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
ventrally. The sesamoid bones (s) are present only in the plantar region. Muscle (m) is present only on the surface and has a pointed ventral surface that is in close apposition to the phalanges (ph). The phalanges (ph) have a rounded dorsal appearance. The ventral tendons (vt) have a rounded appearance while the dorsal tendons (dt) have a flattened appearance. Finally, at a more proximal level, the dorsal limb is devoid of muscle, while the ventral limb has 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. The morphology of the endochondral elements is duplicated ventral arrangement. The phalanges have a ventral narrowing and a pointed end. The dorsal-ventral polarity at the level of the digits are the shape of the phalanges, the presence of sesamoid bones, 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-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.

**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).

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 exhibit a similar appearance to that of lmx1b mutants. (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.
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 fore-limbs 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 ulna, 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 in-between 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 absence of a patella, a straightening of the fibula and an abnormal 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...
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 induces 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., 1998). 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 the dorsal-ventral axis.
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 in 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 mutant mice have an expanded ventral AER leading to a broadened expression of ifg-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 restricted 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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References


