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Limb deformity proteins: role in mesodermal induction of the apical ectodermal ridge

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SUMMARY

During early limb development, distal tip ectoderm is induced by the underlying mesenchyme to form the apical ectodermal ridge. Subsequent limb growth and patterning depend on reciprocal signaling between the mesenchyme and ridge. Mice that are homozygous for mutations at the *limb deformity* (*ld*) locus do not form a proper ridge and the anteroposterior axis of the limb is shortened. Skeletal analyses reveal shortened limbs that involve loss and fusion of distal bones and digits, defects in both anteroposterior and proximodistal patterning.

Using molecular markers and mouse-chick chimeras we examined the ridge-mesenchymal interactions to determine the origin of the *ld* patterning defects. In the *ld* ridge, fibroblast growth factor 8 (*Fgf8*) RNA is decreased and *Fgf4* RNA is not detected. In the *ld* mesenchyme, Sonic hedgehog (*Shh*), *Evx1* and *Wnt5a* expression is decreased. In chimeras between *ld* ectoderm and wild-type mesenchyme, a ridge of normal morphology and function is restored, *Fgf8* and *Shh* are expressed normally, *Fgf4* is induced and a normal skeletal pattern arises.

These results suggest that the *ld* mesenchyme is unable to induce the formation of a completely functional ridge. This primary defect causes a disruption of ridge function and subsequently leads to the patterning defects observed in *ld* limbs. We propose a model in which ridge induction requires at least two phases: an early competence phase, which includes induction of *Fgf8* expression, and a later differentiation phase in which *Fgf4* is induced and a morphological ridge is formed. Ld proteins appear to act during the differentiation phase.

Key words: limb deformity, apical ectodermal ridge, fibroblast growth factor, Sonic hedgehog

INTRODUCTION

Epithelial-mesenchymal interactions direct many aspects of vertebrate limb development. During the early stages of limb development, the mesenchyme induces the overlying distal tip ectoderm to thicken and form a morphologically distinct apical ectodermal ridge. Subsequent interactions between the ridge and the underlying mesenchyme establish proliferation, patterning and outgrowth of the developing limb. The ridge is required for proliferation of the underlying mesenchyme along the proximodistal axis of the limb and the maintenance of the anteroposterior patterning center in the posterior mesenchyme, the zone of polarizing activity (ZPA). Reciprocally, the mesenchyme is required for the maintenance of the ridge (reviewed in Hinchliffe and Johnson, 1980; Tabin, 1991; Tickle and Eichele, 1994). Recent studies focusing on the molecular signals involved in communication between limb ectoderm and mesenchyme indicate that several secreted molecules are important in directing ridge-mesenchyme interactions, including members of the fibroblast growth factor family (FGFs) and sonic hedgehog (SHH). *Fgf4*, *Fgf8* and *Fgf2* are expressed in the ridge and can substitute for the ridge in promoting mesenchymal outgrowth and proximodistal patterning (Niswander et al., 1993; Fallon et al., 1994; Crossley et al., 1996; Vogel et al., 1996 and references therein). FGFs may also direct proliferation of the lateral plate mesoderm in the prospective limb field to initiate early limb development (Cohn et al., 1995; Ohuchi et al., 1995; Crossley et al., 1996; Vogel et al., 1996). SHH, another secreted molecule, localises to the posterior mesenchyme and its ability to polarize the limb implies a role in anteroposterior patterning (Riddle et al., 1993). Expression of *Shh* is dependent on FGF signaling from the ridge and, in turn, *Fgf4* expression appears to be regulated by SHH (Laufer et al., 1994; Niswander et al., 1994). The existence of a positive feedback between *Fgf4* and *Shh* suggests that FGF4 may play a primary role in the maintenance of *Shh*, whereby it indirectly influences anteroposterior patterning. The transcription factor *Evx1* (a murine homologue of *Drosophila even-skipped*) is expressed in the posterior mesenchyme juxtaposed to the *Fgf4* expression domain, and *Evx1* expression is also regulated by FGF from the ridge (Niswander and Martin, 1993b).

Five recessive mouse *ld* alleles have been described that have similar effects on anteroposterior and proximodistal patterning of both the fore- and hindlimbs (Kleinebrecht et al., 1982; Woychik et al., 1985). In addition to limb defects, the

different *ld* alleles exhibit renal defects, which differ in severity depending on the allele. The *Ld* locus encodes a group of novel proteins called formins that are predominantly localized to the nucleus (Woychik et al., 1990b). Alternative splicing and differential polyadenylation of the *Ld* gene produces several mRNA transcripts that are expressed in both the embryo and the adult. In the developing limb, multiple *Ld* transcripts are expressed. Of the four major isoforms (I–IV) described, isoform IV is expressed in the ridge and other minor isoforms are expressed in limb mesenchyme (Jackson-Grusby et al., 1992; Trumpp et al., 1992; Chan et al., 1995). By immunohistochemistry, Ld proteins can be detected in the ridge and posterior mesenchyme during the early stages of limb development and later in the ventral mesenchyme and developing cartilage (Trumpp et al., 1992).

Alterations in limb development first become apparent in *ld* embryos at E10.5, when the ridge normally forms in wild-type mouse embryos (Wanek et al., 1989). *ld* limbs have a shortened anteroposterior axis and a ridge that is correspondingly shortened; the ridge is also dorsoventrally broadened, discontinuous, and less distinct relative to a wild-type ridge (Zeller et al., 1989). The disrupted morphology of the *ld* ridge suggests that there may be a defect in epithelial-mesenchymal communication during its formation. It is unclear whether the defect resides in the mesenchyme such that it is unable to induce ridge differentiation, or in the ectoderm such that it is unable to form a functional ridge in response to a mesenchymal signal. Alternatively, the defect in *ld* may reside in both the mesenchyme and the ectoderm.

Here we demonstrate that, in *ld* limbs, *Fgf4* mRNA is not detectable and *Shh* and *Evx1* expression is greatly reduced, reflecting a disruption in ridge-mesenchyme signaling. *Shh* and *Evx1* expression can be rescued by application of exogenous FGF4 protein, indicating that the *ld* mesenchyme is competent to respond to ridge signals. By generating chimeras between *ld* ectoderm and early wild-type mesenchyme that is competent to induce a ridge, we show that a morphological and functional ridge is induced which can fully support outgrowth and patterning of the mesenchyme. This demonstrates that mutant ectoderm is capable of responding normally to mesenchymal signals necessary for ridge differentiation, and subsequently can appropriately communicate with the mesenchyme to mediate limb patterning. These data indicate that the primary defect resides in the inability of *ld* mesenchyme to induce a fully differentiated apical ridge.

MATERIALS AND METHODS

Embryos at various stages of gestation were obtained from matings of homozygous *limb deformity* mice (translocation-inversion *ld* allele, ld^{In2}; Woychik et al., 1990a) or wild-type (CD1 or B6D2F1) mice. Noon of the day on which the copulation plug was detected was considered E0.5.

Whole-mount RNA in situ hybridizations were performed essentially as described (Wilkinson, 1993; Henrique et al., 1995). Antisense probes were synthesized as described (Gavin et al., 1990; Hébert et al., 1990; Dush and Martin, 1992; Echelard et al., 1993; Crossley and Martin, 1995). Serum-free limb bud cultures were performed on embryos from E10.25-10.5 and cultured for 24 hours in the presence or absence of FGF4 protein (100 ng/ml) as described (Niswander and Martin, 1993a).

Mouse-chick (or -quail) chimeras were constructed as follows: limbs from Hamburger and Hamilton stage 18-21 (Hamburger and Hamilton, 1951) chick (SPAFAS) or quail (Truslow) embryos and E10-10.5 *ld* or wild-type mice were treated with 2% trypsin in PBS. After neutralization in M199 media (Gibco) + 15% fetal calf serum, the ectoderm was separated from the mesenchyme and the chick/quail ectoderm and mouse mesenchyme were discarded. The mouse ectoderm was then placed over the chick/quail limb bud mesenchyme and the chimeric limb bud allowed to heal for >1 hour in culture before it was grafted to a host stage 21-22 chick limb. Embryos were fixed after 7 days in 5% TCA for Alcian blue skeletal preparations, or after 24 hours according to Martin (1990) for scanning electron microscopy. The lack of feather papillae confirmed the presence of mouse ectoderm.

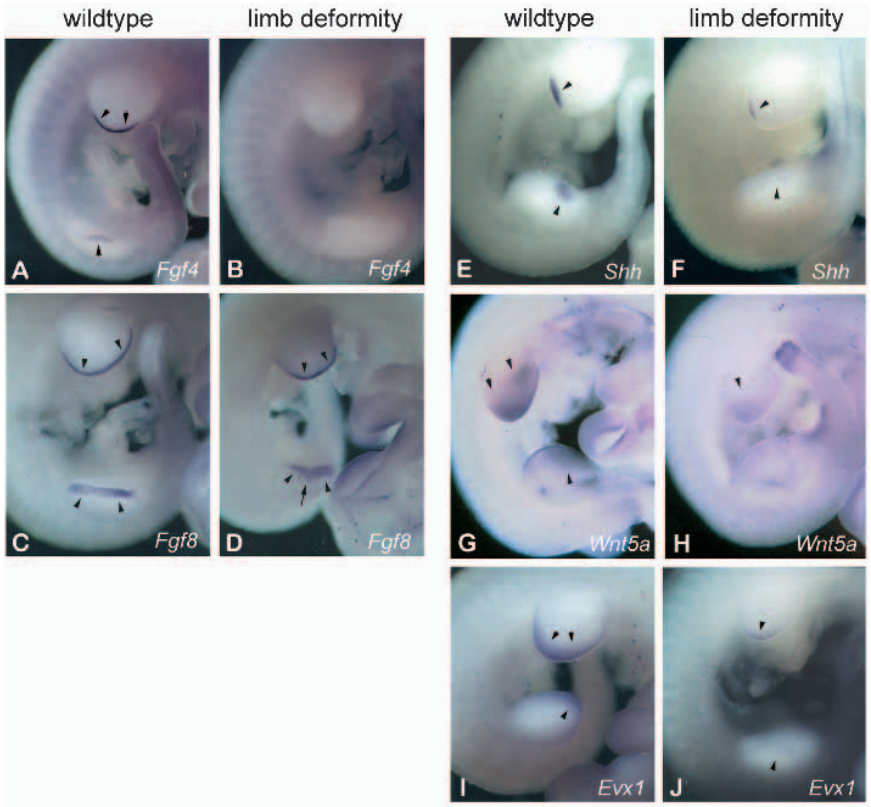

Fig. 1. Gene expression (indicated by arrowheads) in wild-type and *ld* embryos. Whole-mount RNA in situ hybridizations were carried out on E10.5 wild-type (A,C,E,G,I) and *ld* (B,D,F,H,J) embryos. (A,B) Ridge expression of *Fgf4* is detected in wild-type but not *ld* embryos. (C,D) *Fgf8* expression in *ld* ridge shows a slightly decreased and patchy expression compared to wild type (arrow points to a broadening of the *ld* ridge). *Shh* (E,F), *Wnt5a* (G,H) and *Evx1* (I,J) expression is significantly lower in *ld* mesenchyme compared to wild type. Expression patterns similar to those described above were observed at E11.5 (not shown). At E9.5, *Fgf8* RNA levels and distribution in *ld* limbs is comparable to that in wild type (not shown).

RESULTS

Rescue of mesenchymal gene expression in *ld* limbs

Expression patterns of *Fgf4*, *Fgf8*, *Shh*, *Evx1* and *Wnt5a* in wild-type and *ld* embryos (E9.5-11.5) were characterized. *Fgf4* expression was undetectable in the *ld* ridge, as determined by both non-radioactive whole-mount and radioactive section RNA in situ hybridization (compare Fig. 1A and B; Chan et al., 1995; Haramis et al., 1995; and data not shown). At E9.5 prior to ridge formation, *Fgf8* was expressed similarly in wild-type and *ld* ectoderm in a stripe that prefigures the ridge (not shown). In both wild-type and *ld* limbs, *Fgf8* expression continued after ridge formation occurred at E10.5 and was detected in the ridge until at least E12.5. However, in *ld* limbs, *Fgf8* was expressed at slightly reduced levels and often displayed a disorganized and patchy appearance. This decrease may be due to reduced RNA levels or a consequence of the less distinct, discontinuous ridge (Fig. 1D; arrow points to a broadening of the *ld* ridge). *Shh* and *Evx1* are expressed at much lower levels in *ld* limb mesenchyme compared to wild type (Fig. 1E,F,I,J; also reported by Chan et al., 1995; Haramis et al., 1995). In wild-type limbs, *Wnt5a* is expressed in a gradient within the mesenchyme: highest in distal mesenchyme and lower in proximal mesenchyme (Gavin et al., 1990). In *ld* limbs, *Wnt5a* RNA was detected at reduced levels in the mesenchyme, although the remaining mesenchymal expression was still graded (Fig. 1G,H). Non-limb tissues unaffected by the *ld* mutation display normal expression of these genes (not shown).

The absence of *Fgf4* in the *ld* ridge and the known dependence of *Shh* and *Evx1* on FGF signaling prompted us to examine the response of *ld* mesenchyme to an exogenous source of FGF4. A serum-free limb bud culture system was used in which the ridge was removed from one limb (–ridge) of a E10.5 trunk (wild type and *ld*; the opposing limb serves as a control in the same embryo) and the trunks cultured for 24 hours in the presence or absence of FGF4. In the presence of FGF4, *ld* (–ridge) limbs and wild-type (–ridge) limbs each showed similar levels of *Shh* and *Evx1* expression (arrow in Fig. 2A,B and data not shown), whereas in *ld* (+ridge) limbs endogenous *Shh* expression is very low (arrowhead in Fig. 2B). The up-regulation of *Shh* and *Evx1* expression in *ld* limb buds to wild-type levels in the presence of exogenous FGF4 indicates that the mutant *ld* mesenchyme is competent to respond to ridge signals. This confirms the previous results of Haramis et al. (1995), in which *Shh* expression could be rescued when posterior mesodermal tissue was grafted under a wild-type ridge. In the absence of exogenous FGF4, *Shh* and *Evx1* expression was lost in both *ld* and wild-type (–ridge) limbs (Fig. 2C,D). Together with the in situ data, these results indicate that abnormalities in ridge signaling lead to subsequent mesenchymal defects in the *ld* limb that include misregulation of *Shh* and *Evx1* expression.

Wild-type mesenchyme induces *ld* ectoderm to form a functional ridge

In wild-type mouse limbs, limb bud proliferation begins at E9.5, but a ridge does not form until E10.5. From experimental studies in chick, early mesenchyme is capable of inducing a ridge (through stage 19), but then rapidly loses this ability

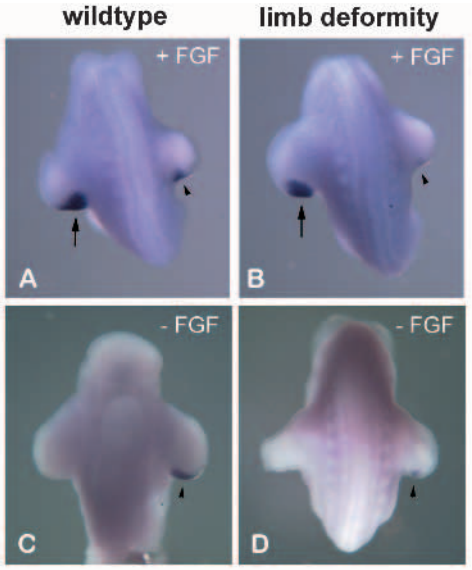

Fig. 2. FGF4 protein rescues *ld* mesenchyme gene expression. The ridge was removed from one limb (–ridge; left limb in each panel) of E10.5 wild-type (A,C) or *ld* (B,D) embryos and the trunks with attached limbs were cultured for 24 hours in the presence or absence of FGF4 protein (100 ng/ml), then processed for whole-mount RNA in situ hybridization. (A,B) In (–ridge) limbs cultured in the presence of exogenous FGF4, *Shh* (arrows) and *Evx1* (data not shown) expression in *ld* mesenchyme (B) is comparable to levels in wild type (A). Proliferation of *ld* mesenchyme was similar to that of wild type (Niswander and Martin, 1993a). (C,D) In (–ridge) limbs cultured in the absence of FGF4, *Shh* and *Evx1* (data not shown) expression is lost in both wild type and *ld*. In contralateral (+ridge; right limb in each panel) *ld* and wild-type limbs, *Shh* and *Evx1* RNA levels were unchanged in the presence or absence of exogenous FGF (arrowheads). As in uncultured limbs (Fig. 1E,F), endogenous *Shh* expression is reduced in *ld* relative to wild type (compare B and D to A and C).

(reviewed in Hinchliffe and Johnson, 1980; limited culturing techniques preclude similar studies in mouse). To test whether the defect in *ld* ridge function is due to the inability of *ld* mesenchyme to induce a functional ridge in the ectoderm or the inability of the *ld* ectoderm to respond to signals from the mesenchyme to form a functional ridge, we recombined early *ld* ectoderm with wild-type chick or quail mesenchyme (shown schematically in Fig. 3A; Jorquera and Pugin, 1971). In such a chimeric limb, a correctly patterned avian skeleton would indicate the presence of a functional ridge. Normal skeletal patterning was observed in control chimeric limbs composed of a wild-type mouse ectoderm and ridge (E10-10.5) combined with wild-type chick mesenchyme from early (stage 18-19: mesenchyme capable of inducing a ridge; Fig. 3B) or late (stage 21; Fig. 3D) limbs. Chimeras between *ld* ectoderm (E10-10.5) and early wild-type mesenchyme (stage 18-19) exhibit complete distal outgrowth and correct digit patterning, similar to chimeras possessing a wild-type ectoderm and ridge (compare Fig. 3C with B; Table 1). In contrast, chimeras made of later stage chick mesenchyme, which is no longer capable of inducing a ridge (stage 21), and *ld* ectoderm (E10-10.5) result in disrupted skeletal patterning (Fig. 3E; Table 1). From these results we conclude that a *ld* ectoderm can be functionally rescued by an early wild-type mesenchyme that is

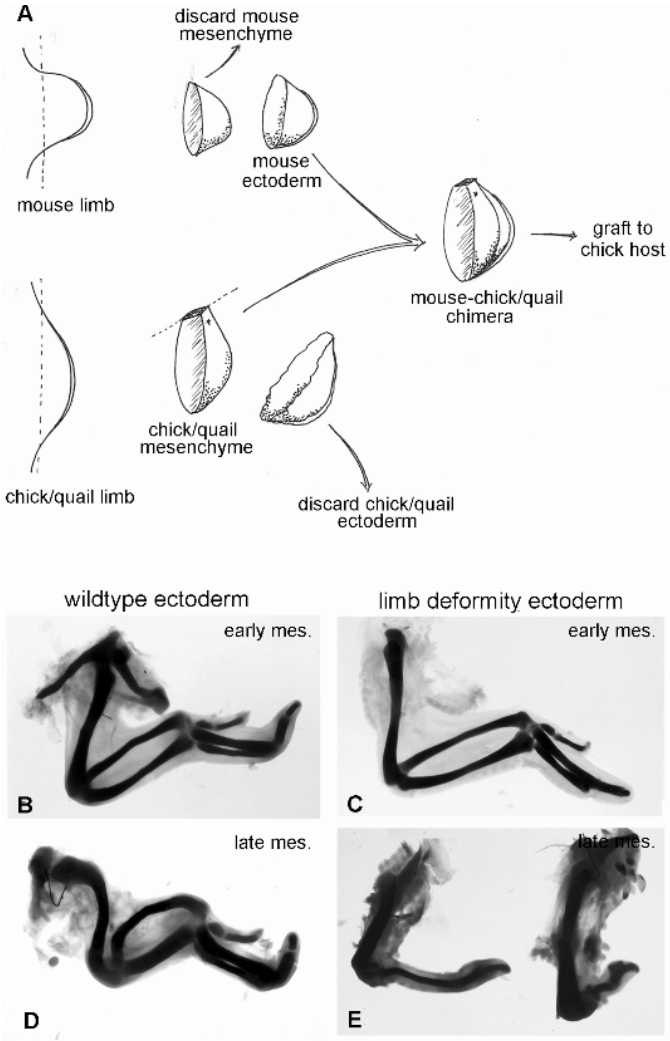

Fig. 3. Wild-type early mesenchyme rescues *ld* ridge function. (A) Schematic diagram of tissue recombinations to produce a chimeric limb bud consisting of mouse ectoderm and chick or quail mesenchyme (mes.). Skeleton of chimeras between (B) wild-type mouse ectoderm and early (stage 18-19 mesenchyme capable of ridge induction) wild-type chick mesenchyme; (C) *ld* mouse ectoderm and early (stage 18-19) wild-type chick mesenchyme; (D) wild-type mouse ectoderm and late (stage 21 mesenchyme no longer capable of ridge induction) wild-type chick mesenchyme. (E) Two examples of skeletons of chimeras between *ld* mouse ectoderm and late (stage 21) wild-type chick mesenchyme. Ectoderm for all chimeras was derived from E10-10.5 mouse limb buds. The skeleton is correctly patterned and distally complete in chimeras between *ld* ectoderm and early wild-type mesenchyme that is competent to induce a ridge (C), similar to chimeras possessing a wild-type ectoderm and ridge (B,D). In contrast, skeletal patterning is severely disrupted in chimeras between *ld* ectoderm and late mesenchyme incapable of ridge induction (E).

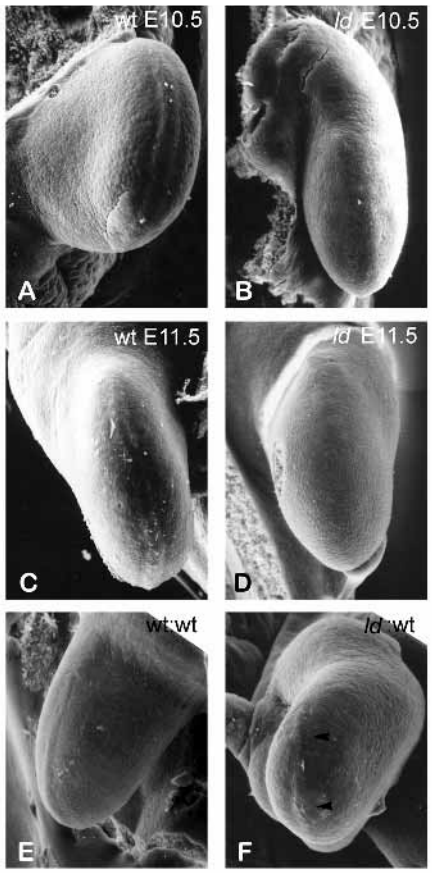

Fig. 4. Wild-type early mesenchyme rescues *ld* ridge morphology. Scanning electron micrographs of wild-type and *ld* mouse embryos and chimeras to demonstrate ridge morphology. Wild-type E10.5 (A) and E11.5 (C) limbs show a distinctive, thickened ridge whereas *ld* E10.5 (B) limbs display a discontinuous, broad and flattened ridge and, by E11.5, the *ld* ridge is barely detectable (D). Chimeras of wild-type ectoderm:early wild-type mesenchyme (E) display a distinctive ridge, as do chimeras of *ld* ectoderm:early wild-type mesenchyme (F). C and D are equivalently staged controls for E and F.

competent to induce a ridge. Moreover, these results indicate that an ectoderm mutant for *ld* is capable of supporting limb growth and patterning.

To determine whether the rescue of skeletal patterning is due to the presence of a morphologically normal ridge, we examined limbs from wild-type and *ld* embryos and from chimeric limbs by scanning electron microscopy. Fig. 4A,C illustrates the morphology of the thickened wild-type ridge at E10.5 and E11.5, respectively, whereas in Fig. 4B,D, the *ld* ridge appears discontinuous and flattened at E10.5 and barely detectable at E11.5 (Zeller et al., 1989). In contrast, chimeras composed of *ld* ectoderm and wild-type mesenchyme display a thickened ridge, comparable to the ridge in chimeras composed of wild-type ectoderm underlaid by wild-type mesenchyme (Fig. 4E,F). Paraffin sections of the chimeric limbs indicate that the *ld* ridge is histologically similar to the wild-type ridge (not shown). These studies indicate that *ld* ectoderm can form a morphologically normal ridge when provided with a wild-type mesenchyme signal, thus supporting the functional results.

To characterise this phenotypic rescue at a molecular level, expression of *Shh*, *Fgf8* and *Fgf4* in the chimeric limbs was examined. In chimeras of *ld* ectoderm:wild-type mesenchyme, we found wild-type levels of *Shh* and *Fgf8* RNA (Fig. 5). Moreover, *Fgf8* expression is less broad and discontinuous than that observed in *ld* limbs (compare Fig. 5D with Fig. 1D). Most strikingly, *Fgf4* expression was rescued in the *ld* ridge

Table 1. Skeletal patterns in different chick/quail:mouse chimeras

	Most distal wing skeletal element present							
Chimera*	Humerus only	Humerus +ulna	Humerus +ulna +radius	Single digit	Two digits	Complete skeleton	Total number of chimeras	% of total complete†
Stage 18-19 wt E10-10.5	1					7	8	87.5
Stage 18-19 *ld* E10-10.5		2	2			8	12	67
Stage 21 wt E10-10.5					1	3	4	75
Stage 21 *ld* E10-10.5	6	4	3	5		6	24	25

*Stage 18-19 or stage 21 refer to the Hamburger and Hamilton (1951) staging of the wild-type chick/quail mesenchyme. E10-10.5 refers to the age of the mouse ectoderm.

†The number of correctly patterned and complete skeletons from the chimeras as a percentage of the total.

(Fig. 5E). This is in contrast to *ld* limbs, in which *Fgf4* is never detected (Fig. 1B). The results of our studies of gene expression patterns, skeletal patterns and the morphology of the ridge show that both ridge morphology and function can be rescued in *ld* ectoderm by a wild-type signal from early stage mesenchyme. The rescued *ld* ridge is then capable of reciprocally signaling the mesenchyme to mediate correct limb patterning.

At present it is not technically feasible to perform the reciprocal experiment testing the ability of *ld* mesenchyme to induce a ridge in wild-type ectoderm, as mouse mesenchyme is not viable when grafted to a chick host. However, two further lines of evidence suggest that the *ld* phenotype cannot be attributed to a primary defect in the ectoderm. First, expression of Ld gene products in *ld* limbs using a ridge-specific transgene does not rescue limb defects (A. Haramis and R. Zeller, personal communication) and second, targeted disruption of *ld* isoform IV, the predominant isoform in the ridge, does not affect murine limb development (Chan et al., 1995). Together our studies implicate a role for Ld proteins as a mesenchymal signal for ridge differentiation. We propose that the primary defect associated with *ld* is the inability of *ld* mesenchyme to induce a fully differentiated ridge. This leads to a secondary defect in which the *ld* ectoderm cannot support normal growth and patterning of the limb.

DISCUSSION

Experimental studies in chick indicate that growth and patterning of the proximal skeletal elements (shoulder/pelvis, humerus/femur) occur prior to ridge differentiation while more distal structures are dependent on a differentiated ridge (Saunders, 1948; Summerbell, 1974). Molecular and morphological evidence indicates that ridge induction may be a multistep process, including an early competence phase and a subsequent differentiation phase. Prior to bud outgrowth in wild-type chick and mouse embryos, *Fgf8* expression can be detected in a strip of cells that prefigures the location of the ridge, suggesting that these cells have acquired the competence to form a ridge. The mesenchyme then begins to proliferate, forming the limb bud. This proliferation results in quite extensive growth of the mouse limb bud prior to ridge differentiation (Wanek et al., 1989), presumably mediated in part by FGF8 in the ectoderm.Subsequently, ridge differentiation takes place as structural and molecular changes result in the formation of a morphologically distinct ridge and induction of *Fgf4*. In the mouse, the time period from early *Fgf8* expression and proliferation of limb mesenchyme to differentiation of the ridge takes approximately 24 hours.

In *ld* limbs, early *Fgf8* expression in the pre-ridge appears normal, as does initial growth of the limb bud, indicating normal progression through the competence phase. This corresponds to the relatively wild-type appearance of the proximal skeleton. However, the ridge differentiation phase appears to be disrupted in *ld* limbs, as neither a thickened ridge nor *Fgf4* expression is detected. This disruption in *ld* ridge function is reflected in the deformities of the distal limb skeleton, including the loss and fusion of distal bones and digits. The chimera results presented here demonstrate that early wild-type mesenchyme rescues ridge morphology and *Fgf4* expression in mutant *ld* ectoderm. Thus, induction of a differentiated ridge appears to require Ld protein in the mesenchyme. In this model, the *ld* mutation discriminates between these two phases of ridge induction. It remains to be determined whether the earlier competence phase also requires a signal from the mesenchyme and, if so, whether Ld plays a role in this phase. As it is not clear whether the available *ld* alleles are complete loss-of-function mutations, gene targeting to generate null alleles of Ld may provide an answer to this question. It is worthwhile noting that immunohistochemical studies of chick limb buds indicate that Ld protein is first detected in posterior limb mesenchyme, but the pattern changes such that it is restricted to ventral mesenchyme by stage 22 (Trumpp et al., 1992). This shift in Ld protein localization occurs around the time the mesenchyme loses the ability to induce a ridge.

Little is known about the molecular pathway by which the mesenchyme induces ridge formation. As described here, Ld activity is required in the mesenchyme for ridge differentiation, indicating a role for Ld in this pathway. Other studies implicate the IGF and FGF signaling pathways in ridge induction and/or maintenance. IGF-I, IGF-II, IGF receptors and IGF binding proteins are expressed in the developing limb bud (Geduspan et al., 1992; Streck et al., 1992; Green et al., 1994). Mutant (*wingless* and *limbless*) chick wing buds that do not form a ridge can be stimulated by IGF treatment to form a thickened

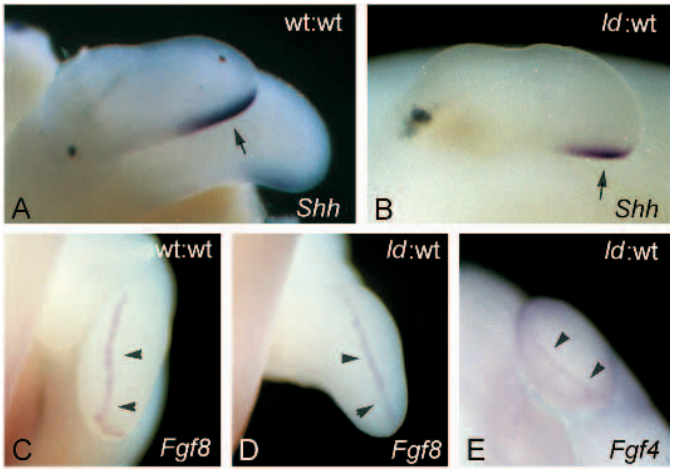

Fig. 5. Wild-type mesenchyme rescues *ld* ridge gene expression. Gene expression in chimeras of early wild-type mesenchyme and wild-type ectoderm (A,C) or *ld* ectoderm (B,D,E). (A,B) *Shh* expression (arrows) is maintained in the mesenchyme of both wild-type and *ld* chimeras. (C,D) *Fgf8* expression (arrowheads) is similar in the ridge of wild-type and *ld* chimeras. Moreover, *Fgf8* expression is less broad and discontinuous than that observed in *ld* embryos. (E) *Fgf4* expression (arrowheads) is detected in the ridge of *ld* chimeras. This is in contrast to its lack of expression in *ld* embryos (Fig. 1B).

ectodermal structure and express *Msx2*, a marker of the ridge (Dealy and Kosher, 1996). FGFs also have the capacity to elicit limb bud proliferation and ridge formation. Application of an FGF bead to the flank stimulates mesenchyme proliferation in the interlimb region and the formation of an ectopic ridge that expresses ridge-specific markers and results in an ectopic limb (Cohn et al., 1995; Ohuchi et al., 1995). However, FGF does not appear to be directly involved in ridge induction (Crossley et al., 1996; Dealy and Kosher, 1996) and instead may act to induce the mesenchyme to express factors such as Ld and IGF, which could influence ridge induction. Further studies should help to determine whether IGF activity is disrupted in *ld* limb mesenchyme.

Lack of detectable *Fgf4* in the *ld* ridge and reduced expression levels of *Shh* in *ld* mesenchyme could in part account for the proximodistal and anteroposterior defects seen in the limbs of these embryos. FGFs appear to be ridge signals required for proximodistal outgrowth and, directly or indirectly, serve to pattern the limb (Niswander et al., 1993; Fallon et al., 1994; Crossley et al., 1996; Vogel et al., 1996 and references therein). Proximodistal patterning of the *ld* limb is only slightly affected (Kleinebrecht et al., 1982; Zeller et al., 1989), which may be a result of the continued expression of *Fgf8* in the *ld* ridge. In contrast, *Fgf4* RNA is not detected in the *ld* ridge; this suggests that FGF4 may play a relatively minor role in proximodistal patterning of the mouse limb. Anteroposterior patterning of the distal skeletal elements is severely affected in *ld* limbs. *Shh* is involved in anteroposterior patterning and its expression is dependent on FGF signaling from the ridge (Riddle et al., 1993; Vogel and Tickle, 1993; Laufer et al., 1994; Niswander et al., 1994). *Shh* expression is dramatically reduced in *ld* limbs. This may be a consequence of the absence of *Fgf4* expression rather than an inherent defect in the mesenchyme, as *Shh* expression, as well as *Evx1* expression, can be restored in *ld* mesenchyme by exogenously supplied FGF4 or by a wild-type ridge (shown here and by Haramis et al., 1995). Considering the interdependence of *Fgf4* and *Shh* expression and their consequences for polarizing activity (Laufer et al., 1994; Niswander et al., 1994), and the apparent disruption of this signaling network in *ld* limbs, we suggest that FGF4 may play a major role in the regulation of *Shh* expression in the mesenchyme. It would be interesting to determine whether exogenous FGF4 can completely rescue the proximodistal and anteroposterior patterning defects of *ld* limbs. If so, it would indicate that Ld activity is not required in the mesenchyme after induction of ridge differentiation or that FGF can compensate for Ld function. However, current limb culturing techniques do not allow explants to survive long enough to determine whether skeletal pattern is rescued. Improved culture conditions or expression of *Fgf4* in the *ld* ectoderm using ridge-specific promoters may assist in answering this question.

In summary, our data demonstrate that wild-type mesenchyme from early limb buds can induce a fully differentiated ridge in *ld* limb ectoderm. Subsequently, this *ld* ridge is capable of sustaining the epithelial-mesenchymal interaction required for normal limb development. We conclude that Ld protein is required in the mesenchyme during early stages of limb development and demonstrate a role for Ld proteins in the molecular pathway necessary for ridge differentiation.

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