tabin, 1997). Vertebrate limbs initially form as buds that protrude at thoracic and lumbar levels to form the forelimb and hindlimb buds. Initially, they are composed of a single columnar epithelium encompassing a loose mesenchyme. The limb bud mesenchyme receives dual contributions from the lateral plate mesoderm and the somitic mesoderm. The lateral plate mesoderm contributes to the connective and skeletal elements of the limb while the muscle of the limb derives solely from somite-derived myoblasts that migrate into the limb bud.

An important step in vertebrate limb pattern is the specification of positional information along the anterior-posterior, dorsal-ventral, and proximal distal axes (Capdevila and Izpisua Belmonte, 2001). Anterior-posterior and proximal-distal patterning is mediated largely by two signaling centers in the early limb bud, the zone of polarizing activity (ZPA) and the apical ectodermal ridge (AER). These signaling centers secrete sonic hedgehog in the case of the ZPA and fibroblast growth factors (FGFs) wnt proteins and bone morphogenetic proteins (BMPs) in the case of the AER that in turn modulate anterior-posterior and proximal distal patterning respectively. The AER is located at the distal end of the limb bud at the interface of dorsal and ventral ectoderm, and is necessary and sufficient for limb bud outgrowth.

Dorsal-ventral limb axis specification in vertebrate embryos occurs through a complex, poorly understood series of epithelial-mesenchymal interactions (Chen and Johnson, 1999). Current models suggest that initially the dorsal-ventral information resides within the mesenchyme of the lateral plate mesoderm. How the lateral plate mesenchyme obtains this information is not clear, but it has been suggested that signals from the somitic mesoderm specify dorsal fates through an inductive mechanism and the

Abbreviations used in this paper: AER, apical ectodermal ridge; BMP, bone morphogenetic protein; FGF, fibroblast growth factor; ZPA, zone of polarizing activity.

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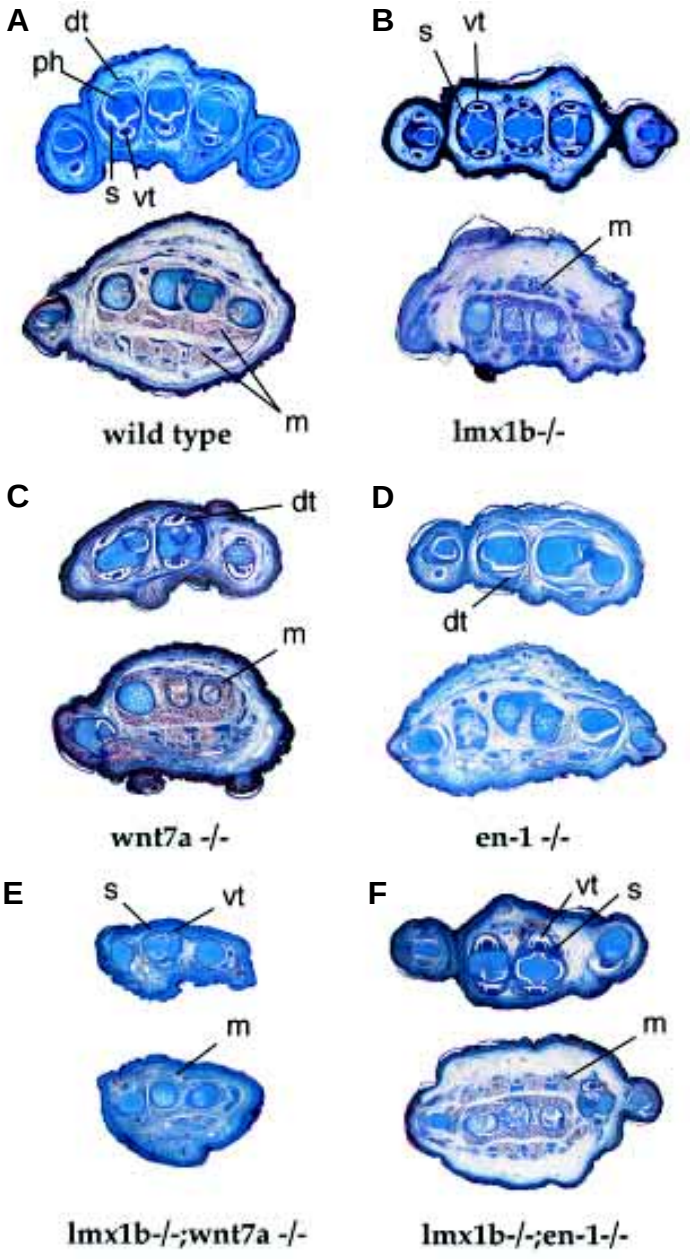

Fig. 1. Histology of wild-type and mutant newborn hindlimbs. *In all panels, dorsal is up and ventral is down.* **(A)** *Sections through the distal phalanges (upper section) and plantar region (lower section) reveal morphological distinctions between the dorsal and ventral portions of the limb. The ventral tendons (vt) have a rounded appearance while the dorsal tendons (dt) have a flattened appearance. The phalanges (ph) have a rounded dorsal surface and a pointed ventral surface that is in close apposition to the sesamoid bones (s). In the plantar region, muscle (m) is present only ventrally.* **(B)** Lmx1b*-/- hindlimbs are symmetric with respect to dorsal-ventral polarity. Note the presence of sesamoid bones and ventral tendons in the dorsal portion of the limb as well as dorsal muscle masses in a duplicated ventral arrangement.* **(C)** Wnt-7a *-/- hindlimbs exhibit a similar appearance to that of* lmx1b *mutants.* **(D)** Engrailed-1 *mutants have the opposite appearance with dorsal tendons in the ventral limb and absence of muscle.* **(E)** Lmx1b/wnt-7a *double homozygotes have a similar appearance to either single mutant but the overall size is smaller.* **(F)** Lmx1b/engrailed-1 *mutants exhibit dorsal-to-ventral transformations very similar to that of* lmx1b *single mutants.*

ventral fate is a default. Subsequently, the dorsal-ventral polarity is transferred to the ectoderm prior to limb bud outgrowth resulting in the expression of *wnt-7a* in the presumptive dorsal limb bud ectoderm and *engrailed-1* in the ventral limb bud ectoderm. In the next step, *wnt-7a,* induces the expression of the LIM-homeodomain transcription factor *lmx1b* specifically in the dorsal mesenchyme of the limb bud. Gain of function experiments in the chick indicated that *lmx1b* is sufficient to specify dorsal limb pattern in ventral limb mesenchyme (Riddle *et al.,* 1995; Vogel *et al.,* 1995). Subsequently, it was shown through gene targeting in the mouse that *lmx1b* activity is necessary to specify dorsal limb pattern (Chen *et al.,* 1998).

The availability of mouse mutants for *lmx1b* (Chen *et al.,* 1998), *wnt-7a* (Parr and McMahon, 1995) and *engrailed-1* (Loomis *et al.,* 1996) has allowed us to explore interactions between these genes by examining the phenotypes of *lmx1b/wnt-7a* and *lmx1b/engrailed-1* double mutant mice. Comparison of the limb phenotypes of single versus double mutants reveals complex genetic interactions that affect both the dorsal-ventral and anterior-posterior axes. Additionally, this analysis reveals roles for *lmx1b* in limb bud growth and patterning that were not previously observed in *lmx1b* single mutants.

Results

Dorsal Ventral Patterning in lmx1b/wnt-7a *and* lmx1b/engrailed-1 *Double Mutants*

Lmx1b and *wnt-7a* homozygotes display a dorsal-to-ventral conversion of the distal limb bud (Chen *et al.,* 1998; Parr and McMahon, 1995). These transformations are most obvious from sections of newborn hindlimbs (Fig. 1). Characteristic features of dorsal-ventral polarity at the level of the digits are the shape of the phalanges, the presence of sesamoid bones, the morphology of tendons, and the arrangement of muscles (Fig. 1A). In the wild-type situation the phalanges have a ventral narrowing and a smooth dorsal surface. Located at sites of ventral tendon attachment are sesamoid bones, small derivatives of the ventral tendons. The ventral tendons exhibit a characteristic rounded appearance, while the dorsal tendons are flattened. Finally, at a more proximal level, the dorsal limb is devoid of muscle, while the ventral limb has a complex muscle pattern. In *lmx1b* homozygotes (Fig. 1B), the limb is mirror-symmetric with a double ventral pattern as assayed by skeleton, tendon, and muscle morphology. *Wnt-7a* mutants exhibit similar dorsal-to-ventral transformation (Fig. 1C), although the transformation is not as complete as seen in the lmx1b mutants. As expected *lmx1b/wnt-7a* double mutant limbs display a dorsal-ventral phenotype similar to that seen in the *lmx1b* single mutants (Fig. 1E).

In contrast to either *lmx1b* or *wnt-7a* mutants, *engrailed-1* mutant mice exhibit a ventral-to-to dorsal conversion of limb tissues (Loomis *et al.,* 1996). Sections through the distal hindlimbs of *engrailed-1* mutants reveal that the characteristic pattern of ventral skeletal and tendon elements is converted to a dorsal phenotype (Fig. 1D). Since *lmx1b* homozygous mutants display an opposite ventralizing phenotype we could determine the epistatic relationship between *lmx1b* and *engrailed-1* by generating the double mutants. *Lmx1b/engrailed-1* double mutant limbs (Fig. 1F) have a phenotype very similar to that seen in *lmx1b* single mutants suggesting that *engrailed-1* is epistatic to *lmx1b* with respect to dorsal-ventral limb patterning.

Skeletal Phenotypes of lmx1b/wnt-7a *and* lmx1b/engrailed *Double Mutants*

To assess the effects of removing either *wnt-7a* or *engrailed-1* in *a lmx1b* mutant background on skeletal patterning, we examined alcian blue/alizarin red skeletal preparations of fore-limbs and hindlimbs dissected from newborn pups (Fig. 2). Wild-type forelimbs are depicted in Fig. 2A. The characteristic skeletal elements that can be seen are the scapula, located at the most proximal portion of the limb, a single long bone, the humerus, in the upper arm, two long bones in the forearm region (also called the zeugopod), the radius and the ulna, followed by the digits at the most distal region. The ulna marks the posterior side of the limb and the radius marks the anterior side of the limbs. In the *lmx1b* single and double mutants, the forelimbs are more severely affected than the hindlimbs and their phenotype is described below. As reported previously (Chen *et al.,* 1998), *lmx1b* mutant forelimbs (Fig. 2B) have a variable loss of the unla, but retain five digits. Similarly, *wnt-7a* (Parr and McMahon, 1995) mutants (Fig. 2C) also have variable ulnar loss and in addition usually lack the posterior-most digit. In contrast, the skeletal pattern of the *engrailed-1* mutant is relatively normal with respect to digit number and ulnar morphology (data not shown).

In the *lmx1b* mutant background, *wnt7a* significantly enhances the lmx1b forelimb skeletal phenotype (Fig. 2D). The number of digits is reduced to two and with full penetrance, there is a single bone in the zeugopodal region. The identity of the digits is difficult to determine, but they probably corresponds to the anterior-most digits judging from their morphology. Similarly, the single long bone in the zeugopodal region is likely to be radius because of the anterior connection the humerus. In addition, elbow joint has failed to separate the humerus and the radius completely, leading to the fusion of the long bones to form a single element. In contrast, engrailed-1 appears to suppress the *lmx1b* phenotype (Fig. 2E). In *lmx1b/engrailed-1* double homozygotes, the ulna is restored to full size and with variable penetrance an extra digit is observed.

The hindlimb phenotype of *lmx1b* mutants and of double mutants is not as pronounced as the forelimb phenotype. However, several interesting features can still be noted. The wild-type hindlimb pattern is shown in Fig. 2F. At the most proximal region is the pelvic girdle followed by the femur in the upper leg. The patella, a derivative of the dorsal patellar tendon, is located at a dorsal position inbetween the femur and the fibula and tibia. Digits are located at the distal end of the hindlimb. *Lmx1b* mutant hindlimbs (Fig. 2G) exhibit a similar overall structure to wild-type hindlimbs. Prominent alterations include the absense of a patella, a straightening of the fibula and an abmormal flexure of the foot, most likely due to a dorsal-ventral patterning defect in the ankle region. In contrast to what was observed for the forelimbs, *lmx1b/wnt-7a* (Fig. 2I) and *lmx1b/engrailed-1* double mutant (Fig. 2J) hindlimbs are very similar to the *lmx1b* single mutants, indicating that there are no observable downstream interactions between these genes in hindlimbs.

Analysis of hoxd11 *Expression in Single and Double Mutants*

The skeletal phenotypes of *lmx1b/wnt7a* and *lmx1b/engrailed-1* mutants suggest that anterior-posterior patterning is affected in these mutants. To gain insight into whether these skeletal defects are preceded by alterations in the anterior-posterior patterning

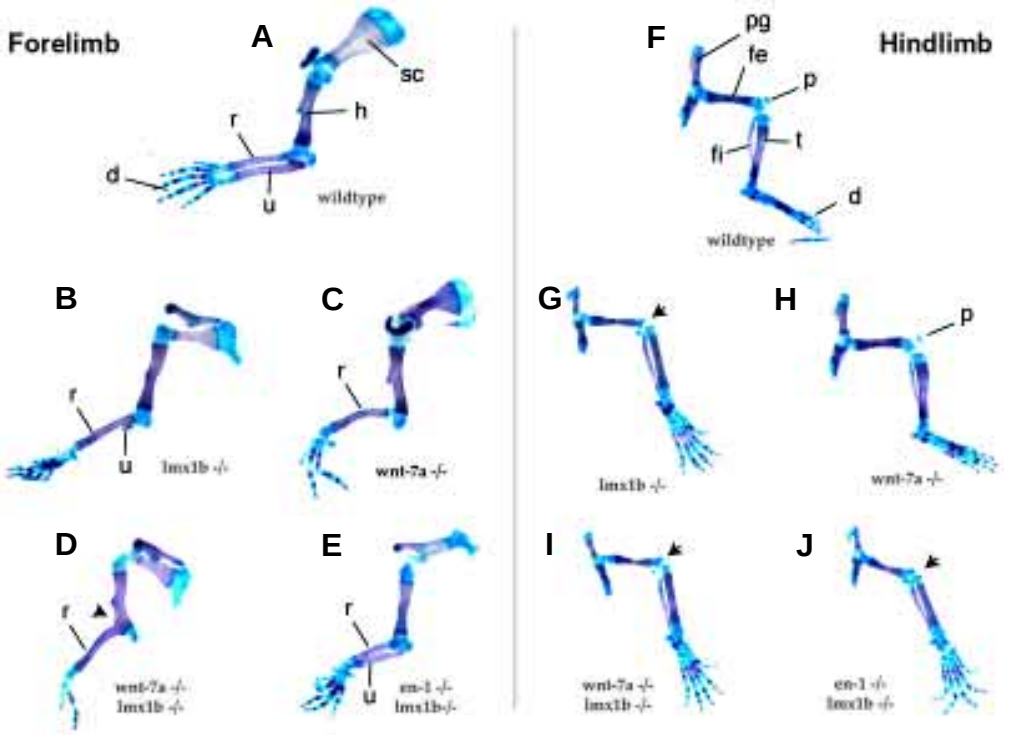

Fig. 2. Skeletal analysis of wild-type and mutant limbs. *Panels A-E are newborn forelimb preparations and panels F-J are newborn hindlimb preparations.* **(A)** *Wild-type forelimb.* **(B)** Lmx1b *mutant forelimb. Note the near-absence of the radius.* **(C)** Wnt-7a *mutant forelimb. Note the absence of the ulna in this specimen and the reduction in digit number.* **(D)** Lmx1b/wnt-7a *double mutant forelimb. Note the further reduction in digit number and the fusion of the radius to the humerus (arrowhead).* **(E)** Lmx1b/engrailed-1 *double mutant forelimb. Note the presence of a relatively normal ulna.* **(F)** *Wild-type hindlimb.* **(G)** Lmx1b *mutant hindlimb. Note that the fibula is much straighter than the wild-type and articulates with the ankle region rather than with the tibia as in wild-type; the tibia is thinner, the patella is absent (arrow), and the digits are rotated to be in plane with the fibula and tibia.* **(H)** Wnt-7a *mutant. The phenotype of the hindlimb is very similar to the wild-type. Note that the presence of the patella, the shape and insertion point of the fibula and the flexure of the foot are all similar to wild-type.* **(I)** Lmx1b/Wnt-7a *double mutant. The hindlimb phenotype is indistinguishable from that of the* lmx1b *single mutant. The arrow points to the absence of the patella.* **(J)** Lmx1b/engrailed-1 *double mutant. The hindlimb phenotype is quite similar to the* lmx1b *single mutant although there are some splaying of the distal ends of the digits. Abbreviations: sc, scapula; h, humerus; r, radius; u, ulna; d, digits; pg, pelvic girdle; fe, femur; p, patella; fi, fibula; ti, tibia.*

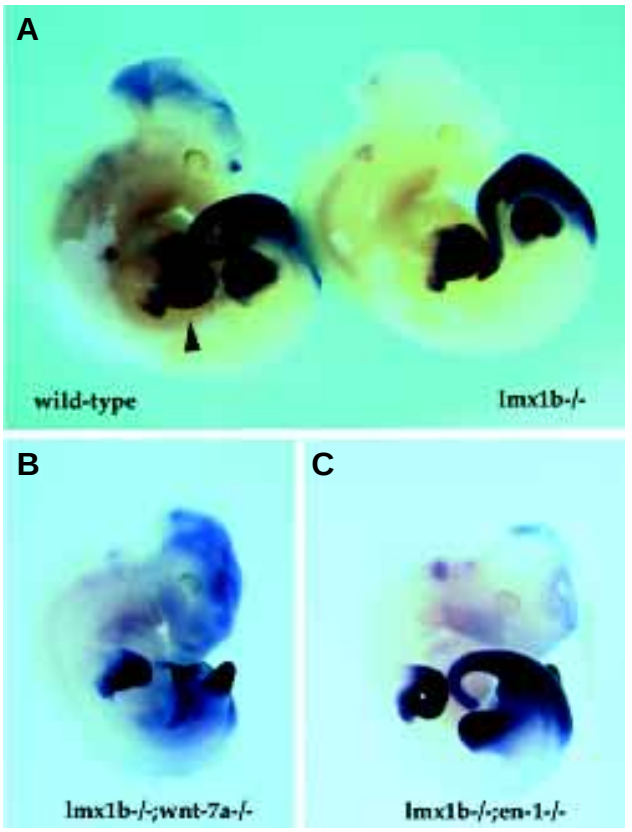

Fig. 3. Expression of *hoxd11* in single and double mutant embryos. **(A)** *Whole-mount* in situ *hybridization of wild-type (left) and* lmx1b *mutant (right) E 11.5 embryos.* Hoxd11 *is expressed in the forelimb bud (arrowhead) in a proximal and distal domain. This pattern is unaltered in* lmx1b *mutants.* **(B)** Hoxd11 *expression in* lmx1b/wnt-7a *double mutants. The two domains of expression are now fused into a single domain that retains posterior restriction. Note that the forelimb is much smaller than wild-type or* lmx1b *single mutants and has a posterior truncation.* **(C)** Hoxd11 *expression in* lmx1b/engrailed-1 *double mutants. The forelimb bud has a relatively normal shape bud and exhibits a single domain of* hoxd11 *expression. Note that this embryo is at a slightly earlier stage than the embryos shown in panel (A).*

pathway, we examined *hoxd11* (Dolle *et al.,* 1989; Izpisua-Belmonte *et al.,* 1991) in single and double mutant limb buds by whole-mount *in situ* hybridization. In wild-type embryos (Fig. 3A), *hoxd11* is expressed in forelimb buds in separate proximal and distal domains. Within each domain, there is stronger expression of *hoxd11* in the posterior portions of the limb bud. In *lmx1b* single mutants there is no evidence of reduction or alteration of *hoxd11* expression. Suprisingly, in the *lmx1b/wnt-7a* double mutant, the expression of *hoxd11* is similar to that of either the *lmx1b* or *wnt-7a* mutant (data not shown), even though the limbs are significantly reduced in size relative to either the *wnt-7a* or *lmx1b* single mutants. Perhaps as a consequence of this smaller size, the proximal and distal domains of *hoxd11* expression are not as distinguished relative to the wild-type. No significant alterations in the expression of *hoxd11* were in *engrailed-1* mutants but in *lmx1b/engrailed-1* double mutants, a fusion of the proximal and distal domains of *hoxd11* expression was observed.

Discussion

The final form of the vertebrate limb is sculpted through a series of inductive events that pattern the three cardinal limb axes (Capdevila and Izpisua Belmonte, 2001; Johnson and Tabin, 1997). The anterior-posterior axis is specified at least in part through the action of the secreted signaling molecule sonic hedgehog. The proximal-distal axis is defined through the antagonism of the AER (mediated by wnt and fgf signaling) and signals emanating from the proximal limb region. The dorsal-ventral limb axis is determined through a series of reciprocal epithelial-mesenchymal interactions between limb bud ectoderm and mesenchyme. In order for the proper shape and function of the adult limb to be achieved, these patterning processes must be coordinated so that positional information along all three axes is seamlessly integrated. We have some knowledge of the pathways that link patterning along the cardinal axes, but our understanding is far from complete.

Known components of dorsal-ventral limb patterning include three factors: *wnt-7a* expressed in the dorsal ectoderm, *engrailed-1* expressed in the ventral ectoderm, and *lmx1b* expressed in the dorsal mesenchyme. Previous studies (Cygan *et al.,* 1997; Loomis *et al.,* 1996; Loomis *et al.,* 1998; Parr and McMahon, 1995; Riddle *et al.,* 1995; Yang and Niswander, 1995) have integrated these genes into a pathway in which *engrailed-1* suppresses the expression of *wnt-7a* in the ventral ectoderm and *wnt-7a* induced the expression of *lmx1b* in the dorsal mesenchyme. Although this simple pathway serves as a useful intellectual framework for understanding dorsal-ventral limb pattern, the situation in the embryo is actually much more complex (Chen and Johnson, 1999). *Wnt-7a* cannot be the only factor regulating dorsal expression of lmx1b because in *wnt-7a* mutants, *lmx1b* expression is only lost from the distal anterior limb mesenchyme (Cygan *et al.,* 1997; Loomis *et al.,* 1998). In addition, *wnt-7a* is thought to mediate integration of dorsal-ventral and anterior-posterior patterning through regulation of levels of *sonic hedgehog* expression (Parr and McMahon, 1995; Yang and Niswander, 1995). Likewise, *engrailed-1* affects both dorsal-ventral polarity and AER positioning (Loomis *et al.,* 1996). Similarly, *lmx1b* mutants display a reduction in the unla along with dorsal-to-ventral conversions of the limb mesenchyme (Chen *et al.,* 1998). Hence, while these three genes, *lmx1b, engrailed-1* and *wnt-7a* may have a predominant role in dorsal-ventral patterning, it is clear that they must have additional roles in patterning of the other limb axes.

The availability of *lmx1b, wnt-7a* and *engrailed-1* mutants has allowed us to test whether the effects of these genes on anterior-posterior and dorsal-ventral patterning occurs via parallel or linear pathways. The simplest and most straightforward phenotype that we observe is that *lmx1b/engrailed-1* mutants display a double-ventral phenotype. Since *lmx1b* and *engrailed-1* mutants have opposite effects on dorsal-ventral polarity with *lmx1b* mutants being ventralized and *engrailed-1* mutants being dorsalized, we can conclude that *lmx1b* acts downstream of *engrailed-1* to control dorsal cell fates. This is in accord with the simple model for dorsal-ventral limb patterning outlined above. However, we can make another strong conclusion from this experiment: *lmx1b* is the only relevant target for *engrailed-1* regulation with respect to dorsal-ventral patterning. A secondary conclusion is that *lmx1b* is the only relevant target for *wnt-7a* in the dorsal mesenchyme with respect

to dorsal cell fate specification. This conclusion is supported by our observation that the *wnt-7a/lmx1b* double mutant phenotype resembles each single mutant with respect to dorsal-ventral polarity.

The situation with respect to the functions of *lmx1b, wnt-7a* and *engrailed-1* on other limb axes is more complex. *Wnt-7a* regulates the expression of *sonic hedgehog,* but apparently this effect is not mediated through the action of *lmx1b* because *lmx1b* single mutants do not have a reduction in *sonic hedgehog* expression nor do they display digit loss. However, both *wnt-7a* and *lmx1b* mutants exhibit a variable loss of the ulna. In the case of *wnt-7a* mutants, this was interpreted to be due to a reduction of sonic hedgehog expression. Do *lmx1b* and *wnt-7a* affect ulnar development by distinct parallel pathways? The phenotype of the *lmx1b/wnt7a* double mutants suggests that this is the case. With complete penetrance, the ulna is lost in these double mutants. It is difficult to determine whether this is simply an additive phenotype or a synergistic phenotype, but in either case it suggests that *lmx1b* regulates a novel pathway within the limb mesenchyme that controls posterior limb development. What might that pathway be? The unexpected observation that loss of *lmx1b* enhances the digit phenotype of *wnt-7a* mutants suggests that *lmx1b* may be involved a general aspect of posterior limb bud growth. One possibility is that *lmx1b* acts to modulate the ability of cells to receive growth promoting signals. A potential mechanism for this could be production of extracellular matrix factors that facilitate growth factor signaling. Pertinent to this argument are the observations that in the eye and kidney, *lmx1b* regulates the composition of the extracellular matrix (Morello *et al.,* 2001; Pressman *et al.,* 2000). Perhaps this function of *lmx1b* could be conserved in most or all tissues in which its activity is required.

In contrast to the enhancement of the anterior-posterior defects seen in the *lmx1b/wnt-7a* mutants, *engrailed-1* suppresses the ulnar defect in *lmx1b* mutants. What might be the mechanism leading to this unexpected observation? *Engrailed-1* mutants have an expanded ventral AER leading to a broadened expression of *fgf-8* and presumably other factors that are produced by the AER. Perhaps this enhanced signaling by the AER makes up for defects in specification and/or growth of ulnar precursors in the *lmx1b* mutants. In this regard, it is interesting to note that *lmx1b* expression is restriced to the dorsal mesenchyme of the limb while *engrailed-1* expression is limited to the ventral ectoderm. Hence it is difficult to imagine how *engrailed-1* dependent factors interact with *lmx1b*-dependent factors. Perhaps this information is integrated in at the distal part of the limb where the dorsal mesenchyme and the ventral ectoderm are fairly closely apposed. Indeed, it has been proposed that dorsal-ventral positional information is specified within this distal mesenchyme (Akita, 1996). The determination of the exact mechanism of *engrailed-1* suppression of the *lmx1b* ulnar phenotype will require a detailed knowledge of the pathways regulated by *engrailed-1* and *lmx1b*.

Materials and Methods

Mice were housed in a conventional colony and genotyped by PCR using DNA extracted from tail biopsies according to the methods outlined in Chen *et al.* (1998) for *lmx1b*, Parr and McMahon (1995) for *wnt-7a*, and Loomis *et al.* (1996) for *engrailed-1*. Timed pregnant matings were set between *lmx1b/wnt7a* and *lmx1b/engrailed-1* double heterozygotes and the date of plug set as 0.5 dpc. Newborn mice were sacrificed and skeletal preparations prepared according to Chen *et al.* (1998). For histological studies, newborn forelimbs or hindlimbs were immersed in Bouin's fixative overnight and processed for paraffin sectioning. Seven micron sections were stained with Mallory's trichrome according to Pressman *et al.* (2000). *In situ* hybridization was carried out according to Chen *et al.* (1998).

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