The mouse polydactylous mutation, luxate (lx), causes anterior shift of the anteroposterior border in the developing hindlimb bud

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ABSTRACT Pattern formation along the anterior-posterior axis of the vertebrate limb is established upon activation of Sonic Hedgehog (SHH) in the zone of polarizing activity (ZPA). Since many mouse mutants with preaxial polydactyly show ectopic expression of Shh at the anterior margin of the limb buds, it has been thought to be a primary defect caused by these mutations. We show here that the mouse mutation luxate (lx) exhibits dose-dependent reduction in the size of the Fgf8 expression domain in the ectoderm from the initial stage of limb development. This aberration was independent of Fgf10 expression in the limb mesenchyme. Shh was induced in the mesenchyme underlying the posterior end of the Fgf8 expression domain, indicating an anterior shift of Shh expression in lx hindlimb buds. Prior to the ectopic induction of Shh, the expression domains of genes downstream from Shh, namely dHAND, Gli1, Ptc and Gre, which are normally expressed in posterior mesenchyme of limb buds, expanded anteriorly on the lx hindlimb buds. Conversely, the expression domains of anterior mesenchymal markers such as Gli3 and Alx4 decreased in size. Thus, ectopic Shh is not a primary defect of the lx mutation. Rather, our results indicate that the lx mutation affects the positioning of the anteroposterior border in developing hindlimb buds.

KEY WORDS: luxate, limb development, Shh, Fgf-8, d-HAND

Introduction

Limb development is one of the classical model systems that have been used for studying vertebrate morphogenesis. Pattern formation of limbs requires coordinate signals along the anteroposterior (A-P) and the proximodistal (P-D) axes. The A-P axis is controlled by the zone of polarizing activity (ZPA), located at the posterior margin of the limb bud. Grafting of the ZPA to the anterior margin of chick limb bud results in a mirror-image duplication of the anterior digits (Saunders and Gasseling, 1968; Tickle et al., 1975; Tickle, 1981). Sonic hedgehog (Shh) is expressed in the region corresponding to the ZPA and is thought to mediate ZPA activity (Riddle et al., 1993).

Pattern formation and continued outgrowth along the P-D axis is controlled by the thickened epithelium located at the distal tip of limb buds, called the apical ectodermal ridge (AER) (Saunders, 1948). Previous studies suggested that several members of the Fgf family expressed in the AER control limb outgrowth (Niswander et al., 1993; Crossley et al., 1996). When limb outgrowth is initiated, Fgf8 is activated in the intermediate mesoderm by unknown signal(s), and Fgf8 induces Fgf10 in the lateral plate mesoderm. The mesenchyme expressing Fgf10 bulges from the trunk and forms limb buds. Fgf10 in the limb mesenchyme then induces the expression of Fgf8 in the distal epithelium and subsequently in the AER (Ohuchi et al., 1997).

Formation of the proper limb axis requires interaction between ZPA and AER signals. Fgf4, Fgf9 and Fgf17 are expressed in the posterior two-thirds of the AER, and their expression is maintained by Shh. In conditional mutants of Fgf4, Fgf9 and Fgf17, expression of Shh and morphology of the resulting limbs are normal (Colvin et al., 1999; Sun et al., 2000). On the other hand, conditional disruption of Fgf8 in the AER results in reduced limb size and a delay of Shh expression (Lewandoski et al., 2000; Moon et al., 2000). These data suggest that a positive feedback loop exists between Shh and the combined activities of two or more Fgfs, and that Fgf8 alone has a distinct role in promoting cell proliferation and inducing Shh expression in normal limb development.

Gremlin (Gre) is a member of the DAN family of BMP antagonists and blocks BMP2, BMP4 and BMP7 signaling (Hsu et al.,...
In developing limb buds, *Gre* maintains AER and *Fgf4* expression through inhibition of BMP signaling, while *Gre* is maintained by *Shh* (Capdevila et al., 1999; Merino et al., 1999; Zúñiga et al., 1999). Thus, *Gre* mediates the feedback loop between *Shh* and *Fgf4*.

Many genes are known to be involved in restricting *Shh* expression to the posterior side of limb buds. *Gli3* and *Alx4* are expressed in the anterior region of the mesenchyme, which is complementary to the *Shh* expression domain. These two genes are thought to repress *Shh* in normal limb development (Masuya et al., 1997; Qu et al., 1997). Disruption of *Gli3* and *Alx4* causes ectopic expression of *Shh* and polydactylous phenotypes in extra-toes-Jackson (*Xt J*) and Strong's luxoid (*lst*) (Hui and Joyner, 1993; Masuya et al., 1997; Qu et al., 1998). *dHAND* is expressed in posterior mesenchyme and activates *Shh* expression. Overexpression of *dHAND* in the whole mouse limb bud causes ectopic induction of *Shh* and activation of *Shh* downstream genes, which consequently results in preaxial polydactyly (Charité et al., 2000).

Mutants with preaxial polydactyly compose a major form of limb abnormalities in mouse. Ectopic expression of *Shh* and *Fgf4* is a general phenomenon in mutants such as *Xt J*, *lst*, Recombinant induced mutant 4 (*Rim4*), Hemimelic extra toe (*Hx*) and X-linked polydactyly (*Xpl*) (Masuya et al., 1995; Masuya et al., 1997). Although these genes likely have a function to repress *Shh* expression in normal limb development, mechanism of the suppression is poorly understood. Moreover, it remains open by what mechanism the expression domains of *Gli3* and *Alx4* are restricted to the anterior side in the limb mesenchyme.

**Luxate** (*lx*) is a spontaneous limb mutant that has been mapped to the proximal region of Chromosome 5 (Lane, 1967). *lx* mutants show preaxial polydactyly, which is restricted to the hindlimbs. Homozygotes show polydactyly, oligodactyly, hemimelia with shortened tibias, and sacralization of the 26th vertebra (Carter, 1951). In addition to the skeletal anomalies, they have various kidney defects such as horseshoe kidney, polycystic kidney and hydronephrosis (Carter, 1953). *lx* mutants have been reported to exhibit ectopic *Shh* expression in the hindlimb buds (Masuya et al., 1997). To elucidate the defect responsible for the phenotype in *lx* mutants and to study the interaction between the *lx* gene and other key genes known to function in limb development, we examined the expression of these key genes in early stage *lx* mutant embryos. In this study, we show that *lx* mutants exhibit anterior-restricted expression domain of *Fgf8* in the ectoderm of the hindlimb bud. The expression domain of *Gre* in the mesenchyme is also shifted to the anterior side in the early stage of limb development. In addition, the expression domains of *Gli1*, *ptc* and *dHAND*, which are restricted to the posterior side of wild-type limb buds, are expanded anteriorly. At later embryonic stages, ectopic *Shh* expression was observed at the anterior margin of the limb bud. Our results indicate that induction of ectopic *Shh* is not the primary defect of the *lx* mutation, but rather that the *lx* mutation affects the positioning of anteroposterior border in developing hindlimb buds. These results suggest that the *lx* gene functions to regulate the initial anteroposterior polarization of hindlimb buds, perhaps through defining the boundary of *Fgf8* expression domain in the surface ectoderm.
Results

Skeletal Phenotype of *lx*

The skeletal phenotype of *lx* mutants has been described in detail (Carter, 1951; Carter, 1953; Carter, 1954). The phenotype, however, is known to vary depending on the genetic background (Masuya et al., 1997). We therefore established a *lx* homozygous line on the genetic background of the C57BL/6J strain by intercrossing of C57BL/6J-*lx*/*+* KitW-v mice (see materials and methods). Since all progeny generated from the line showed hemimelia with shortened or missing tibia on their hindlimbs, the line was confirmed as homozygous for the *lx* mutation. Most of the *lx* homozygotes exhibited tibial hemimelia only on the right hindlimb (Fig. 1 A,B), with only a few mice displaying it bilaterally. They also showed hyperphalangy or anterior duplication of the first digit, either unilaterally or bilaterally in the hindlimbs (Fig. 1B). In addition to the limb defects, transformation of the 26th vertebra (hindmost lumber; L6) into the first sacral form was reproducibly observed. This posterior transformation caused the anterior shift in leg position (Fig. 1 C,D).

Heterozygous *lx* mice never showed the tibial hemimelia or the vertebral defects. They, however, did have an extra digit in the preaxial side of the hindlimbs (data not shown). No forelimb abnormalities were observed in either *lx* homozygotes or heterozygotes.

Shh-Fgf4 Feedback Loop in *lx* Mutant Mice

In many preaxial polydactylous mouse mutants including *lx*, ectopic expression of Shh and Fgf4 is often observed at the anterior margin of developing limb buds (Masuya et al., 1995; Masuya et al., 1997). To clarify the precise patterns of Shh and Fgf4 expression during *lx* limb development, we performed *in situ* hybridization using *lx* homozygous embryos. In wild-type hindlimb buds, Shh expression is first detected at the posterior margin of the mesenchyme at E10.5 (Fig. 2A), and is more strengthened in the developing ZPA at E11.5 (Fig. 2C). It becomes undetectable by E12.5. In *lx* hindlimb buds, Shh expression was first detected at E10.5 (Fig. 2B). Compared with wild-type mice, however, expression was notably more anterior in location. At E11.5, *lx* formed the narrow limb buds. At this stage, Shh was detected in the ZPA, but its expression domain was extended anteriorly (Fig. 2D). In addition, at this stage, a small focus of Shh expression was observed in the anterior margin of the hindlimb bud (arrowheads in Fig. 2D,E). At E12.5, Shh was nearly undetectable in the posterior side of the *lx* hindlimb buds, whereas strong ectopic expression of Shh was detected in the anterior mesenchyme with ectopic outgrowth of the anterior tissue (data not shown). Activation of ectopic Shh in *lx* mutants showed a one-day lag compared with endogenous Shh.

Expression of Fgf4 is induced by Shh, and Fgf4, in turn, maintains Shh expression during normal limb development (Laufer Fig. 2. Expression patterns of Shh, Fgf8, Fgf4 and Gre in wild-type and *lx* homozygous embryos. (A-E) Double-la-beled *in situ* hybridization shows Fgf8 expression in the AER (purple) and Shh expression in the mesenchyme (blue). Arrows mark the posterior margin of the Fgf8 expression domain. In wild-type embryos, Fgf8 expression extends throughout the edge of the limb, and Shh is seen in the posterior margin at E10.5 (A) and at E11.5 (C). In *lx* homozygotes embryos, Fgf8 expression is restricted to the anterior side of the ectoderm, associated with the anteriorized Shh expression at its margin at E10.5 (B) and E11.5 (D). Fgf8 expression extends throughout the edge of the limb in *lx*/*lx*. Ectopic Shh is expressed in the anterior mesenchyme (arrowhead in D). (E) Anterior view of the Shh and Fgf8 expression domain in *lx*/*lx*. Shh is expressed in the mesenchyme underlying the anterior end of the Fgf8 expression domain. (F-I) Expression of Fgf4 at E10.5 (F,G) and E11.5 (H,I). In *lx*/*lx*, Fgf4 is shifted anteriorly (G, I) compared with wild type (F, H). Arrowheads indicate the posterior end of the Fgf4 expression domain. (J-M) Expression of Gre in wild-type (J,L) and *lx*/*lx* (K,M) embryos. At E10.5 (J,K), Gre expression underlies Fgf4 expression in the ectoderm of wild-type embryos. In E11.5 *lx* embryos (M), Gre expression is expanded to the anterior margin of the limb bud (arrowhead in M), overlapping with the ectopic Shh expression. Scale bar, 0.1 mm.
arrows indicate the posterior margin of the Fgf8 expression domain. The extent of Fgf8 expression depends on the dose of the lx mutant gene mesenchyme in E10.5 wild-type (A,D), lx/+ (B,E) and lx/lx (C,F) embryos. Fgf10 expression domains are not affected (D-F). Scale bar, 0.1 mm.

et al., 1994; Niswander et al., 1994). In E10.5 wild-type embryos, Fgf4 expression is initiated in the middle of the apical ectoderm (Fig. 2F). In the hindlimb of E10.5 lx embryos, Fgf4 expression domain was shifted anteriorly in the apical ectoderm (Fig. 2G). In E11.5 wild-type embryos, Fgf4 is detected in the posterior two-thirds of the AER, and the expression domain is directly overlaid by Shh expression domain in the mesenchyme (Fig. 2H). In E11.5 lx embryos, Fgf4 localized in the anterior AER, and was hardly detectable on the posterior side (Fig. 2I).

In normal limb development, Gremlin (Gre) is thought to maintain the positive feedback loop between Shh and Fgf4. In wild-type embryos, Gre is expressed in the posterior half of the mesenchyme in a region underlying Fgf4 expression in the AER (Fig. 2 J,L). In E10.5 lx hindlimb, the expression domains of both Shh and Fgf4 were biased towards the anterior, and Gre was also expressed in the anterior half of the limb mesenchyme in parallel with the more anterior localization of Fgf4 in the AER (Fig. 2 G,K). In E11.5 lx hindlimb, Gre expression was activated in a wide range of the limb mesenchyme, where endogenous and ectopic Shh were expressed (Fig. 2M). This indicates that the signal between ectopic Shh and the AER is mediated through Gre, as is the case in normal development. Furthermore, in lx hindlimbs, Shh, Fgf4 and Gre were shifted towards the anterior side of the limb bud in the initial stage of development.

Spatial Relationship of Shh and Fgf8 Expression Domains

In normal limb development, the ZPA interacts with the AER to form the proper A-P and P-D axes. Fgf8 is expressed throughout the whole AER, and the posterior end of the expression domain overlaid the Shh expression domain in the ZPA (Fig. 2 A,C). In E10.5 lx hindlimb buds, the Fgf8 expression domain was reduced in size and was restricted to the anterior side of the apical ectoderm (Fig. 2B). The posterior end of the Fgf8 expression domain overlaid the Shh expression domain. In E11.5 lx embryos, Fgf8 was detected in the whole AER with the highest expression in the most anterior end (Fig. 2D). Both ends of the Fgf8 expression domain overlaid the Shh expression domain (Fig. 2 D,E). In E11.5 lx hindlimbs, although Fgf4 was not expressed in the posterior side (Fig. 2I), the Fgf8 expression domain was overlapped with Shh in the posterior AER. Thus, Fgf8, but not Fgf4, likely functions to maintain the expression of Shh in lx.

Dose Dependent Effect of the lx Mutant Gene on Fgf8 Expression

To control the initial outgrowth of hindlimbs, Fgf10 in the mesenchyme induces Fgf8 in the apical ectoderm. In the hindlimb buds of lx homozygotes, we detected anterior-restricted expression domain of Fgf8. To address the possibility of a dose-dependent effect of the lx mutant gene, expression of Fgf8 was compared in lx heterozygotes, lx homozygotes, and wild-type embryos. The embryos studied were littermates derived from intercrossing lx heterozygotes. In wild-type hindlimbs, Fgf8 expression in the apical ectoderm extended approximately 3 somites in width (Fig. 3A). In lx heterozygotes and homozygotes, Fgf8 expression extended about 2.5 (Fig. 3B) and 2 somites in width (Fig. 3C), respectively. These data indicate that the Fgf8 expression domain in the apical ectoderm of the hindlimb depends on the expression of the lx mutant gene in a dose-dependent manner. On the other hand, Fgf10 was expressed similarly in whole limb mesoderm in the embryos regardless of lx genotype (Fig. 3 D,E,F).

Excess Cell Death in lx Hindlimb Buds

Although lx mutants had normal hindlimb bud size at E10.5, at later stages, the hindlimb bud narrowed. In E10.5 lx hindlimbs, Fgf8 was not expressed in the posterior side of the apical ectoderm raising the possibility that lx hindlimbs are defective in AER formation. Using E10.75 embryos, we performed Nile blue staining to detect cell death in the hindlimb buds. In wild type, no prominent cell death was observed in the AER (Fig. 4A). By contrast, lx mutants showed evidence of excess cell death in the posterior side of the apical ectoderm (Fig. 4B). The regression of the AER on the posterior side may cause hypoplasia of the hindlimb buds in lx.

A-P Axis Formation in lx Hindlimb Mesenchyme

Since the A-P axis was altered in lx hindlimb ectoderm, we examined axial formation in early stage limb mesenchyme. In E10.5 wild-type embryos, Gli1 and the SHH receptor Ptc are expressed in the posterior side of the limb mesenchyme (Fig. 5A). In E11.5 lx hindlimb buds, Gli1 and Ptc were expressed in the posterior side of the limb mesenchyme (Fig. 5B). However, in E10.75 lx homozygotes, Gli1 and Ptc were not expressed in the posterior side of the limb mesenchyme (Fig. 5C). This indicates that the A-P axis is altered in lx hindlimbs.
A,C), and their expression domains overlaps with Fgf4 expression. In E10.5 lx hindlimb buds, Ptc and Gli1 expression was expanded to the whole mesenchyme (Fig. 5 B,D). In wild-type embryos, Gli3 and Alx4 are expressed at the anterior side of the limb mesenchyme (Fig. 5 E,G). In E10.5 lx hindlimb buds, the Gli3 and Alx4 expression domains in the anterior mesenchyme were slightly narrower than in wild type (Fig. 5 F,H). Our data indicate that the extent of the anterior mesenchyme was reduced in lx hindlimb buds from an early stage of the development. To confirm a reduction of the anterior mesenchyme in lx, we examined the expression of dHAND, which acts upstream of Shh and is expressed in the posterior mesenchyme of limb buds (Charité et al., 2000). At E10.5, the dHAND expression domain was expanded in lx hindlimb buds compared with wild type (Fig. 6 A,B).

These results suggest that lx limb mesenchyme is composed from the narrowed anterior region and the expanded posterior region from the beginning of limb development. To elucidate the effects of Alx4 and Gli3 on dHAND expression, we examined the expression of dHAND in lst and Xtr, which are loss-of-function mutants of Alx4 and Gli3, respectively (Hui and Joyner, 1993; Qu et al., 1998). In E10.5 lst limb buds, dHAND expression in the mesenchyme was normal (Fig. 6C). In Xtr embryos, however, dHAND expression was expanded to the anterior region of the limb mesenchyme (Fig. 6D).

**Discussion**

**Primary Defects of the lx Mutation**

In E10.5 lx homozygote embryos, we observed anterior-restricted expression domain of Fgf8 in the ectoderm of the hindlimb, as well as anteriorised expression domain of Shh (Fig. 2B). These were the earliest abnormalities recognized in lx homozygous embryos. Altered expression patterns of Fgf8 and Shh, both of which are known to play key roles in outgrowth and patterning of limb buds, likely causes hemimelia and preaxial polydactyly of lx mutants displayed at later stages. We also observed that lx homozygotes reproducibly exhibited posterior transformation of the 6th lumber vertebrae into a sacral form (Fig. 1). Previous reports have shown that the lx mutation alters hindlimb positioning, leading to an anterior shift equivalent to the distance of 0.5-1 somite (Carter, 1954). Thus, the lx mutation appears to cause anterior positioning of the initial hindlimb field. A similar anterior shift of hindlimb position was observed in mouse mutants with disrupted expression of Hoxd-11 and Hoxd-10 in the trunk mesoderm (Gerard et al., 1996), and in gdf11 knockout mice (McPherron et al., 1999). Notably, these other mutants did not show digital abnormality along the A-P axis, indicating that an anterior shift of limb position is not always associated with abnormalities in A-P axis formation within limb buds. Thus, the lx mutation is associated with two distinct defects in limb development. Firstly, the expression domains of Fgf8 and Shh are altered in lx mutants. Secondly, the lx mutation affects positioning of the initial hindlimb field along the body axis.

In normal limb development, Fgf10 expression in the lateral plate mesoderm induces Fgf8 expression in the apical ectoderm of limb buds (Ohuchi et al., 1997). Fgf8 is the only gene among Fgf family members which is expressed before the induction of Shh (Crossley et al., 1996). Shh knockouts show no alteration of Fgf8 expression pattern (Sun et al., 2000). Conversely, a conditional mutant lacking Fgf8 in the AER shows delayed Shh expression (Lewandoski et al., 2000). These reports suggest that Fgf8 normally acts to induce Shh expression. Consideration of past results and the findings in this study raise the possibility that the defect underlying the phenotype in the lx mutant is due to altered regulation of Fgf8 expression in the apical ectoderm of the limb bud. Since we observed no alteration in Fgf10 expression in the mesoderm of the lx hindlimb buds, the primary defect caused by the lx mutation is likely to be in the regulation of Fgf8 expression, possibly in receiving the Fgf10 signal. In normal development, Fgf8 in the intermediate mesoderm determines presumptive limb territory, and induces Fgf10 in the lateral plate mesoderm. Thus, the anterior shift of the initial limb field in lx mutants might be due to a defect in Fgf8 expression in the intermediate mesoderm in the earlier developmental stage.

**Fig. 5.** Expression patterns of Gli1 (A,B), Ptc (C,D), Alx4 (E,F) and Gli3 (G,H) in E10.5 wild-type (A,C,E,G) and lx/lx (B,D,F,H) hindlimb buds. Arrows indicate the anterior limits of Gli1 and Ptc or the posterior limits of Alx4 and Gli3 in the limb mesenchyme. In lx / lx, Gli1 (B) and Ptc (D) expression domains are extended to the anterior side, while the expression domains of Alx4 (F) and Gli3 (H) reduced in size. Scale bar, 0.1 mm.
Induction of Shh and Margins of the Fgf8 Expression Domain

By simultaneous detection of the expression of Shh and Fgf8 in this study, we observed that Shh was induced in the mesenchyme underlying the posterior end of the Fgf8 expression domain in wild-type limb buds (Fig. 2A). A similar spatial relationship between the posterior end of Fgf8 expression domain and the position of Shh induction was also observed in lx mutants, despite the more anterior localization of both molecules was observed (Fig. 2B). Likewise, we also observed that in E11.5 lx hindlimb buds, ectopic Shh expression was induced in the mesenchyme under the anterior end of the Fgf8 expression domain (Fig. 2D). Thus, both margins of Fgf8 expression domain appear to have the potential to induce Shh expression, although additional factors may also be involved.

Control of Limb Bud Size by Fgf8

In this study, we found that the size of the hindlimb bud is normal in lx homozygous and heterozygous embryos when they first emerge from the trunk, suggesting that the initial hindlimb territory of lx mutants contains the same mass of mesenchyme as wild-type mice. In fact, Fgf10 is expressed throughout the whole limb mesenchyme in lx limb buds. In later stages, lx mutants developed narrowed hindlimb buds due to excess cell death, which is probably caused by the absence of Fgf8 expression in the posterior apical ectoderm (Fig. 4). Therefore, while the expression of Fgf8 in surface ectoderm is not essential for the initiation of hindlimb outgrowth and determination of hindlimb size, it is required to promote cell proliferation. This is also supported by the previous studies of mice lacking Fgf8 expression in limb ectoderm. These mutants develop normally sized limbs in the early stage, which then become narrow in later stages (Lewandoski et al., 2000).

Function of dHAND and Gli3 in A-P Border Formation in Limb Mesenchyme

The A-P axis in lx hindlimb bud was shifted to the anterior side in both the ectoderm and the mesenchyme prior to ectopic Shh expression. Transgenic mice overexpressing dHAND throughout the hindlimb bud show ectopic induction of Shh (Charité et al., 2000), suggesting that dHAND functions to induce Shh in normal limb development. On the other hand, Gli3 and Alx4 are negative regulators of Shh (Masuya et al., 1995; Qu et al., 1997; Qu et al., 1998). Ectopic expression of dHAND results in repression of Gli3 expression in chick limbs (Fernandez-Teran et al., 2000). In this study, we found expansion of dHAND expression domain in lx mutants, accompanied with a reduction of the size of Gli3 and the Alx4 expression domains (Fig. 6B). In addition, the Xl' mutant displayed dHAND expression throughout the limb bud in the absence of Gli3 (Fig. 6D). Gli3 and Alx4 have been reported to act in parallel pathways in limb formation (Takahashi et al., 1998). Therefore, in normal limb development, Gli3 and dHAND likely down-regulate one another reciprocally, which may contribute to determination of the A-P border in limb mesenchyme and to the formation of the proper A-P axis.

Fig. 6. Expression of dHAND in hindlimb buds of E10.5 wild type (A), lx homozygote (B), lst homozygote (C) and Xt homozygote (D). Arrowheads indicate the anterior margin of the dHAND expression domain in the limb bud. The dHAND expression domain is expanded in lx / lx and Xt / Xt, but unaffected in lst / lst. Scale bar, 0.1 mm.

Fig. 7. Summary of expression patterns of several key genes in limb development of lx embryos. (Left) In the wild-type limb mesenchyme, Alx4 and Gli3 are expressed in the anterior side. Shh expressed in the ZPA activates downstream genes such as Ptc, Gli1 and Gre. Gre mediates the signal between the ZPA and the AER. The expression domains of dHAND and Gli3 are controlled by reciprocal downregulation. (Right) lx homozygotes have normal hindlimb bud size during early stages of development, but their position is shifted to the anterior side along the body axis. The A-P axis of the limb bud is altered both in the mesenchyme and the ectoderm. Shh is induced in the mesenchyme underlying the anteriorly shifted end of the Fgf8 expression domain (blue arrow). dHAND expression is expanded throughout the hindlimb bud. The lx gene likely plays at least two distinct roles in positioning of the hindlimb bud along the body axis and regulation of Fgf8 expression in the surface ectoderm (indicated by gray arrows).
In addition to a role as an upstream activator of Shh, ectopic expression of dHAND induces expression of Shh downstream genes, such as Gli1 and Ptc, without activation of Shh in the chick limbs (Fernandez-Teran et al., 2000). In lx mutants, Gli1 and Ptc were activated throughout the limb mesenchyme prior to the ectopic activation of Shh, and their expression domains were overlapped with the dHAND expression domain (Fig. 5). Thus, our data suggest that dHAND has the potential to activate the Shh downstream genes Gli1 and Ptc, independent of Shh. On the other hand, Gre expression was reported to be normal in the absence of dHAND (Charité et al., 2000). The present study indicates that Gre is not expressed in the posterior side of lx hindlimb bud (Fig. 2L). Gre expression, therefore, is not influenced by dHAND. All available data suggest the presence of at least two signaling pathways for the induction of Shh downstream genes, dHAND-dependent and independent ones.

**Polydactyly in lx Homozygotes**

In preaxial polydactyly mouse mutants, ectopic expression of Shh is often observed in the anterior margin of their limb buds. The mutated genes have been proposed to repress Shh expression in the anterior mesenchyme of limb buds (Masuya et al., 1997). In the case of the lx mutation, the earliest ectopic expression of Shh was detected at E11.5. At this stage the reduced limb size, phenotype characteristic of lx, had already been apparent. Thus, ectopic Shh is unlikely to be the primary defect. Within the lx hindlimb bud, the A-P border was shifted to the anterior side from the initial stage of limb development, as summarized in Fig. 7. As a result, genes that determine the posterior identity of the limb had expanded in the lx hindlimb bud. Among them, dHAND induces Shh in normal limb development, and its overexpression causes ectopic Shh expression, leading to preaxial polydactyly and tibial hemimelia (Charité et al., 2000). Thus, expanded expression of dHAND in the anterior mesenchyme might induce ectopic Shh expression, leading to the altered skeletal patterning of lx hindlimbs.

In the mouse polydactyly mutant Dominant hemimelia (Dh), Fgf8 domain is shifted anteriorly in the apical ectoderm and activation of ectopic Shh occurs at E12.5 (Lettice et al., 1999). The gene expression patterns shown in Dh heterozygotes resemble those of lx homozygotes. Dh heterozygotes show tibial hemimelia and reduction of the lumbar vertebrae (Searle, 1964), which is similar to the skeletal phenotype of lx homozygotes. Furthermore, Dh hindlimb buds are narrower than those of wild-type mice and the limb position is shifted to the anterior by 2-3 somites. Thus, Dh and lx may be located in the same signaling pathway to establish the A-P axis of hindlimb. In this context, we await the examination of whether Dh shows anterior expansion of dHAND expression.

**Materials and Methods**

**Ix mice**

C57BL/6J-1x/C heterozygous mice were purchased from The Jackson Laboratory (Bar Harbor, Me., USA), and maintained by backcrossing to C57BL/6J (B6) in the Genetic Strain Resource Center, National Institute of Genetics (NIG) (Mishima, Japan). To generate a homozygous lx line, we obtained progeny from the intercross of B6-1x/C-KitW-v mice and selected for progeny showing hemimelia. After 10 generations of repeating sib-mating, the homozygous line was established. All progeny generated from the line show hemimelia. For whole-mount in situ hybridization, embryos were obtained from intercrossing of the homozygotes.

**Consomic Strain and Generation of lx Heterozygotes**

A consomic strain, B6.MSM-Chr5, was established at NIG. In this strain, Chromosome 5 derived from the MSM strain (Japanese wild-mouse origin) was introgressed into the genetic background of B6. The chromosome was maintained in the heterozygous state by backcrossing to B6. Mice heterozygous for the lx mutation were obtained from crosses of the lx homozygous line and B6.Chr5-MSM. In the resultant progeny, the genotype of the region flanking the lx locus was determined based on polymorphisms of microsatellite markers. For in situ hybridization, embryos were obtained from intercrosses of the heterozygotes. The genotype of the lx-linked region in the progeny was determined using genomic DNA, which was PCR-amplified with the primers, D5Mit146, D5Mit15, D5Mit19, and D5Mit287. The genomic DNA was isolated from adult ears or fetal yolk sacs using a protocol modified from Laird et al. (1991).

**lst Mice**

B6C3-a/a-lx/+ mice were purchased from The Jackson Laboratory. For whole-mount in situ hybridization, embryos were obtained by intercrossing heterozygotes. Genomic DNA was isolated from fetal yolk sacs. Embryos were genotyped by PCR-amplification with primers flanking the mutated region of Alx4 gene (primers: 5'-GCTTGGAAAGTTCTCCAGAG-3' and 5'-AGTGGGTAAATGCTC-3') (Qu et al., 1998).

**Skeletal Preparations**

Double staining of the skeleton with alcian blue and alizarin red was performed essentially as described elsewhere (Wallin et al., 1994).

**Whole-Mount In Situ Hybridization**

Single-labeled whole-mount in situ hybridization using digoxigenin-UTP (Roche) labeled RNA probes was carried out as described by Wilkinson et al. (1992). For signal detection, a color reaction using NBT and/or BCIP (Roche) was performed. Simultaneous detection of two transcripts using double-color in situ hybridization was carried out as described by Hecksher-Sørensen et al. (1998). Digoxigenin-UTP and fluorescein-UTP (Roche) labeled RNA probes were hybridized at the same time and detected using BCIP and NBT. The dHAND probe was generated from the entire murine dHAND coding region (Srivastra et al., 1995). The Alx4 probe is a 648bp fragment including the paired tail domain (nucleotides 1077-1724). Photographs were taken using Keyence (Osaka, Japan) VH-8000 system.

**Nile Blue Sulphate Staining of Dead Cells**

Dead cells were stained with Nile blue sulphate (MERCK) as described by A. S. W. Shum et al. (1999). Embryos were explanted into PBS, bathed in Nile blue sulphate dissolved in lacted Ringer’s solution (1 / 50,000) for 15 min at 37°C, and then washed in Ringer’s solution.

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