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Short contribution

Shh, Fgf4 and *Hoxd* gene expression in the mouse limb mutant hypodactyly

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ABSTRACT The semidominant mouse mutation hypodactyly (*Hd*), caused by a deletion within the *Hoxa13* gene, results in reduced digits; heterozygotes lack digit I in the hindlimb and homozygotes have only one digit on each limb. We investigated expression of *Shh* and *Fgf4* signaling molecules involved in digit specification in mutant limb buds. *Shh* and *Fgf4* are expressed in the posterior part of the limb buds as normal but expression may be slightly prolonged. The extent of digit reduction in hypodactyly is much more severe than in the *Hoxa13* deficient mouse and resembles that in the *Hoxa13*$^{-/-}$/*Hoxd13*$^{-/-}$ double mutant mouse. We found that the pattern of *Hoxd13* and *Hoxd11* transcripts was not markedly different in the mutant compared with the normal limbs even though the mutant limbs are narrower. Therefore *Hoxd* genes are transcribed as normal in the mutant. This makes it likely that the severe digit reductions in hypodactyly are caused by interference with *Hoxd13* function at the protein level. Similar interactions between mutant and normal *HOX* gene products have been suggested to occur in the human semidominant disorder, synpolydactyly, caused by mutations in *HOXD13*.

KEY WORDS: *Shh, Fgf4, Hoxd, mouse limb, hypodactyly*

The semidominant mouse mutation hypodactyly *(Hd)* affects the digits (Hummel, 1970). Mice heterozygous for *Hd* show variable reduction of hindlimb digit I whereas homozygous animals (*Hd/Hd*) have just one digit on each of the four limbs. Analysis of the skeleton revealed that only distal limb structures are affected and, consistent with this, early limb development in the mutant appears normal (Robertson *et al.*, 1996). A narrowing of the limb bud can be detected as the digital plate begins to form and coincides with an increase in cell death and a gap between the apical ridge and the mesenchyme. In addition, *Hd/Hd* mesenchyme cells in culture show a reduction in chondrogenesis (Robertson *et al.*, 1996).

The molecular basis of the *Hd* mutation has been shown to be caused by a 50 nucleotide deletion in exon 1 of the *Hoxa13* gene which results in a shortened transcript (Mortlock *et al.*, 1996). *Hoxa13* is expressed at the tip of the limb bud and, together with transcripts of another *Hox* gene *Hoxd13*, becomes confined to the hand/foot plate (Yokouchi *et al.*, 1991). The pattern of *Hox* gene expression in the limb bud is established as a result of positional signaling. In normal limb development, positional signaling involves a cascade of signals produced by posterior mesenchyme in the polarizing region comprising retinoic acid, SHH and BMPs. Outgrowth signals are provided by the apical ectodermal ridge and include FGF4. *Shh* expression in the mesenchyme and *Fgf4* expression in the ridge are mutually maintained during limb bud outgrowth (Laufer *et al.*, 1994; Niswander *et al.*, 1994). There is evidence that retinoic acid, SHH and BMPs each in concert with FGFs can activate *Hoxd13* expression (Laufer *et al.*, 1994; Niswander *et al.*, 1994; Duprez *et al.*, 1996) and these signals may also activate *Hoxa13* expression initially in posterior distal cells.

Digit reduction in *Hd/Hd* embryos is much more severe than that reported for the *Hoxa13*$^{-/-}$ mouse (Fromental-Ramain *et al.*, 1996) suggesting that *Hd* is not a null allele of *Hoxa13*. Instead the phenotype of *Hd/Hd* mutant limbs is more similar to that of the *Hoxa13*$^{-/-}$/*Hoxd13*$^{-/-}$ double mutant (Fromental-Ramain *et al.*, 1996). Hypodactyly is of added interest because of a very recent report identifying a *HOXA13* nonsense mutation in a family with hand-foot-genital syndrome (Mortlock and Innis, 1997). Here we examine the expression patterns of *Shh* and *Fgf4* in hypodactyly limb buds and whether *Hoxd* genes are expressed normally.

Shh and Fgf4 expression

At 11.5 dpc *Shh* and *Fgf4* transcripts are detected in the posterior mesenchyme and apical ridge respectively in limb buds of normal (Fig 1A, C) and *Hd/Hd* embryos (Fig. 1B, D). Thus the

Abbreviations used in this paper: Hd, hypodactyly; dpc, days *post coitum*.

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feedback loop which mutually maintains *Fgf4* and *Shh* expression is initiated normally and this is consistent with the normal development of the proximal parts of the limb in mutant embryos. At 12.5 dpc, expression of *Shh* and *Fgf4* is no longer present in limbs of +/+ (Fig. 1I, K) and *Hd*/+ embryos, but we still detect expression of both genes in the hindlimbs but not forelimbs of *Hd/Hd* embryos (Fig 1J, L). It is not clear whether this is due to delayed development in the mutant or reflects a consequence of abnormal *Hoxa13* product. In other mutants, loss of digits is associated with attenuated signaling of the polarizing region. In the limb deformity (*ld*) mutant, *Shh* expression is prematurely terminated and *Fgf4* expression is absent (Haramis *et al.*, 1995) and a reduction in *Shh* expression is also associated with loss of posterior digits in the *Wnt7a* knockout mouse (Parr and McMahon, 1995). Thus digit loss can result either from changes in signal production as illustrated by the *ld* and *Wnt7a*$^{-/-}$ mutants or from changes in response to polarizing signals as seems to be the case for hypodactyly.

Hoxd11 and Hoxd13 expression

The extent of digit reduction in hypodactyly is much more marked than in the *Hoxa13*$^{-/-}$ mouse and resembles more closely the phenotype of the *Hoxa13*$^{-/-}$/*Hoxd13*$^{-/-}$ double mutant mouse. One possibility is that *Hoxd13* (and other *Hoxd* genes) is not transcribed in the mutant and we therefore investigated expression of *Hoxd13* and *Hoxd11*. During the time when the distal limb is being patterned, expression of *Hoxd13* is seen in limb buds of both wild-type and mutant embryos. At 11.5 dpc, *Hoxd13* expression in normal embryos is localized to posterior distal mesenchyme as previously described (Dollé *et al.*, 1991; Fig. 1E) and covers approximately 41% of the total distal limb area (Table 1). The limb buds of *Hd/Hd* are much narrower than normal limb buds but the expression domain in *Hd/Hd* (Fig. 1F) appears only slightly reduced when size is taken into account (Table 1). A slight reduction in expression of *Hoxd13* is again seen at 12.5 dpc in *Hd/Hd* mutants (compare Fig. 1M with 1N, Table 1).

There is evidence of interactions between *Hox* genes in the same cluster (Zákány and Duboule, 1996) and therefore we examined *Hoxd11* expression in hypodactyly. At 11.5 dpc, *Hoxd11* expression in +/+ embryos is found in two regions, one distal the other more proximal, both running antero-posteriorly across the developing hand/foot plate (Izpisúa-Belmonte *et al.*, 1991; Fig.1G). However, in *Hd/Hd* embryos, there is only a single region of expression which covers most of the distal limb (Fig.1H) and covers a larger percentage area than that in normal limbs (Table 1). By 12.5 dpc, we can begin to distinguish two domains of *Hoxd11* expression in *Hd/Hd* limbs. These domains are less well defined than in normal buds and the extent of expression appears somewhat reduced (Fig 1O, P).

Our analysis shows that the percentage of the limb bud expressing *Hoxd13* and *Hoxd11* at 11.5 dpc and 12.5 dpc is slightly altered in the mutant. It seems unlikely that these small alterations in *Hoxd* gene transcript patterns at these stages would be sufficient to account for the differences in morphology between hypodactyly and the *Hoxa13* deficient mouse. However, it cannot be excluded that reduced expression of *Hoxd11* contributes to the severity of the phenotype. A human disorder, synpolydactyly, has recently been shown to be caused by a mutation in *HOXD13* (Muragaki *et al.*, 1996). Like hypodactyly, synpolydactyly is semidominant and has a more severe phenotype than that of the *Hoxd13* deficient mouse (Dollé *et al.*, 1993). A triple knockout of *Hoxd11*, *Hoxd12* and *Hoxd13* in mice gives a phenotype that resembles synpolydactyly suggesting that, in the human condition, mutant *HOXD13* blocks functioning of other *HOX* genes (Zákány and Duboule, 1996). A similar explanation could account for the severity of the digit reductions in hypodactyly. Our results show that *Hoxd13* is still transcribed in the *Hd/Hd* mutant but the abnormal *Hoxa13* product could interfere with the normal functioning of *Hoxd13*. This would explain why the severe phenotype of hypodactyly resembles that of the *Hoxa13*$^{-/-}$/*Hoxd13*$^{-/-}$ double mutant mouse.

TABLE 1.

AREA OF EXPRESSION OF *HOXD13* AND *HOXD11* IN DEVELOPING LIMB BUDS OF NORMAL AND MUTANT EMBRYOS

	11.5 dpc		12.5 dpc	
	+/+	Hd/Hd	+/+	Hd/Hd
Total area of distal limb[a]	0.56±0.1 (66)	0.46±0.07 (17)	0.91±0.12 (24)	0.55±0.04 (5)
Area of *Hoxd13* expression in distal limb	0.23±0.03 (34)	0.16±0.02 (9)	0.46±0.06 (12)	0.24±0.01 (3)
Area of *Hoxd13* expression as % of distal limb	41%	35%	51%	44%
Area of *Hoxd11* expression in distal limb	0.32±0.07 (32)	0.35±0.06 (8)	0.54±0.14 (12)	0.17±0.21 (2)
Area of *Hoxd11* expression as % of distal limb	57%	76%	59%	31%

[a] Area expressed in arbitrary units; number of limbs analyzed in brackets. Note area of distal limb increases much more in +/+ than in *Hd/Hd* embryos.

Experimental Procedures

Preparation of embryonic material

Mice carrying the *Hd* mutation on a (C57BL/6J x C3HeB/FeJLe-a)F_1 background were obtained from the Jackson Laboratory and maintained at University College, London. Timed matings were set up between heterozygous (*Hd*/+) mice, midday of vaginal plug appearance was designated 0.5 days *post coitum* (dpc). Embryos were removed and putative *Hd/Hd*, *Hd*/+ and +/+ littermates identified on the basis of their limb morphology (Robertson *et al.*, 1996). At 11.5 dpc, *Hd/Hd* embryos had narrow, pointed limb buds while +/+ or *Hd*/+ embryos had normal paddle shaped buds. By 12.5 dpc *Hd*/+ embryos can be distinguished from +/+ embryos due to loss of anterior tissue in hindlimb buds. Phenotyped embryos were fixed in 4% paraformaldehyde overnight and subsequently split down the midline using a sharp tungsten needle.

Whole-mount in situ hybridization

Whole-mount *in situ* hybridization was carried out on each half embryo essentially as described by Nieto *et al.* (1996). The riboprobes have all been described previously; *Shh* (Echelard *et al.*, 1993), *Fgf4* (Niswander and Martin, 1992), *Hoxd11* (Izpisúa-Belmonte *et al.*, 1991) and *Hoxd13* (Dollé *et al.*, 1991). After

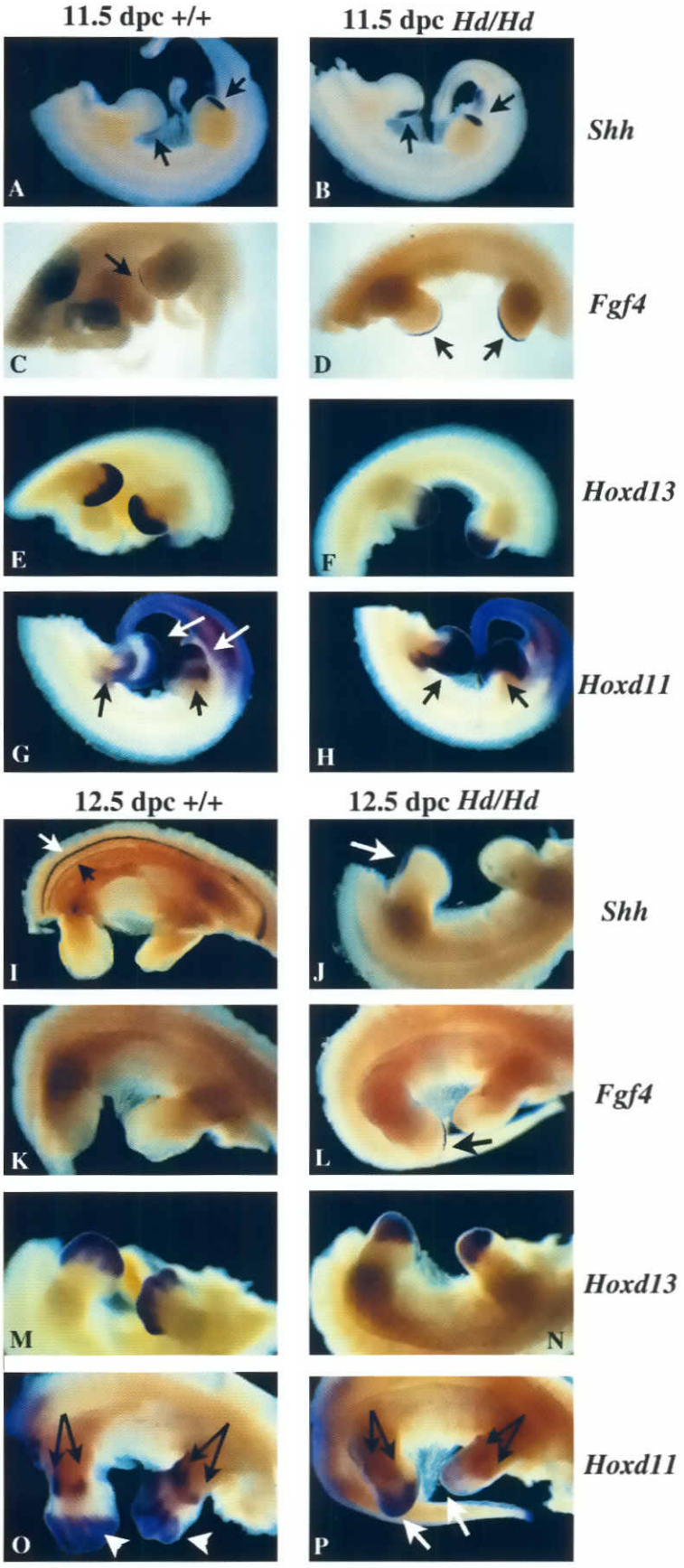

Fig. 1. Expression patterns of *Shh, Fgf4, Hoxd11* and *Hoxd13* genes during development of normal (+/+) and hypodactyly (*Hd/Hd*) mouse limbs. **(A)** *11.5 dpc +/+ embryo; Shh is expressed in posterior mesenchyme of both fore and hindlimb buds; expression in forelimb bud (arrow head) is less pronounced than in hindlimb (arrow).* **(B)** *11.5 dpc Hd/Hd embryo; strong expression of Shh in posterior mesenchyme of fore and hindlimb buds (arrows).* **(C)** *12.5 dpc +/+ embryo; Shh expression in limb buds not detectable, but can still be seen in neural tube (arrow heads) and notochord (arrows).* **(D)** *12.5 dpc Hd/Hd embryo; Shh is still expressed in posterior mesenchyme of hindlimb bud (arrow), but no longer detected in forelimb bud.* **(E)** *11.5 dpc +/+ embryo; expression of Fgf4 detectable in apical ectodermal ridge on hindlimb bud (arrow).* **(F)** *11.5 dpc Hd/Hd embryo; Fgf4 expressed strongly in apical ectodermal ridge of both fore(arrows) and hindlimb (arrow heads) buds.* **(G)** *12.5 dpc +/+ embryo; Fgf4 expression not detected in either of the limb buds.* **(H)** *12.5 dpc Hd/Hd embryo; Fgf4 expressed in apical ectodermal ridge of hindlimb bud (arrow). No expression detected in forelimb bud.* **(I)** *11.5 dpc +/+ embryo; strong expression of Hoxd13 detected in mesenchyme of fore and hindlimb buds (arrows).* **(J)** *11.5 dpc Hd/Hd embryo; strong expression of Hoxd13 in both fore and hindlimb buds (arrows).* **(K)** *12.5 dpc +/+ embryo; expression of Hoxd13 in forelimb and hindlimb bud distal mesenchyme.* **(L)** *12.5 dpc Hd/Hd embryo; expression of Hoxd13 in forelimb (white arrow) and hindlimb bud distal mesenchyme (black arrow).* **(M)** *11.5 dpc +/+ embryo; Hoxd11 expression found in two regions (black arrows indicate proximal domains and white arrows distal domains) in mesenchyme of fore and hindlimb buds.* **(N)** *11.5 dpc Hd/Hd embryo; strong expression of Hoxd11 found in a single domain in both fore and hindlimb buds (arrows).* **(O)** *12.5 dpc +/+ embryo; Hoxd11 continues to be expressed in two domains (black arrows indicate proximal domains, white arrow heads distal domains) in fore and hindlimb buds.* **(P)** *12.5 dpc Hd/Hd embryo; Hoxd11 detected in two domains in fore and hindlimb buds similar to those in +/+ embryos (black arrows indicate proximal domains, white arrows distal domains).*

colour detection, embryos were photographed using a Zeiss SV2 dissecting microscope and attached camera. To estimate area of gene expression as a proportion of total distal limb, individual limbs were dissected away from the body wall and the ventral surface re-photographed and drawn. Distal limb area was measured from the proximal edge of the hand/foot plate at the indentation of the presumptive wrist/ankle. Images were scanned and subsequently analyzed using NIH image analysis 5.8 to obtain a quantitative figure in arbitrary units.

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