Lef1 expression is activated by BMP-4 and regulates inductive tissue interactions in tooth and hair development

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Targeted inactivation of the murine gene encoding the transcription factor LEF-1 abrogates the formation of organs that depend on epithelial–mesenchymal tissue interactions. In this study we have recombined epithelial and mesenchymal tissues from normal and LEF-1-deficient embryos at different stages of development to define the LEF-1-dependent steps in tooth and whisker organogenesis. At the initiation of organ development, formation of the epithelial primordium of the whisker but not tooth is dependent on mesenchymal *Lef1* gene expression. Subsequent formation of a whisker and tooth mesenchymal papilla and completion of organogenesis require transient expression of *Lef1* in the epithelium. These experiments indicate that the effect of *Lef1* expression is transmitted from one tissue to the other. In addition, the finding that the expression of *Lef1* can be activated by bone morphogenetic protein 4 (BMP-4) suggests a regulatory role of this transcription factor in BMP-mediated inductive tissue interactions.

[*Key Words*: *Lef1*; BMP-4; epithelial–mesenchymal interactions; tooth and whisker development]

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Vertebrate organs are typically composed of two dissimilar tissues, most commonly an epithelium and mesenchyme, which influence each other during organogenesis. Inductive interactions between these tissues govern the initiation of organ development, subsequent morphogenesis and terminal cytodifferentiation (for review, see Grobstein 1967; Gurdon 1992). Initiation of morphologically visible organ formation is frequently marked by local thickening of the epithelium and condensation of subjacent mesenchymal cells. Morphogenesis subsequently involves complex growth of the epithelium, which either branches within the mesenchyme or folds around the mesenchymal condensation forming a papillary structure. Finally, terminal differentiation of cells of both epithelial and mesenchymal lineages governs the synthesis of gene products specific for organ function.

The importance of tissue interactions for organ morphogenesis has been demonstrated by classical embryological experiments involving the separation of epithelial and mesenchymal tissues and subsequent recombination with heterologous partner tissue (Grobstein 1967; Saxén 1977; Kollar 1983; Kratochwil 1986). These studies indicated that epithelial–mesenchymal interactions are reciprocal and sequential, and that either component may play the dominant role in organogenesis, depending on the organ system and the developmental stage. Development of teeth and skin appendages (whiskers, hairs, feathers, and scales) has been studied extensively and shown to share several features (for review, see Slavkin 1974; Sengel 1976; Thesleff and Humerinta 1981; Kollar 1983; Thesleff et al. 1995). In both tooth and whisker development, an ectodermally derived epithelium interacts with mesenchyme that originates from neural crest cells of the first branchial arch (LeDouarin 1982; Lumsden 1988; Noden 1988). In each case, the invaginating epithelium embraces part of the condensed mesenchyme which forms a characteristic mesenchymal papilla during early morphogenesis. Studies aimed at gaining insight into the mechanisms of tooth and hair follicle development have focused on the analysis of the expression patterns of candidate regulatory genes encoding transcription factors, cell surface molecules, and growth factors (Panaretto 1993; Thesleff et al. 1995). Many of these genes were found to be expressed in spatially and temporally defined patterns that reflect inductive tissue interactions (Jones et al. 1991; Lyons et al. 1991; MacKenzie et al. 1991; Jowett et al. 1993; Vainio et al. 1993; Hogan et al. 1994; Parr and McMahon 1994). Moreover, some growth factors, such as bone morphogenetic protein 4 (BMP-4) and fibroblast growth factor-4 (FGF-4), have been found to mimic the effects of dental epithelium by inducing the expression of specific genes in the mesenchyme of tooth germs cultured in vitro (Vainio et

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al. 1993; Jernvall et al. 1994). Recently, experimentally induced mutations in the transcription factor genes *Msx1* and *Lef1* revealed a role of these regulatory genes in tooth development (Satokata and Maas 1994; van Genderen et al. 1994).

Lymphoid enhancer-binding factor 1 (LEF-1) is a cell type-specific transcription factor expressed in lymphocytes of the adult mouse, and in the neural crest, mesencephalon, tooth germs, whisker follicles, and other sites during embryogenesis (Travis et al. 1991; Waterman et al. 1991; Oosterwegel et al. 1993; van Genderen et al. 1994; Zhou et al. 1995). LEF-1 is a member of the family of high mobility group (HMG) proteins which has the capacity to induce a sharp bend in the DNA helix (Giese et al. 1992; Love et al. 1995). In addition, LEF-1 activates transcription only in collaboration with other DNA-binding proteins (Giese and Grosschedl 1993; Carlsson et al. 1993). In the context of the T-cell receptor α enhancer, LEF-1 protein appears to play an architectural role, promoting the assembly of a higher-order nucleoprotein complex by juxtaposing nonadjacent factor binding sites (Giese et al. 1995). In addition, LEF-1 has been shown to activate the human immunodeficiency virus-1 enhancer in the context of nucleosomal DNA templates suggesting a role of this transcription factor in nucleosomal derepression (Sheridan et al. 1995).

Targeted inactivation of the *Lef1* gene in the mouse germ line resulted in a pleiotropic phenotype in which the development of teeth, whiskers, hair follicles, and mammary glands was found to be severely impaired (van Genderen et al. 1994). Tooth development is initiated in *Lef1*$^{-/-}$ embryos; however it is arrested before the formation of a mesenchymal dental papilla. Likewise, development of body hair follicles and mammary glands is incomplete or abrogated before morphogenesis. All organs that are affected by the mutation in the *Lef1* gene share a requirement for tissue interactions between ectoderm-derived epithelium and mesenchyme. Thus, the phenotype of this mutant mouse raised the interesting possibility that LEF-1 plays a general regulatory role in ectodermal–mesenchymal tissue interactions (van Genderen et al. 1994). Moreover, forced expression of LEF-1 in the skin of transgenic mice was recently found to result in abnormalities in the orientation of hair follicles and occasionally in ectopic formation of hair follicles (Zhou et al. 1995).

In the present study, we applied classical tissue-recombination techniques to define the function of *Lef1* in tooth and whisker development. Experimental combinations of epithelial and mesenchymal tissues from normal and *Lef1*$^{-/-}$ mutant embryos allowed us to determine the tissue type and developmental stages in which *Lef1* is critical for morphogenesis of both organs. These experiments indicated that *Lef1* expression is required only transiently in one tissue to induce a specific morphogenetic event in the other tissue. Moreover, expression of *Lef1* is activated in presumptive dental mesenchyme by BMP-4, suggesting that this transcription factor acts in a BMP-mediated signaling pathway.

Results

Lef1 *is expressed in a stage- and tissue-specific pattern during tooth development*

We analyzed *Lef1* gene expression in embryos between days 10 and 16 of gestation (E10–E16) by in situ hybridization and immunohistochemistry. Consistent with previous observations (Oosterwegel et al. 1993), *Lef1* was found strongly expressed in the thickened oral epithelium at the initiation of visible tooth development between E10 and E11 (Fig. 1A). At E12, expression of *Lef1* shifts to the condensing mesenchyme around the invaginated epithelial tooth bud (Fig. 1B). Beginning morphogenesis at E13 is accompanied by expression of *Lef1* both in the mesenchymal condensation and in the immediately adjacent basal cells of the epithelium (Fig. 1C). These epithelial cells differ in their proliferation and signaling properties from other epithelial cells and form the future enamel knot (Jernvall et al. 1994; Vaahtokari et al. 1996). During the subsequent cap and bell stages of tooth development (E14–E16), *Lef1* transcripts are continuously detected in both tissues, including the mesenchymal papilla and preodontoblasts, and in the epithelium-derived preameloblasts (data not shown). This pattern of expression was confirmed by immunohistochemistry (Fig. 1D).

Although *Lef1* is expressed at the earliest stages of tooth development, *Lef1*$^{-/-}$ mouse embryos initiate the formation of tooth germs (van Genderen et al. 1994). The first visible defect in tooth development of *Lef1*-deficient embryos can be detected at the late bud stage around E13 when the dental papilla fails to form (Fig. 1E,F). In particular, the mutant dental epithelium does not form the enamel knot and fails to adopt the shape of a cap at later stages (van Genderen et al. 1994), although the mutant tooth bud persists at least until birth. This mutant phenotype suggests that E13 may be the critical stage for LEF-1 action in tooth development.

Formation of the mesenchymal dental papilla depends on epithelial Lef1 *expression*

The sequential shifts in the pattern of gene expression between epithelial and mesenchymal tissues and the arrest in tooth development at the bud stage suggested a regulatory role for *Lef1* in odontogenesis. To define the *Lef1*-dependent step in this process, we performed tissue combinations of presumptive dental epithelium and mesenchyme from normal and homozygous mutant (−/−) embryos. Normal tissues were obtained from wild-type and heterozygous embryos which are phenotypically indistinguishable. For the tissue recombinations, we dissected tooth anlagen or tooth germs from embryos at different stages of development (E10–E17), separated the epithelial and mesenchymal components and reassociated normal and mutant partner tissues in both combinations. The tissue recombinants were initially incubated in vitro but subsequently transplanted under the kidney capsule for completion of organogenesis and terminal cytodifferentiation. Tooth development

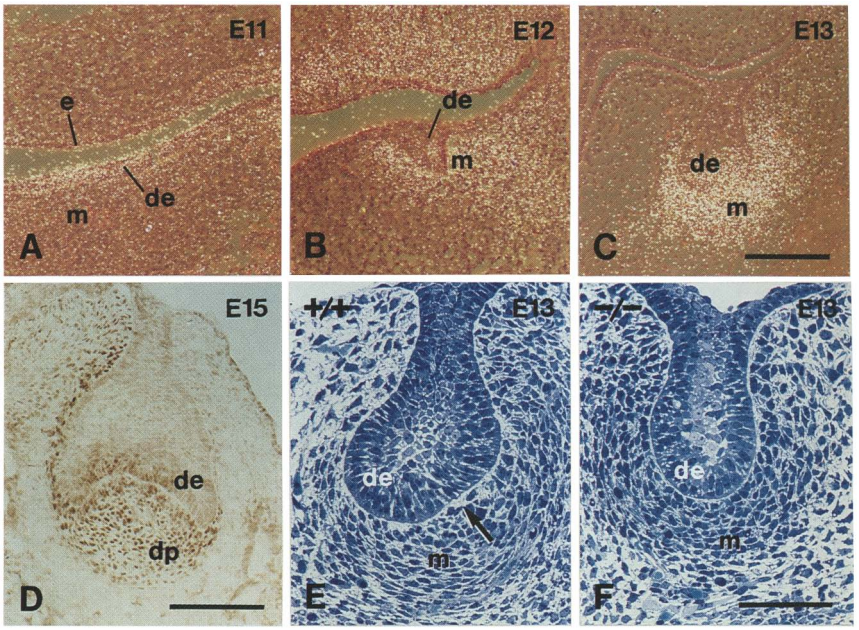

Figure 1. Pattern of *Lef1* expression and *Lef1*$^{-/-}$ phenotype in early tooth development. Coronal sections of E11 to E15 wild-type embryos were either hybridized with a *Lef1*-specific RNA probe or immunostained with anti-LEF-1 antibodies. (*A*) Oral cavity of an E11 embryo showing *Lef1* transcription in the thickened presumptive dental epithelium (de). (e) Oral epithelium; (m) mesenchyme. (*B*) Invagination of dental epithelium at E12 and shift of *Lef1* expression to the underlying mesenchyme (m). (*C*) E13 molar tooth germ containing abundant *Lef1* transcripts in the condensing dental mesenchyme (m) and in epithelial cells at the base of the tooth bud. (*D*) Molar tooth germ at the cap stage (E15), in which LEF-1 is expressed in the nuclei of cells in the dental papilla (dp) and epithelial cells of the enamel knot. (*E,F*) Plastic sections through the molar anlage of a late E13 wild-type (+/+) embryo (*E*) and mutant (−/−) littermate (*F*). Cells of the mesenchymal condensation arrange for the future dental papilla in the wild-type tooth (*E*), but not in the mutant (*F*) in which the mesenchyme (m) seems more condensed and undifferentiated. The dental epithelium (de) remains bud-shaped in the mutant (*F*), whereas it begins basal flattening (arrow) for the eventual formation of the cap in the wild-type tooth (*E*). Bars, (*A–D*) 100 μm; (*E–F*) 50 μm.

was assessed in histological sections of explanted tissue recombinants.

All combinations of normal epithelium and mutant mesenchyme produced teeth at high frequency, irrespective of the stage of the donor embryos (Fig. 2A). Tooth morphogenesis was normal and both tissues completed cytodifferentiation including the secretion of dentin by mutant odontoblasts and enamel by ameloblasts (Fig. 2C,D). By contrast, odontogenesis in reciprocal associations of mutant epithelium and normal mesenchyme was dependent on the stage of the donor embryos (Fig. 2B). No teeth developed from tissues of E10–E12 embryos (Fig. 2F), the mutant epithelium instead forming only large keratinizing cysts and the normal mesenchyme empty alveolar bone. Recombinations of E13 dental tissues developed very few tooth-like structures, whereas those of E14–E17 tissues yielded morphologically normal teeth at high frequency (Fig. 2B,G). Notably, the mutant dental epithelium differentiated into a normal ameloblast cell layer. These tissue combinations indicate that *Lef1* expression is required only in the epithelium to allow for morphologically normal tooth development. However, dental mesenchyme from normal E14 or older embryos which was exposed to *Lef1*-expressing epithelium before tissue separation was capable of developing teeth in association with mutant epithelium. Control transplants of unmanipulated mutant tooth germs never developed teeth in organ culture or as a subrenal graft suggesting that soluble factors present in serum or in wild-type mice can not compensate for the *Lef1* deficiency. Taken together, these data suggest that epithelial *Lef1* expression is necessary for the induction of the mesenchyme, presumably for the formation of the dental papilla, but is dispensable for further cytodifferentiation.

Previous recombination studies with normal tissues indicated that the different shapes of the incisor and molar teeth are determined by the mesenchyme after E11 (Kollar and Baird 1969; Lumsden 1979; Kollar and Mina 1991). To examine whether *Lef1* participates in the determination of tooth shape, we combined normal epithelium of the E13 incisor anlage with mutant mesenchyme of an E13 molar anlage and vice versa. In both types of recombinations, the *Lef1*-deficient mesenchyme was still capable of determining the final shape of the tooth (Fig. 2E,H), indicating that specification of the type of the dental papilla is independent of *Lef1*.

Lef1 *gene expression during whisker development*

Whiskers (vibrissae) are specialized skin appendages that differ from body hair by their size, morphology, and function (Davidson and Hardy 1952; Hardy 1992). Before visible whisker development, LEF-1 protein is detected by immunohistochemistry exclusively in the mesenchyme of the whisker pad of E11 embryos (Fig. 3A). Initiation of whisker development at E12 is accompanied by additional LEF-1 protein expression in the epithelial placodes (Fig. 3B). With the formation of invaginated whisker pegs at E13, both cells of the condensed mesenchyme and the immediately adjacent epithelial cell layer express LEF-1 protein (Fig. 3C). In stage E15 embryos, LEF-1 is expressed in cells of the mesenchymally derived dermal papilla and in matrix cells of the forming epithelial hair

		E10	E11	E12	E13	E14	E15	E17
A	+ epithelium / − mesenchyme	4/5	2/4	15/21	20/21	11/11	6/6	2/3
B	− epithelium / + mesenchyme	0/5	0/5	0/19	3/23	10/16	7/7	2/2

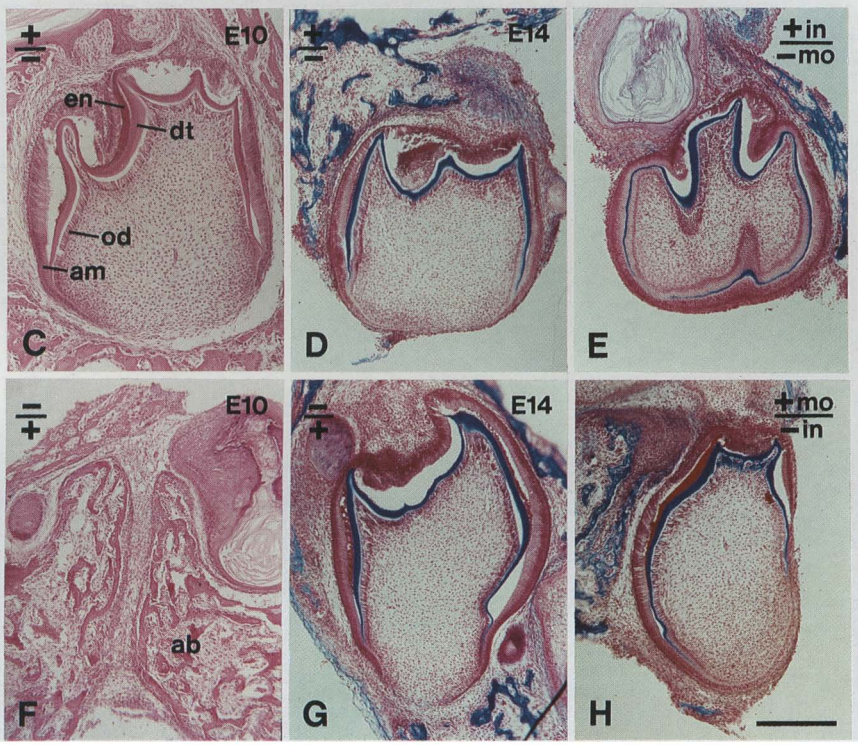

Figure 2. Tooth development in experimental combinations of dental tissues from normal (+) and *Lef1*$^{-/-}$ mutant (−) embryos. Presumptive dental epithelium and mesenchyme were separated at the stages indicated (E10–E17) and associated in both combinations (*A*,*B*). The numbers in the boxes indicate the number of experiments that yielded well formed teeth out of the total number of recombinations performed. (*A*) Combinations of normal epithelium with mutant mesenchyme yielded teeth, irrespective of the stage of the donor embryo. (*B*) In the reciprocal recombination, teeth formed only with tissues from embryos older than E13. (*C*,*D*,*F*,*G*) Representative sections through recombined tissues after complete development as subrenal grafts. (*C*,*D*) Molars formed in combinations of normal epithelium with mutant mesenchyme from E10 (*C*) and E14 (*D*) embryos. Note typical cytodifferentiation of mutant odontoblasts (od) secreting predentin (dt). (am) Ameloblasts; (en) enamel. (*F*,*G*) Development of combinations of mutant epithelium with normal mesenchyme. Tissue recombinant from E10 embryos (*F*) showing an empty alveolar bone (ab) and keratinizing epithelial cysts, and from E14 embryos (*G*) showing normal tooth morphology, including differentiation of an ameloblast layer by mutant epithelium. (*E*) Typical molar tooth produced in a recombination of normal E13 epithelium of the incisor anlage with mutant mesenchyme of the molar anlage (+in/−mo). (*H*) The combination of normal epithelium of the molar anlage with mutant mesenchyme of the incisor anlage (+mo/−in) produces incisors with the characteristic simpler crown morphology and the characteristic asymmetric deposition of dentin and enamel. Sections in *C* and *F* were stained with hematoxylin–eosin, and all others with the Azan dichromic stain to differentially stain dentin (blue) and enamel (red). Bar, 200 μm.

bulb (Fig. 3D). In addition, LEF-1 expression can be detected in cells of the basal layer of the epidermis at this stage (Fig. 3D). The pattern of LEF-1 protein expression in the primordia of body hair follicles of E15 embryos is very similar to that of early whisker pegs (Fig 3E,F), consistent with the recently reported distribution of *Lef1* transcripts (Zhou et al. 1995).

In contrast to tooth development, which is arrested after formation of the primordium, whisker development is not even initiated in *Lef1*$^{-/-}$ embryos. No whisker placodes and whisker pegs can be detected in mutant embryos at E12 and E13 (Fig. 3H), whereas in normal embryos the local condensations of the mesenchyme and invagination of the epithelium are clearly visible (Fig. 3G). Mutant embryos, however, develop rudimentary body hair follicles at a reduced number (van Genderen et al. 1994).

Initiation of whisker development requires mesenchymal Lef1 *expression*

To define the dependence of whisker development on *Lef1* function, we combined epithelium and mesenchyme of normal and mutant whisker pads at embryonic stages E11–E13. The appearance of whisker placodes and pegs was monitored in vitro. Some recombined tissues were subsequently transplanted under the kidney capsule to allow for complete whisker development. Between E11 and E13, all combinations of normal whisker pad mesenchyme with mutant epithelium developed characteristic whisker follicles at high frequency, together with pelage hair follicles (Fig. 4A). The morphology of the whisker follicles was normal, including the formation of mesenchymally derived dermal papilla, dermal sheath and blood sinus, and the epidermally derived root sheaths and hair shaft (Fig. 4C,D). As a control, unmanipulated snout pads from mutant embryos did not form whiskers in subrenal grafts although they developed some pelage hair (data not shown).

The development of reciprocal tissue combinations in which mutant whisker pad mesenchyme was associated with normal epithelium was dependent on the stage of the embryo (Fig. 4B). Recombinations with tissues from E12 and E13 embryos developed complete whisker follicles (Fig. 4E,F), but none of the tissue recombinations using E11 epithelium formed whiskers. In these recom-

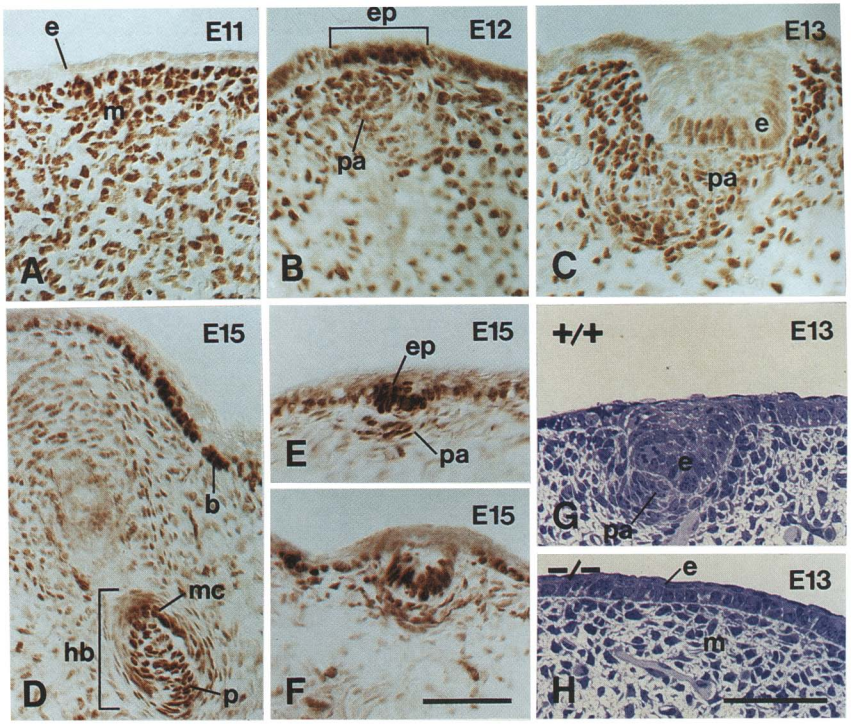

Figure 3. Pattern of expression of LEF-1 and *Lef1*$^{-/-}$ phenotype in whisker development. Coronal sections through the whisker pad were immunostained with anti-LEF-1 antibodies. (*A*) At E11, before the appearance of whisker anlagen, LEF-1 is restricted to the mesenchyme (m), with no protein detectable in the epithelium (e). (*B*) Expression of LEF-1 protein at E12 is still present in mesenchymal cells, especially in those condensing under the epithelium to form a papilla anlage (pa). In addition, strong expression is seen in the nuclei of cells in the epithelial thickenings or placodes (ep). (*C*) Invaginating whisker peg of an E13 embryo showing intensive staining for LEF-1 in mesenchymal cells of the developing whisker papilla (pa) and in adjacent basal epithelial (e) cells. (*D*) Whisker follicle from an E15 embryo, expressing LEF-1 in the hair bulb (hb), including mesenchymal cells of the dermal whisker papilla (p) and proliferating matrix cells of the epithelium (mc). At this stage, cells in the basal layer of the epidermis (b) also express LEF-1. (*E,F*) E15 body hair follicles at different stages of development express LEF-1 both in the epithelial placode (ep) and in the mesenchymal papilla anlage (pa) that is condensating immediately underneath. (*G,H*) Plastic sections through whisker pads of E13 wild-type (+/+, *G*) and mutant (−/−, *H*) embryos. Invagination of the epithelium (e) and organization of the mesenchymal papilla anlage (pa) are only seen in the wild-type embryo. Bars, 50 μm.

binations not even placodes or pegs were detected. Thus, mesenchymal *Lef1* expression appears to be required for the initiation of whisker development between E11 and E12. After the formation of whisker primordia, either tissue is competent to mediate organogenesis in combination with a *Lef1*-deficient partner tissue. Notably, both tissues can undergo full cytodifferentiation in the absence of a functional *Lef1* gene.

Transient requirement of epithelial Lef1 *function in tooth and whisker development*

To further define the role of *Lef1* in inductive tissue interactions, we designed double recombination experiments in which either mutant mesenchyme or mutant epithelium was associated with normal partner tissue transiently, during the critical *Lef1*-dependent period of organ development (Fig. 5). Toward this goal, epithelium and mesenchyme from normal and mutant embryos were first associated in either combination. After 36 or 48 hr of in vitro culture, the tissues were separated again and the normal tissue was replaced with the equivalent mutant tissue. Thus, the resulting tissue recombinants were completely mutant, but one component had been exposed for a short time to normal partner tissue.

Double recombinations of tooth germs, in which mutant E14 molar mesenchyme was transiently associated with normal E14 molar epithelium and subsequently combined with mutant epithelium, developed in subrenal grafts morphogically normal teeth in 6 out of 10 combinations (Fig. 5A). The reciprocal double recombination, in which mutant E14 epithelium was transiently associated with normal E14 dental mesenchyme, failed to develop teeth and formed only keratinized cysts within alveolar bone (Fig. 5B). Double recombinations of whisker pads, in which mutant E12 mesenchyme was exposed to normal E12 epithelium and subsequently associated with mutant epithelium, developed whisker follicles of relatively normal morphology (Fig. 5C). The reciprocal double recombination, in which mutant E12 epithelium was transiently exposed to normal E12 mesenchyme formed only rudimentary whiskers. Short and unstructured epidermal ingrowths with a fully differentiated sebaceous gland were surrounded by a dermal capsule and occasionally by a small blood sinus, whereas the most essential structures of the follicle, the root sheaths, hair shaft and dermal papilla, were missing (Fig. 5D). Control double recombinations, in which mutant epithelium was combined with normal mesenchyme in both primary and secondary association, developed typical whisker follicles, indicating that the double manipulation did not affect the developmental capacity of the explants (data not shown).

These data, together with those from the single recombinations, indicate that transient expression of *Lef1* in the epithelium is sufficient for both tooth and whisker morphogenesis after the initiation of organogenesis. Presumably, epithelial *Lef1* expression provides a develop-

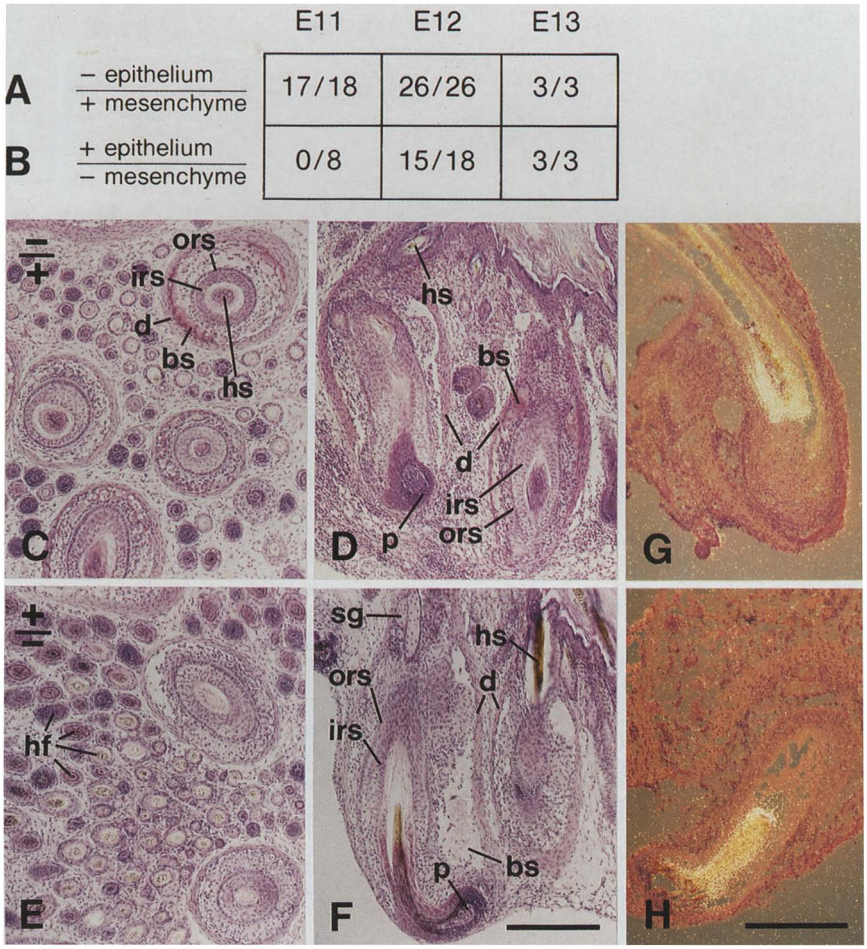

Figure 4. Whisker development in experimental combinations of whisker pad epithelium and mesenchyme from normal (+) and *Lef1*$^{-/-}$ mutant (–) embryos. Tissues were separated at the stages indicated (E11–E13) and recombined in both ways (*A*,*B*). The numbers in the boxes indicate the number of experiments that produced whiskers out of the total number of recombinations performed. (*A*) All combinations containing normal mesenchyme developed whiskers. (*B*) In the reciprocal recombination, whisker formation only occurred if the donor embryo was at least 12 days old. (*C–F*) Sections of E12 tissue combinations that were allowed to develop completely. (*C*,*D*,*G*) Mutant epithelium combined with normal mesenchyme. Whisker follicles, among body hair follicles, shown in cross and in longitudinal section exhibit characteristic structures such as inner (irs) and outer (ors) root sheaths, hair shaft (hs), sebaceous gland (sg), blood sinus (bs), outer dermal sheath (d), hair bulb and dermal papilla (p). (*E*,*F*,*H*) Combinations of normal epithelium with mutant mesenchyme (both E12) develop normal follicles in which dermal structures, including papilla (p) are formed by mutant mesenchyme. (*G*,*H*) Whisker sections of tissue recombinants hybridized with a probe for hair keratin A1 (HK-A1) showing intense transcription also in cells derived from *Lef1*$^{-/-}$ epithelium (*G*). Bars, 200 μm.

mental signal that is required for the formation of the dental and dermal papilla respectively. Although we can not rule out the formal possibility that the normal epithelium deposits extracellular matrix components on the mutant mesenchyme which then allows development of the mutant epithelium in the second recombination, we consider it unlikely that such material withstands enzymatic tissue separation. In contrast to tooth development, additional *Lef1* expression in the mesenchyme is necessary for the initiation of whisker development. Although continuous mesenchymal *Lef1* expression can also allow for further whisker morphogenesis, even in combination with mutant epithelium, transient mesenchymal *Lef1* expression alone is not sufficient for complete organ formation.

Epidermal cytodifferentiation in the absence of functional Lef1

In addition to whiskers, normal pelage hair developed in both types of epithelial–mesenchymal tissue combinations. The number of hair follicles was significantly lower in combinations of mutant epithelium with normal mesenchyme as compared to the reciprocal recombination (Fig. 4). This is consistent with the previously reported reduction of the number of body hair follicles in *Lef1*$^{-/-}$ mice to about one-third relative to wild type (van Genderen et al. 1994). Moreover, keratinization of hair shafts appeared to be less pronounced in the combination of mutant epithelium and normal mesenchyme, indicating that epithelial *Lef1* expression may have an additional function in pelage hair development. Hair keratins are synthesized during terminal differentiation of epithelial cortex cells (Kopan and Fuchs 1989; Powell et al. 1991). A putative role of LEF-1 protein in the regulation of hair keratin genes was inferred from the identification of multiple LEF-1 binding sites in the promoters of these genes (Zhou et al. 1995). Therefore, we examined the whisker follicles formed in both recombinations for the expression of hair keratin (HK)-A1 by in situ hybridization (Fig. 4G,H). HK-A1 expression was detected in both types of recombinations suggesting that LEF-1 may be dispensable for this particular terminal cytodifferentiation. The rudimentary body hair follicles of *Lef1*$^{-/-}$ mice, however, fail to express HK-A1 at detectable levels (data not shown), presumably because of incomplete organogenesis.

Lef1 *expression is activated by BMP-4*

Many genes encoding transcription factors, growth fac-

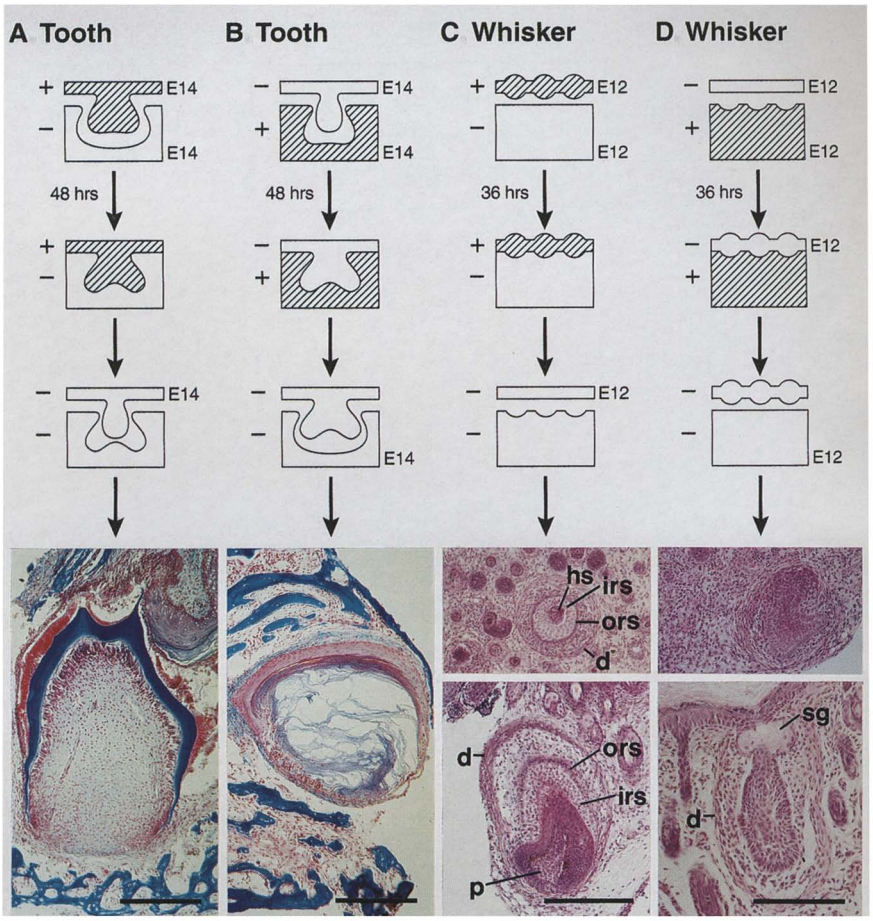

Figure 5. Transient expression of *Lef1* in the epithelium is sufficient for tooth and whisker morphogenesis. Schematic representation and results of double recombination experiments. In the first recombination, the normal tissue (crosshatched) was combined with the mutant tissue. The genotype of the tissues, [normal (+); *Lef1*$^{-/-}$ mutant (−)], and the embryonic stages are indicated. In the second recombination the normal tissue was replaced with an equivalent mutant tissue. (*A*,*B*) Tooth development; (*C*,*D*) whisker development. (*A*) Mutant E14 dental mesenchyme was first cultured in association with E14 normal epithelium for 48 hr, then recombined with E13 or E14 mutant dental epithelium. Although neither tissue contains a functional *Lef1* gene, these combinations yielded well-formed teeth. (*B*) The reciprocal double combination of dental tissues (mutant epithelium transiently exposed to normal mesenchyme, then recombined with mutant mesenchyme) failed to form teeth and developed only keratinizing cysts within alveolar bone. (*C*) Mutant E12 whisker pad mesenchyme was first associated with normal E12 whisker epidermis (carrying whisker pegs), then recombined with mutant epidermis. Normal whisker structures developed from this type of double recombination. (hs) Hair shaft; (irs) inner root sheath; (ors) outer root sheath; (d) dermal sheath; (p) papilla. (*D*) Mutant E12 snout epidermis first combined with normal E12 whisker pad mesenchyme. After 36 hr (and formation of epidermal whisker pegs), the normal mesenchyme was replaced by mutant E12 mesenchyme. Whisker development continued to some extent (note outer dermal sheath) but remained incomplete (note absence of epidermal differentiation and of a dermal papilla). (d) dermal sheath; (sg) sebaceous gland. Bars, (*A*–*C*) 200 μm; (*D*) 100 μm.

tors, and extracellular matrix molecules have previously been shown to be expressed in spatial and temporal patterns consistent with inductive tissue interactions during organogenesis (Vainio et al. 1989, 1993; Jones et al. 1991; Lyons et al. 1991; MacKenzie et al. 1991; Jowett et al. 1993; Bitgood and McMahon 1995). With the aim of gaining insight into a putative relationship of some of these molecules with *Lef1*, we initially examined their expression in *Lef1*$^{-/-}$ embryos. Transcripts of the transcription factor gene *Msx1*, whose targeted inactivation also results in an arrest of tooth development at E13 (Satokata and Maas 1994), were detected at a similar level in normal and *Lef1*$^{-/-}$ tooth germs (Fig. 6A). Thus, *Msx1* may act either upstream of *Lef1* in a putative genetic hierarchy or, alternatively, in a different pathway. Moreover, as shown in Figure 6A, the *Lef1*$^{-/-}$ mutation did not alter the expression of *Bmp4*, encoding a TGF-β-like signaling molecule that had been previously shown to activate *Msx1* expression in presumptive dental mesenchyme (Vainio et al. 1993). Likewise, expression of the related *Bmp2* gene can be detected in the dental epithelium of *Lef1*$^{-/-}$ embryos at E13 (data not shown). In addition, we examined the expression of the transcription factor genes *Msx2*, *AP2*, and that of another TGF-β-like signaling molecule, activin βA, which has been recently shown to regulate tooth development (Matzuk et al. 1995). Expression of these genes in *Lef1*$^{-/-}$ tooth germs was unchanged relative to normal embryos (data not shown).

BMP-4 has been previously proposed to represent a morphogenetic signal that mediates epithelial–mesenchymal interactions during tooth development (Vainio et al. 1993). Local application of BMP-4-containing agarose beads to presumptive dental mesenchyme of E11 embryos mediates morphogenetic and molecular changes that resemble the effects of dental epithelium, including the transcriptional activation of the *Msx1* and *Msx2* genes (Vainio et al. 1993). *Lef1* is expressed during early tooth development in a pattern similar to that of *Bmp2* and *Bmp4*, suggesting that these genes may participate in a common regulatory pathway (Vainio et al. 1993; van Genderen et al. 1994). The apparently normal expression of both *Bmp2* and *Bmp4* in *Lef1*-deficient embryos, however, suggested that BMPs may not be regu-

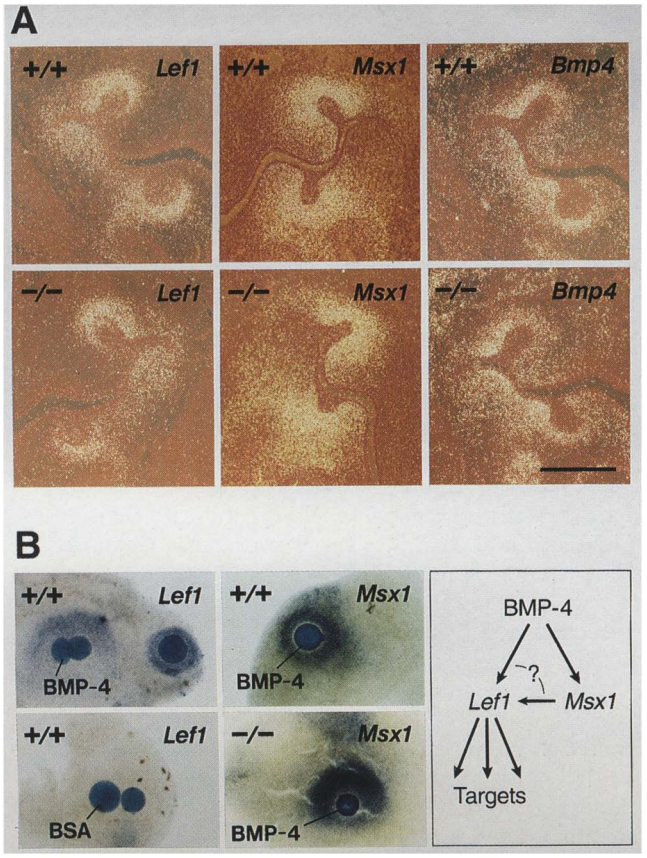

Figure 6. *Lef1* may act downstream of *Bmp4*. (*A*) Expression of *Lef1*, *Msx1*, and *Bmp4* in wild-type (+/+) and mutant (−/−) tooth germs of E13 embryos. Coronal sections were hybridized with specific RNA probes. Prominent expression of all three genes is detected in the condensing mesenchyme around the tooth buds of wild type and mutant embryos. Bar, 200 μm. (*B*) BMP-4 activates *Lef1* gene expression in dental mesenchyme. The presumptive dental mesenchyme (E11) was cultured for 18–24 hr with beads soaked in BMP-4 (100 ng/ml) or BSA (100 ng/ml). Expression of *Lef1* and *Msx1* was examined by whole-mount in situ hybridization. Diameter of beads (75–100 μm). A scheme of the putative regulatory hierarchy of *Bmp4*, *Lef1*, and *Msx1* is shown on the *right*.

lated by *Lef1*. Therefore, we examined the possibility that BMPs regulate the expression of *Lef1*. *Bmp4* is expressed in the dental epithelium of E10 embryos and mesenchyme of E13 and older embryos, whereas dental epithelial cells of the enamel knot express both *Bmp2* and *Bmp4* at E13/E14 (Vaahtokari et al. 1996). Although our tissue recombinations indicated that *Lef1* function is restricted to the epithelium, we could not examine the effects of BMP-containing beads on *Lef1* expression in dental epithelium because this tissue cannot be cultured in the absence of mesenchyme. For this reason, we examined the potential of recombinant BMP-4 to induce *Lef1* expression in presumptive dental mesenchyme of E11 embryos (Fig. 6B). *Lef1* transcripts were detected by whole mount in situ hybridization in the area around the BMP-4 beads. *Lef1* was never detected in E11 mesenchyme cultures exposed to BSA-containing agarose beads, indicating that the effect of BMP-4 on *Lef1* expression is specific. To examine the relationship between *Lef1* and *Msx1* in this BMP-4 mediated signaling pathway, we analyzed the BMP-4-induced expression of *Msx1* in presumptive dental mesenchyme from normal and *Lef1*$^{-/-}$ embryos. *Msx1* transcripts were induced irrespective of the genotype of the embryo (Fig. 6B). Taken together, these data suggest that *Lef1* acts downstream of a BMP-4 signal and may be activated either via *Msx1* or via an independent pathway.

Developmental decisions are often stabilized by positive feedback loops in which cell type-specific transcription or growth factors autoregulate their own expression (Bienz 1992). In particular, BMP-4 was shown to autoregulate its own expression (Vainio et al. 1993). To examine whether LEF-1 protein regulates the synthesis of its mRNA, we analyzed normal and mutant E13 embryos for the presence of *Lef1* transcripts by in situ hybridization. In the *Lef1*$^{-/-}$ mice, the insertion of the *neo*^{R} gene into an exon encoding part of the HMG domain of LEF-1 interferes with protein expression and function (van Genderen et al. 1994), but allows for accumulation of detectable *Lef1* transcripts from the mutant allele. Similar levels of *Lef1* transcripts were detected in normal and mutant tooth germs (Fig. 6A), and no change in the distribution of *Lef1* transcripts in whole embryo sections was detectable (data not shown). Thus, the developmental regulation of *Lef1* gene expression is independent of the synthesis of functional LEF-1 protein.

Discussion

We have previously shown by targeted gene inactivation in the mouse that *Lef1* is essential for the development of teeth, hair follicles, and mammary glands (van Genderen et al. 1994). The dependence of the development of these organs on interactions between epithelial and mesenchymal tissues raised the possibility that *Lef1* participates in the control of inductive tissue interactions. In this study, we have performed heterologous combinations between normal and *Lef1*$^{-/-}$ tissues to identify the tissue type and the developmental stage of functionally important *Lef1* expression in tooth and whisker organogenesis. The results of these experiments provide strong evidence for a direct role of *Lef1* in the transcriptional control of inductive tissue interactions. First, tooth and whisker formation is dependent on *Lef1* expression in only one tissue although LEF-1 protein can be detected in both the epithelium and the mesenchyme throughout organogenesis. Tooth and whisker development depends on *Lef1* expression in the epithelium, whereas *Lef1* expression in the mesenchyme is also essential for the initiation of whisker development. Second, after the critical stage of *Lef1* expression in one tissue, the partner tissue acquires the capacity to form normal organs in combination with mutant tissue, suggesting that the effect of this transcription factor has been transmitted from one tissue to the other. Moreover,

the *Lef1*-dependent events in both tooth and whisker development, that is, the formation of the mesenchymal dental and dermal papillae and of epidermal whisker placodes, occur in the tissues in which the endogenous *Lef1* gene is dispensable for organogenesis. Finally, *Lef1* expression is required only transiently during specific inductive events in the initiation of organ development and/or morphogenesis but is dispensable for cytodifferentiation of either tissue.

Role of Lef1 *in tooth development*

Targeted inactivation of the *Lef1* gene resulted in an arrest of early tooth development at E13, after formation of the epithelial tooth bud and mesenchymal condensation but before morphogenesis, that is, folding of the epithelium and formation of the mesenchymal papilla (van Genderen et al. 1994). Our tissue recombination experiments now indicate that *Lef1* expression in the dental epithelium is necessary and sufficient to overcome the developmental arrest in odontogenesis. However, after the formation of a mesenchymal dental papilla between E13 and E14, epithelial *Lef1* expression is no longer needed for further morphogenesis. Moreover, the development of morphologically normal teeth in double recombinations, in which both tissues are eventually *Lef1*-deficient, demonstrates that the requirement for LEF-1 action is both transient and non-cell-autonomous. Together, these observations suggest that *Lef1* expression in the epithelium is critical for the induction of the mesenchyme between E13 and E14 to form a dental papilla, but is dispensable for both the initiation of tooth development and the epithelial and mesenchymal cytodifferentiation.

Classical tissue recombination experiments have revealed sequential and reciprocal epithelial–mesenchymal interactions in tooth development (Fig. 7A; for review, see Thesleff and Hurmerinta 1981; Lumsden 1988). It is generally assumed that odontogenesis is initiated by signals from oral epithelium to the neural crest-derived mesenchyme of the first branchial arch. Thereafter, the mesenchyme remains committed to its odontogenic fate, and capable of dictating further tooth development as shown by its ability to induce formation of an enamel organ in nonodontogenic epithelium (Mina and Kollar 1987). This transition from epithelial to mesenchymal dominance takes place around E11/E12, well before the mesenchyme becomes independent of *Lef1*-expressing epithelium (Mina and Kollar 1987; Lumsden 1988). It is therefore unlikely that *Lef1* is involved in this early action of the epithelium on the mesenchyme. Moreover, the results of our E13 molar/incisor combinations with mutant mesenchyme indicate that mesenchymal commitment for the type of tooth (i.e., molar vs. incisor) had occurred either before, or at least independently of, the *Lef1*-dependent epithelial induction of the mesenchymal dental papilla.

The requirement for *Lef1* function in the developing tooth germ is much more limited than anticipated from the complex spatial and temporal expression pattern of the gene. From E10 to E12, *Lef1* transcripts are detected initially in the epithelium and subsequently in the mesenchyme, consistent with the change in the developmental dominance of these tissues (schematically summarized in Fig. 7A). However, an essential function for *Lef1* expression could be demonstrated only in the dental epithelium between E13 and E14, which coincides with the presence of *Lef1* transcripts in the most basal cells of the epithelial tooth bud, the future enamel knot. This structure has been proposed to function as a signaling

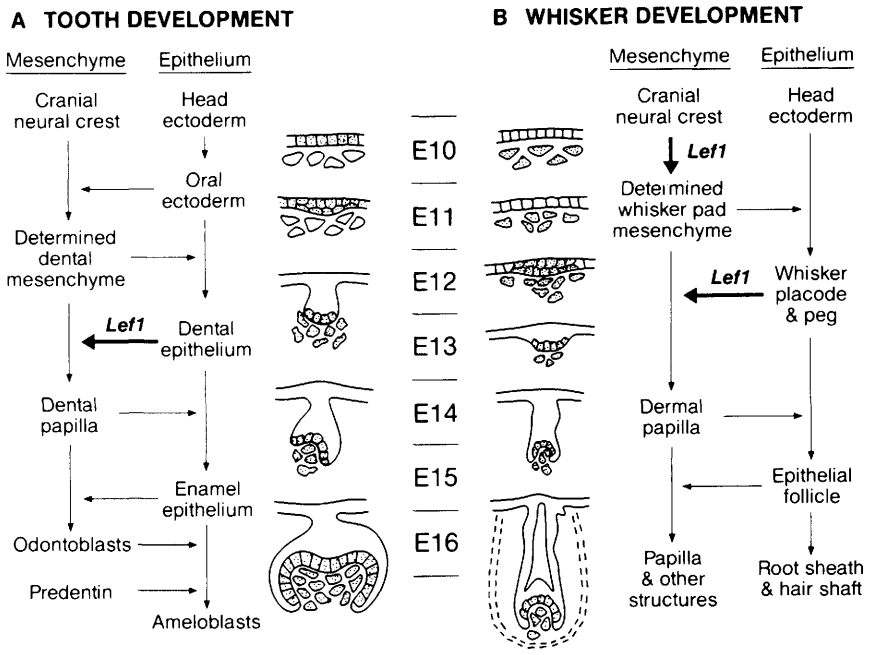

Figure 7. Schematic diagram of tissue interactions and *Lef1* function during tooth and whisker follicle development. Vertical lines indicate progressive organogenesis. Horizontal lines indicate tissue interactions with the directionality of the inductive events shown. The change in the shape of epithelial and mesenchymal tissues during organogenesis is schematically presented. Expression of the *Lef1* gene in epithelial and/or mesenchymal cells is shown by shading. (*A*) Scheme of putative tissue interactions during tooth development (based on Thesleff and Hurmerinta 1981, and Lumsden 1988). The *Lef1*-dependent induction of the dental papilla by basal cells in the epithelium is shown by a thick arrow. (*B*) Scheme of suggested tissue interactions during whisker follicle development. The determination of whisker pad mesenchyme and the induction of the dermal papilla are *Lef1*-dependent.

center for tooth morphogenesis (Jernvall et al. 1994; Vaahtokari et al. 1996), and our data suggest that LEF-1 may regulate this signaling activity.

Role of Lef1 *in whisker development*

The absence of visible whisker placodes and pegs in *Lef1*$^{-/-}$ embryos indicates that *Lef1* is essential for the initiation of whisker development. Our tissue recombinations have now identified the mesenchyme as the tissue which has to express *Lef1* for the initiation of whisker development. At E11, whisker follicles were obtained only in combinations containing normal mesenchyme, indicating that mesenchymal *Lef1* expression is sufficient at this early stage. This finding is consistent with results of classical experiments which suggested that the dermis initiates the development of skin appendages by induction of the epidermis (for review, see Sengel 1976). Dermal condensations within the whisker pad are observed before the formation of epidermal placodes and represent the first morphologically visible event in whisker development (Wessells and Roessner 1965). Moreover, mesenchymal condensations can induce hair follicle formation in transplantation under hairless epithelium (Kollar 1970). Thereafter, the epidermal placodes interact with subjacent mesenchyme by inducing the formation of dermal papillae as demonstrated by combining epidermal feather placodes of chick embryos with either immature or genetically incompetent dermis (Sengel 1958; Sengel and Abbott 1963).

In the initiation of whisker development, *Lef1* could be involved either in the determination and organization of the mesenchyme or directly in the induction of the epithelium to form placodes. The results of the double recombination in which mutant mesenchyme was transiently exposed to normal E12 epithelium and subsequently combined with mutant epithelium indicate that induced mesenchyme can mediate whisker formation in the absence of functional LEF-1 protein. Therefore, it is unlikely that mesenchymal *Lef1* expression is directly involved in the induction of epithelial whisker placodes, and we favor the view that *Lef1* participates in the preceding determination of the mesenchyme.

The full development of whisker follicles, however, is dependent on at least one other step that is controlled by *Lef1*. The double recombination in which normal mesenchyme was replaced by mutant tissue, after induction of the epithelial whisker pegs, allowed only for incomplete morphogenesis. In addition, the reciprocal double recombination in which mutant E12 mesenchyme was transiently exposed to normal E12 epithelial whisker pegs developed relatively normal whisker follicles, revealing a requirement for epithelial *Lef1* expression. In contrast, single recombinations in which normal mesenchyme was associated with mutant epithelium developed normal whiskers. Thus, epithelial induction of the mesenchyme to form a whisker papilla requires either continuous LEF-1 action in the mesenchyme or *Lef1* expression in the epithelial placodes and pegs (Fig. 7B). Taken together, these observations suggest that in normal whisker development a second *Lef1*-mediated signal from the epithelium may help to stabilize the mesenchymal commitment to form a whisker papilla.

The functions of *Lef1* in tooth and whisker development appear to be similar in several aspects. In both organs, *Lef1* is required for the induction of a mesenchymal papilla by an invaginated epithelial bud without a need for mesenchymal *Lef1* expression. Moreover, epithelia in both cases express *Lef1* in their basal cell layer which is in immediate contact with a condensed mesenchyme. Finally, in both organs LEF-1 independence is reached with the formation of a mesenchymal papilla, and further morphogenesis and cytodifferentiation do not require *Lef1* despite its continuing expression. However, the role of *Lef1* in tooth and whisker organogenesis differs in at least one aspect. Formation of the epithelial whisker placodes and pegs is completely dependent upon prior expression of *Lef1* in the mesenchyme, whereas epithelial tooth buds are formed in *Lef1*$^{-/-}$ embryos. In this regard, tooth development resembles the formation of mammary glands and of body hair follicles, which are arrested at early stages (van Genderen et al. 1994). The difference in the requirement for *Lef1* expression in the initiation of organogenesis is unclear but it may reflect a partial redundancy of *Lef1* in some organ systems. For example, *Lef1* may be redundant with the closely related *Tcf1* gene (Travis et al. 1991; Waterman et al. 1991; van de Wetering et al. 1991). In developing tooth germs, however, the expression of *Tcf1* does not overlap with that of *Lef1* and, therefore, the initiation of tooth development may be independent of both *Lef1* and *Tcf1*.

Lef1 *is part of a BMP-mediated pathway in inductive tissue interactions*

Three mechanisms have been proposed for the transmission of inductive signals in organogenetic tissue interactions: diffusible factors, cell–cell contacts, and interactions mediated by the extracellular matrix (for review, see Grobstein 1967; Saxén et al. 1976; Birchmeier and Birchmeier 1993). Our tissue recombination experiments indicate that LEF-1 regulates an inductive process in tooth and whisker organogenesis, although the elucidation of the precise mechanism of LEF-1 action will have to await the identification of genetic targets of *Lef1*. The observation that *Lef1* expression can be activated in presumptive dental mesenchyme by the signaling molecule BMP-4 suggests that *Lef1* may function downstream of *Bmp4* in a putative regulatory pathway. However, we cannot rule out the possibility that *Lef1* also regulates the expression of *Bmp4* in a feedback loop. According to this view, *Lef1* would be redundant with another transcription factor because *Bmp4* transcripts can be detected in the dental mesenchyme of *Lef1*-deficient embryos at normal levels. BMP-4 was also shown to activate the expression of the transcription factor gene *Msx1* (Vainio et al. 1993), whose inactivation results in arrest of tooth development at precisely the same stage as in *Lef1*$^{-/-}$ embryos (Satokata and Maas 1994). Thus, BMPs may act on both *Lef1* and *Msx1*. In this scheme, *Msx1*

may function upstream of *Lef1* or in parallel because the expression of *Msx1* is activated by BMP-4 in *Lef1*$^{-/-}$ embryos.

A role of BMPs in organogenesis was inferred from experiments in which *Bmp4* was misexpressed in the outer sheath of hair and whisker follicles, resulting in the perturbation of hair follicle development and loss of cell proliferation (Blessing et al. 1993). In tooth development, *Bmp2* and *Bmp4* are expressed in spatial and temporal patterns which together could account for the developmental expression pattern of *Lef1* (Lyons et al. 1990; Jones et al. 1991; Vainio et al. 1993; Vaahtokari et al. 1996). BMP-2 and BMP-4 have been shown to have overlapping and complementary functions in various developmental processes. BMP-4 can act as a posterior–ventralizing factor in mesoderm induction (Dale et al. 1992; Jones et al. 1992; Graff et al. 1994; Winnier et al. 1995) whereas BMP-2 may provide an epithelial signal in limb development (Niswander and Martin 1993; Francis et al. 1994). The identification of the functionally important BMP in *Lef1*-mediated regulation of tooth development, however, will require further in vivo analysis because BMP-2 and BMP-4 are interchangeable in stimulating gene expression in vitro (Vainio et al. 1993). Moreover, the mechanism of BMP-mediated signaling in organogenesis is unclear, but it may involve diffusion in the responding tissue or propagation of the inductive signal by a relay-like series of cell–cell interactions (Gurdon 1992). In conclusion, our data suggests that *Lef1* may function in a BMP-mediated signaling pathway and we have now formally identified LEF-1 as a transcription factor that regulates inductive tissue interactions in at least two epithelial–mesenchymal organ systems.

Materials and methods

Mice

The generation and analysis of the LEF-1 null mutant mice has been described previously (van Genderen et al. 1994). Timed embryos were obtained by crossing *Lef1*$^{+/-}$ heterozygous mice counting the vaginal plug as day 0.5. The embryonic stages were confirmed by morphological criteria. Homozygous mutant mice can easily be identified by E12 because they lack characteristic whisker hillocks on the snout. The genotypes of all embryos were confirmed by polymerase chain reaction (PCR) analysis as described (van Genderen et al. 1994). Both wild-type and heterozygous embryos were used as donors for normal tissue.

Tissue dissection and recombination

Embryos were dissected in Dulbecco's PBS. Tooth anlagen were taken from the lower jaw. From E10 to E12, the entire jaw (oral face) was used, at E13 the incisor pair and the individual molar rudiments were prepared separately. From E14 onwards, only the molar anlagen were used because of the difficulties in isolating the deeply invaginated incisor epithelium from normal embryos. Dental epithelium and mesenchyme were mechanically separated after incubation in 0.1% crude collagenase (Sigma type I) in Dulbecco's minimal essential medium (DMEM) for 20 min at 37°C. Whisker pads were taken from E11 to E13 embryos; E13 is the latest stage at which clean dermal–epidermal separation can be achieved in normal embryos (due to the rapid ingrowth of the whisker). The tissues were separated after incubation in 2.25% crude trypsin (Sigma type II) and 0.75% pancreatin (Difco N.F.) in PBS for 15–30 min on ice; protease digestion was stopped in 30% horse serum. For recombination, epithelium and mesenchyme were aligned in the right orientation on top of Nuclepore membrane filters (pore size 0.1 μm, Costar), and subsequently cultured in DMEM supplemented with 10% fetal calf serum and 10% chick embryo extract. After 2 days in vitro, the recombinations were transplanted under the kidney capsule of adult mice for 8–12 days, to allow for full development of teeth or whiskers.

Histological procedures

The explanted tissue recombinants were fixed in Bouin's solution, embedded in paraffin, serially sectioned at 7 μm, and stained with hematoxylin–eosin or Azan stain according to standard procedures. For plastic sections, dental areas and whisker pads from wild-type and mutant animals were fixed in 4% paraformaldehyde and 2% glutaraldehyde in 0.1M phosphate buffer, postfixed in 2% osmium tetroxide, embedded in epoxi resin, sectioned at 1 μm, and stained with 1% toluidine blue.

In situ hybridization and immunocytochemistry

For in situ hybridization, embryos or recombination explants were fixed in 4% paraformaldehyde in PBS, embedded in paraffin, and sectioned at 7 μm. The preparation of the RNA probes and the hybridization conditions have been described previously (van Genderen et al. 1994). The *Lef1* probe included nucleotides 1158–1517 (Travis et al. 1991). The *Bmp4* probe consisted of a 285-bp *Pst*I–*Eco*RI fragment (Wozney et al. 1988; Vainio et al. 1993), the *Msx1* probe of a 700-bp *Eco*RI–*Bgl*II fragment (MacKenzie et al. 1991). The probe for hair keratin A1 was prepared by reverse transcriptase-based PCR and cloning and comprised of nucleotides 1009–1127 (Kaytes et al. 1991).

Immunocytochemistry was performed on 10-μm-thick cryosections from embryos fixed in 4% paraformaldehyde in PBS, cryoprotected in 30% sucrose/PBS, and frozen with dry ice. Rabbit anti-murine LEF-1 antibodies were used at 1:100, and rabbit anti-syndecan (Pharmingen) at 1:1000. The ABC method was used for immunodetection as described (van Genderen et al. 1994).

Incubation of tissue explants with agarose beads

Freshly dissected presumptive dental mesenchyme from the lower jaw of E11 embryos was incubated for 18–24 hr with Affigel blue agarose beads (100–200 mesh, 75–150 μm diam.; Bio-Rad) soaked with recombinant BMP-4 protein (100 ng/ml; gift of E. Wang, Genetics Institute) or BSA (100 ng/ml) as described by Vainio et al. (1993). The tissue explants were subsequently fixed in 100% methanol and the expression of *Lef1* and *Msx1* was determined by whole-mount in situ hybridization (Wilkinson and Nieto 1993).

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