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Development of several organs that require inductive epithelial–mesenchymal interactions is impaired in *LEF-1*-deficient mice

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Lymphoid enhancer factor 1 (LEF-1) is a sequence-specific DNA-binding protein that is expressed in pre-B and T lymphocytes of adult mice, and in the neural crest, mesencephalon, tooth germs, whisker follicles, and other sites during embryogenesis. We have generated mice carrying a homozygous germ-line mutation in the *LEF-1* gene that eliminates its protein expression and causes postnatal lethality. The mutant mice lack teeth, mammary glands, whiskers, and hair but show no obvious defects in lymphoid cell populations at birth. The *LEF-1*-deficient mice also lack the mesencephalic nucleus of the trigeminal nerve, the only neural crest-derived neurons normally present within the brain, but no deficiency can be detected in other neural crest-derived neuronal populations. Together, the pattern of these defects suggest an essential role for *LEF-1* in the formation of several organs and structures that require inductive tissue interactions.

[*Key Words*: Lymphoid enhancer factor 1; neural crest; organogenesis; mouse development]

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Naturally occurring and experimentally induced mutations have revealed that many developmental processes are controlled by transcriptional regulatory proteins. In particular, DNA-binding proteins that are expressed in specific cell lineages, or at specific stages of differentiation, participate in the developmental regulation of gene expression (for review, see Gruss and Walther 1992; Voss and Rosenfeld 1992; Weintraub 1993). Although some transcriptional regulators are restricted to single cell lineages, most are expressed in multiple lineages and often by phenotypically unrelated cell types. Such pleiotropic expression may indicate that a transcriptional regulator can subserve multiple functions through combinatorial association with distinct proteins in different cell types (Herskowitz 1989). Alternatively, pleiotropic expression may suggest that a given factor participates in a developmental process that is common to many different cell types or tissues.

Lymphoid enhancer-binding factor 1 (LEF-1) was cloned initially as a pre-B and T lymphoid-specific gene encoding a DNA-binding protein of the family of high mobility group (HMG) proteins (Travis et al. 1991; Waterman et al. 1991). LEF-1 is encoded by a gene located on chromosome 3 in the mouse and chromosome 4 in humans (Milatovich et al. 1991). Sequence-specific recognition of DNA by LEF-1 protein was found to be governed by an 85-amino-acid region, termed the HMG domain, that displays sequence homology with other members of this family of proteins (Giese et al. 1991). Functional and biochemical characterization of LEF-1 indicated that this protein participates in the regulation of the enhancer associated with the T-cell receptor (TCR)α gene (Travis et al. 1991; Waterman et al. 1991). LEF-1 protein has the capacity to induce a sharp bend in the DNA helix and is dependent on other enhancer-bound proteins to activate transcription (Giese et al. 1992). Together with the requirement for a particular arrangement of factor-binding sites in the TCRα enhancer, these observations were interpreted to suggest an "architectural" role for LEF-1 in the assembly of a higher order nucleoprotein complex (Grosschedl et al. 1994; Tjian and Maniatis 1994). In addition, LEF-1 contains a transcriptional activation domain that is enhancer context specific and may mediate, directly or indirectly, the association with other enhancer-bound proteins (Carlsson et al. 1993, Giese and Grosschedl 1993).

Despite these insights into the biochemical properties of LEF-1, its biological role is still obscure. In the adult mouse, the *LEF-1* gene is expressed specifically in lymphoid tissues (Travis et al. 1991; Waterman et al. 1991). During mouse embryogenesis, however, a wider pattern of RNA expression from the *LEF-1* gene was detected by in situ hybridization analysis (Oosterwegel et al. 1993). At day 10.5 of embryogenesis (E10.5), *LEF-1* is expressed

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in the neural crest, branchial arches, and limb buds. At E14.5, expression of *LEF-1* was found in the thymus, lung, kidney, tooth germs, brain, and inner ear. This developmentally regulated pattern of expression indicated that *LEF-1* is a marker for the formation of various organs.

Morphogenesis of vertebrate organs depends on reciprocal inductive interactions between different tissues that result in differentiation and organization of cells into organs (Spemann and Mangold 1924; Gurdon 1991). In particular, inductive interactions between epithelial and subjacent mesenchymal tissues have been implicated in mediating organogenesis (Grobstein 1967). Whereas the mesenchymal component of such organs is usually of mesodermal origin, the mesenchyme of the head and the neck is derived from the cranial neural crest ("mesectoderm") (Le Douarin 1982; Noden 1984, 1992). Cranial neural crest-derived cells form the connective tissue and the visceral skeleton of head and neck, and these cells interact with ectoderm or endoderm in the formation of organs such as teeth, whiskers, or salivary glands. During these types of tissue interactions, the expression of several genes encoding transcription factors, growth factors, cell surface molecules, and components of the extracellular matrix has been shown to be regulated (Robert et al. 1989; Lyons et al. 1990; Jones et al. 1991; MacKenzie et al. 1991, 1992; Vainio et al. 1991, 1993; Jowett et al. 1993). However, the functional roles of these genes in organogenesis remain to be established.

In this study, we have extended the analysis of the developmental pattern of *LEF-1* gene expression by in situ hybridization and immunohistochemistry, and have examined the role of *LEF-1* in murine development by targeted gene inactivation. We find that mice homozygous for the mutation in the *LEF-1* gene die shortly after birth and show deficiencies in some but not all organs that express *LEF-1* during their formation. Specifically, the mutant mice lack teeth, mammary glands, whiskers, and body hair, and show an absence of the mesencephalic nucleus of the trigeminal nerve (TMN). Together these findings suggest a crucial role for LEF-1 in the formation of organs that require inductive interactions between epithelial cells of ectodermal origin and mesenchymal cells.

Results

Expression of LEF-1 *in the developing mouse*

We examined the expression of *LEF-1* between E12.5 and E16.5 by in situ hybridization of embryo sections with an ^{35}S-labeled *LEF-1* anti-sense RNA probe. In a sagittal section of an E12.5 embryo, abundant expression of *LEF-1* was detected in the mesencephalon, in the mesenchyme of the snout, and at multiple sites of organogenesis including the ear and dental placodes (Fig. 1A). In a mid-sagittal section of an E14.5 embryo, major sites of *LEF-1* gene expression were identified in the mesencephalon, tooth germs, whisker follicles, pituitary gland, and kidney (Fig. 1B). The abundance of *LEF-1* gene expression is reduced markedly at E16.5, but persists at high levels in the whisker follicles (Fig. 1F) and thymus (data not shown).

Previous in situ hybridization studies at E10.5 identified abundant *LEF-1* transcripts in the presumptive dental epithelium (Oosterwegel et al. 1993). We also observed prominent *LEF-1* mRNA expression in the dental placodes of day 12.5 embryos, but found that at this stage it was confined predominantly to the condensed neural crest-derived mesenchyme underlying the ectodermally derived dental epithelium (Fig. 1C). Moreover, in the cap stage of tooth development at E14.5, expression of *LEF-1* could be detected in both dental epithelium and dental papilla mesenchyme (Fig. 1D). The pattern of *LEF-1* expression in the developing tooth was also confirmed at the cellular level by immunohistochemistry with purified antibodies directed against LEF-1 protein (Fig. 1E). This spatial and temporal expression pattern of *LEF-1* coincides with inductive interactions between epithelial and mesenchymal cells during tooth development (Slavkin 1974; Thesleff and Hurmerinta 1981; Kollar 1983). Specifically, heterologous and heterochronic tissue recombination studies have shown that the presumptive dental epithelium has potential to induce ectopic partner tissue to form a tooth until day 12 of embryogenesis, after which time this developmental potential shifts to the condensing mesenchyme (Kollar and Baird 1970; Mina and Kollar 1987).

Higher magnification of an E12.5 embryo revealed hybridization in a defined region of the midbrain containing cells that constitute the TMN (Fig. 1G). These neurons have distinctive size and morphology and have been shown, by lineage tracing experiments in birds, to derive from the cranial neural crest (Narayanan and Narayanan 1978). Immunohistochemistry of E12.5 embryo sections with antibodies directed against LEF-1 demonstrated that the protein was expressed in cells within the TMN and immunostaining was localized predominantly to the nuclei of these cells (Fig. 1H).

Targeted inactivation of the LEF-1 *gene*

Using an 8-kb genomic *LEF-1* DNA fragment, we generated an insertion-type targeting gene construct in which the *phosphoglycerokinase* (*PGK*)–*neo* gene was inserted into the second exon of the HMG domain of LEF-1 (Fig. 2A). Previous analysis of amino- and carboxy-terminal LEF-1 polypeptides indicated that the HMG domain of LEF-1 is essential for DNA binding (Giese et al. 1991). To enrich for homologous recombinants, we also included a *PGK–thymidine kinase* (*tk*) gene in the plasmid (Thomas and Capecchi 1987). The *LEF–neo–tk* targeting construct was linearized and electroporated into D3 embryonic stem (ES) cells, and clones were selected doubly for the presence of the *neo* gene and the loss of the *tk* gene (Ramírez-Solís et al. 1993). Clones were analyzed by digesting genomic DNA with *Bam*HI and hybridizing DNA blots with a radiolabeled probe derived from genomic sequences 5′ of the *LEF-1* gene in the targeting vector. A 4.9-kb fragment, diagnostic of homologous recom-

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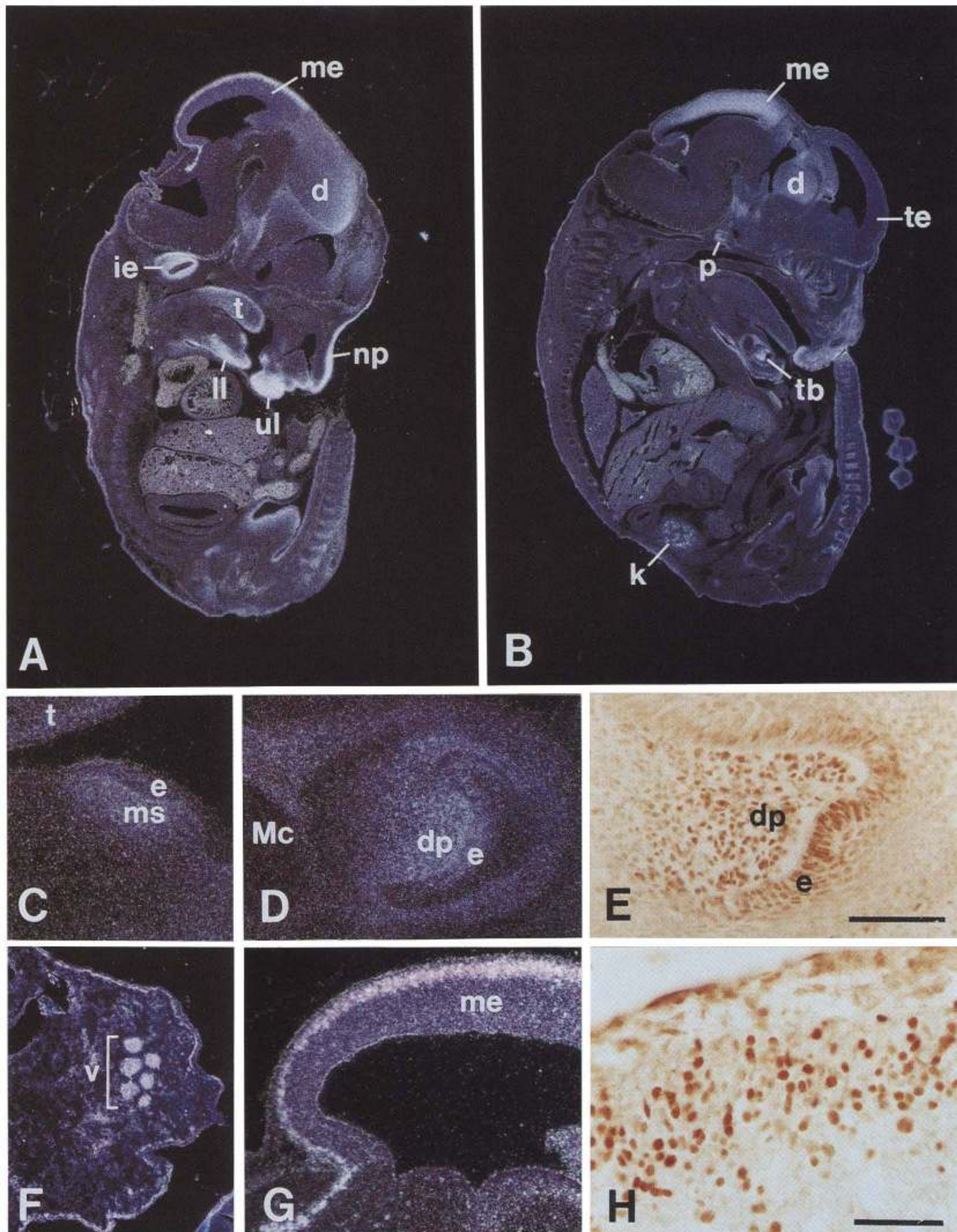

Figure 1. Pattern of expression of *LEF-1* during mouse development. In situ hybridization and immunohistochemistry analysis of sagittal sections of frozen mouse embryos at days 12.5, 14.5, and 16.5 of gestation. Embryo sections were hybridized with a ^{35}S-labeled *LEF-1* anti-sense probe, and sites of expression were visualized by dark-field illumination. The specificity of hybridization was confirmed by using a *LEF-1* sense RNA probe (data not shown). (*A*) Sagittal section of an E12.5 embryo. Sites of abundant *LEF-1* expression include mesencephalon (me), diencephalon (d), inner ear (ie), tongue (t), lower and upper lips (ll, ul), nasal process (np). (*B*) Mid-sagittal section of an E14.5 embryo. Sites of *LEF-1* expression include pituitary gland (p), tooth buds (tb), and the kidney (k). (te) Telencephalon. (*C,D*) Higher magnification of a mandibular tooth germ at early bud stage (E12.5) and at cap stage (E14.5), respectively. At E12.5, *LEF-1* expression is localized predominantly to the mesenchyme (ms) underlying the presumptive dental epithelium (e), but later (E14.5) it is localized to both the dental papilla mesenchyme (dp) and dental epithelium (e). The position of Meckel's cartilage (Mc) is indicated. (*E*) Protein expression of LEF-1 in a mandibular tooth germ at the cap stage (E14.5) was visualized by immunohistochemistry. Bar, 100 μm. (*F*) *LEF-1* RNA expression in the vibrissae (v) of an E16.5 embryo. (*G*) *LEF-1* RNA expression in the mesencephalon (me) of an E12.5 embryo. (*H*) Localization of LEF-1 protein in the mesencephalon of an E12.5 embryo by immunohistochemistry. LEF-1 is expressed in the nuclei of large cells that constitute the developing TMN. Bar, 50 μm.

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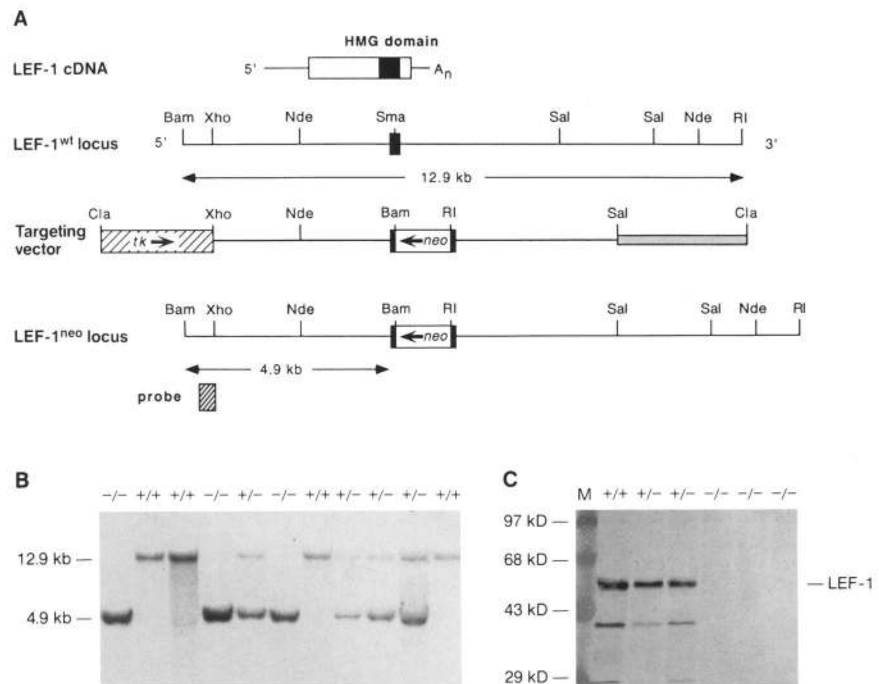

Figure 2. Targeted disruption of the murine *LEF-1* locus. (*A*) The *LEF-1* cDNA with the HMG domain shown as a solid box is indicated at the *top*. The line below represents the wild-type *LEF-1* locus in the region of the HMG domain. The targeting vector includes a *PGK–neo*^{r} gene, inserted into the *Sma*I site residing in the 3′ exon of the HMG domain, and a *tk* gene. Transcriptional polarity of the *neo*^{r} and *tk* genes are indicated by arrows. pBR vector sequences are represented by a shaded box. Sites for restriction enzymes are indicated above the lines, and the length of the fragments generated by a *Bam*HI–*Eco*RI digest that hybridize with a genomic flanking probe are indicated below. (*B*) DNA blot analysis of genomic DNA from a representative litter generated by the mating of heterozygous mutant *LEF-1*-deficient mice. DNA was digested with *Bam*HI–*Eco*RI and probed with a fragment shown in *A*. The 12.9- and 4.9-kb DNA fragments are generated from the wild-type and mutant *LEF-1* allele, respectively. (*C*) Immunoblot analysis of nuclear extract from pre-B cells that were obtained from wild-type, heterozygous, and homozygous mutant *LEF-1*-deficient mice by transformation with Abelson MuLV. The blot was incubated with purified polyclonal antibodies directed against LEF-1 as described in Travis et al. (1991), and LEF-1 was visualized by an alkaline phosphatase secondary antibody. The position of the predominant 55 kD LEF-1 polypeptide is indicated. A minor 39-kD LEF-1 polypeptide is visible, which may be a degradation product or a product of an alternatively spliced *LEF-1* transcript. Equivalent amounts of nuclear extract were used in each lane as confirmed by staining a parallel blot with Ponceau S (data not shown).

bination in the *LEF-1* locus, was detected at a frequency of ~1 in 50 clones (data not shown). Accuracy of the recombination events was also confirmed by DNA blot analysis of *Bam*HI- and *Nde*I-digested DNA that confirmed the integrity of the 3′ homologous arms and presence of a single *neo* integrant (data not shown).

Targeted ES clones were injected into C57BL/6 blastocysts, and resulting chimeric mice with ES cell contribution exceeding 70% were crossed with C57BL/6 wild-type mice. Multiple chimeras from one clone transmitted the targeted allele through the germ line. Crossing the *LEF-1* heterozygous mutant mice, which showed no obvious defects, generated 74 litters with 23% of the offspring homozygous for the mutation. The genotypes of the offspring were determined by DNA blot analysis of genomic DNA digested with *Bam*HI and *Eco*RI and hybridized with a flanking probe (Fig. 2B). Homozygous mutant (−/−) mice had a drastically impaired viability with only 63% surviving the first week after birth and only 5% surviving the second week. No homozygous mutant survived to weaning, indicating that they carry a recessive mutation in an essential gene.

Expression of LEF-1 *in mutant mice*

We have shown previously that *LEF-1* is expressed in pre-B cell lines (Travis et al. 1991). To confirm that the targeted *LEF-1* allele does not produce functional LEF-1 protein, we derived pre-B cell lines from the bone marrow of 10-day-old wild-type (+/+), heterozygous (+/−), and homozygous mutant (−/−) mice by transformation with Abelson murine leukemia virus (MuLV). We prepared nuclear extracts from the pre-B cell lines and assayed for the presence of LEF-1 protein by immunoblot analysis (Fig. 2C). Incubation of the immobilized protein extracts with antibodies directed against LEF-1 protein allowed for detection of 55- and 39-kD LEF-1 polypeptides in pre-B cells from +/+ and +/− mice. In contrast, these polypeptides were not detected in pre-B cell extracts from −/− mice, indicating that the mutation in the *LEF-1* gene eliminates its protein expression.

Arrested follicle development in body hair and whiskers

The most obvious phenotype of the *LEF-1*-deficient mice was the lack of body hair and vibrissae (whiskers) (Fig. 3). In homozygous mutant mice, some rudimentary hair without pigmentation became visible at approximately day 9 after birth but was progressively lost after day 12. Moreover, the mutant mice had a pointed snout and were significantly smaller than sibling heterozygous and wild-type mice.

Hair follicle development in the mouse normally starts between E13 and E14 with the formation of small focal epidermal thickenings, termed placodes, in associ-

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Figure 3. Phenotype of a *LEF-1*-deficient mouse. The homozygous mutant mouse (*left*) at day 16 after birth lacks whiskers and body hair. Moreover, the snout has a pointed appearance and the animal is significantly smaller compared to a wild-type sibling mouse (*right*).

ation with small dense aggregates of mesenchyme, termed dermal papilla (Sengel 1976; Hardy 1992). Subsequently, the epidermal placodes grow into the underlying dermis and by E18, most nascent follicles have acquired characteristics of a mature follicle.

Histological examination of the skin of *LEF-1*-deficient embryos at E14 and E16 revealed that follicle formation began at the expected time and proceeded through the early stages, but the number of follicles was reduced to one-third of that found in wild-type mice (data not shown). In 3-day-old homozygous mutant mice, however, virtually all follicles present were short and rudimentary, suggesting an arrest in development at a stage corresponding to E17 (Fig. 4A–D). Moreover, the skin of the mutant mice was deficient in dermal fat and the follicles lacked melanin. Because *LEF-1* is expressed in neural crest-derived cells, we examined the skin for the presence of melanocytes, which are of neural crest origin. We performed immunohistochemistry on sections of skin with an antibody directed against the melanocyte-specific antigen c-kit (Nishikawa et al. 1991). Melanocytes were detected in both wild-type and mutant skin (Fig. 4E,F), suggesting that the absence of melanin in the skin of *LEF-1*-deficient mice may represent a functional defect secondary to the arrest in hair follicle development (Billingham and Silvers 1969).

The defect in hair development was most pronounced in the whiskers, which normally are much larger and develop 2–3 days earlier than other hair on the body. No whiskers were detected in postnatal mutant animals at day 3 (Fig. 5C,D). The lack of whiskers and their associated dermal fat seemed to account for the pointed appearance of the animals' snouts, because the mandible and maxillae were structurally normal.

We also extended the histological examination to other epidermal appendages such as nails and sweat glands. Nails and histologically normal sweat glands were detected in the *LEF-1* −/− mice at 10 days after birth (data not shown) indicating that only some skin structures are affected by mutation of the *LEF-1* gene.

Failure of tooth formation

Radiographic examination of homozygous mutant mice at 10 days after birth revealed the absence of the upper and lower incisors, and molars as compared to wild-type littermates (Fig. 5A,B). Bone structure of the jaws appeared relatively normal in the mutant mice, except that the alveolar portions (the bony ridges that support the dental roots and whose formation is known to depend on normal tooth development) were absent from both the mandible and the maxilla. We examined a series of parasagittal sections of wild-type and mutant heads of three-day-old animals. In *LEF-1*-deficient mice, no incisor or molar teeth were detected, whereas these structures were clearly visible in comparable sections of wild-type mice (Fig. 5C,D). To distinguish between a failure of initiation and a failure of completion of tooth development, we performed histologic analysis of the mandible of wild-type and mutant mice at E13 and E15. Tooth development is initiated normally between embryonic days 11 and 12 by invagination of ectodermally derived oral epithelium into the underlying cranial neural crest-derived mesenchyme, generating a tooth germ (Slavkin 1974; Kollar 1983). At E15, the mesenchymal cells condense and form the dental papilla surrounded by a cap-like epithelium. In *LEF-1*-deficient mice at E13, tooth germs at the bud stage were detected that were almost indistinguishable from those of sibling wild-type embryos (data not shown). At E15, however, wild-type mice displayed well-advanced tooth germs at the late cap stage, whereas tooth development in mutant littermates had not proceeded beyond the bud stage (Fig. 5E,F). Thus, tooth development is initiated in the *LEF-1*-deficient mice, but becomes arrested at the bud stage in E13 embryos.

Lack of mammary glands

Mammary glands share with both hair follicle and tooth development the involvement of inductive epithelial–mesenchymal interactions (Kratochwil 1969, 1986; Sakakura et al. 1976). Mammary glands normally develop between E11 and E12 in both female and male mouse embryos, before sexual differentiation of gonads (for review, see Sakakura 1987). At E13, five pairs of mammary buds, three thoracic and two inguinal, can be detected as thickenings of the epidermis on the ventral side of the embryo. Further development of the mammary epithelium into ductal structures is specific to female embryos, and occurs in response to an interaction between the epithelium, the mammary mesenchyme, and the fat pad precursor (Sakakura et al. 1982, 1987).

We examined developing mammary glands for the expression of *LEF-1* by in situ hybridization using cryosections of ventral skin from a wild-type mouse embryo at E13.5 (Fig. 6A). Abundant mRNA expression was de-

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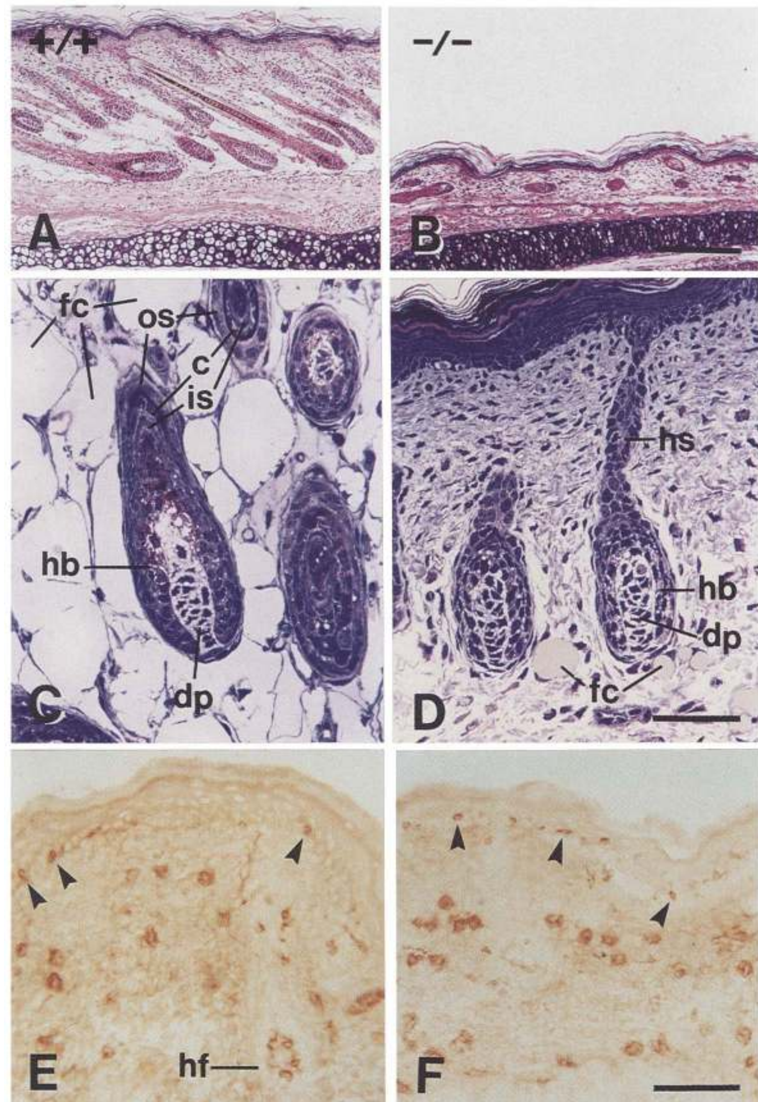

Figure 4. Skin from wild-type (+/+) and mutant (−/−) postnatal day 3 mice. (*A*,*B*) Paraffin sections through the skin in the head of a wild-type (*A*) and mutant (*B*) animal stained with hematoxylin–eosin. The skin from the mutant is considerably thinner, lacks dermal fat, and has fewer hair follicles. Bar, 200 μm. (*C*,*D*) Plastic sections, stained with toluidine blue, showing the morphology of wild-type (*C*) and mutant (*D*) hair follicles. Hair follicles in the mutant are rudimentary and fail to project as deeply into the dermis as normal follicles. The hair sheath is not fully differentiated. The epidermis of the mutant, however, is histologically normal. It shows an apparently normal layering and keratinization. In contrast, the dermal adipocytes in the mutant are severely underrepresented. The brown pigment in wild-type hair follicles (*A*,*C*) corresponds to melanin granules not seen in mutant follicles (*B*,*D*). (c) Cortex; (dp) dermal papilla; (fc) fat cell; (hb) hair bulb; (hs) hair sheath; (is) inner sheath; (os) outer sheath. Bar, 50 μm. (*E*,*F*) Sections from wild-type (*E*) and mutant (*F*) mice immunostained with antibodies to c-kit, a marker for melanocytes. Note the normal presence of both epidermal (arrowheads) and dermal melanocytes. The lack of melanin in the mutant hair follicles is not attributable to a lack of melanocytes but to a failed melanogenic activity caused by the arrested hair growth cycle. (hf) Hair follicle. Bar, 100 μm.

tected in the epidermis of the skin and in the mammary bud. This pattern of expression was confirmed by immunohistochemistry with antibodies directed against LEF-1 protein (Fig. 6B), indicating that LEF-1 is expressed in developing mammary glands.

Examination of newborn *LEF-1* −/− females by histological staining of whole mounts revealed a lack of mammary glands (data not shown). To determine whether mammary gland development is initiated in the mutant mice, we examined the skin of embryos at E13.5 for the presence of mammary buds (Fig. 6C,D). In a partial whole mount of ventral skin from a wild-type embryo, four mammary buds were clearly visible. In contrast, a severely reduced number of rudimentary mammary buds was detected in an equivalent whole mount of skin from a sibling homozygous mutant embryo. This defect was consistently observed in eight mutant embryos examined. Thus, the formation of mammary glands and hair follicles in *LEF-1*-deficient mice shows a similar reduction in the number of anlagen and an arrest at an early stage in organogenesis.

Examination of other organs that depend on inductive tissue interaction during development, such as the gastrointestinal system whose epithelium is endodermal in origin (Haffen et al. 1987), revealed no obvious abnormalities. Likewise, both the endodermal and the mesodermal components of the urogenital system (Saxen 1987; Cunha et al. 1992) appeared normal (data not shown). Thus, the development of organs that involve inductive interactions between epithelium of ectodermal origin and mesenchyme is specifically impaired in the *LEF-1*-deficient mice.

Absence of the TMN

In situ hybridization analysis and immunohistochemistry of mouse embryos indicated that the expression of *LEF-1* in the brain is remarkably restricted to the area in

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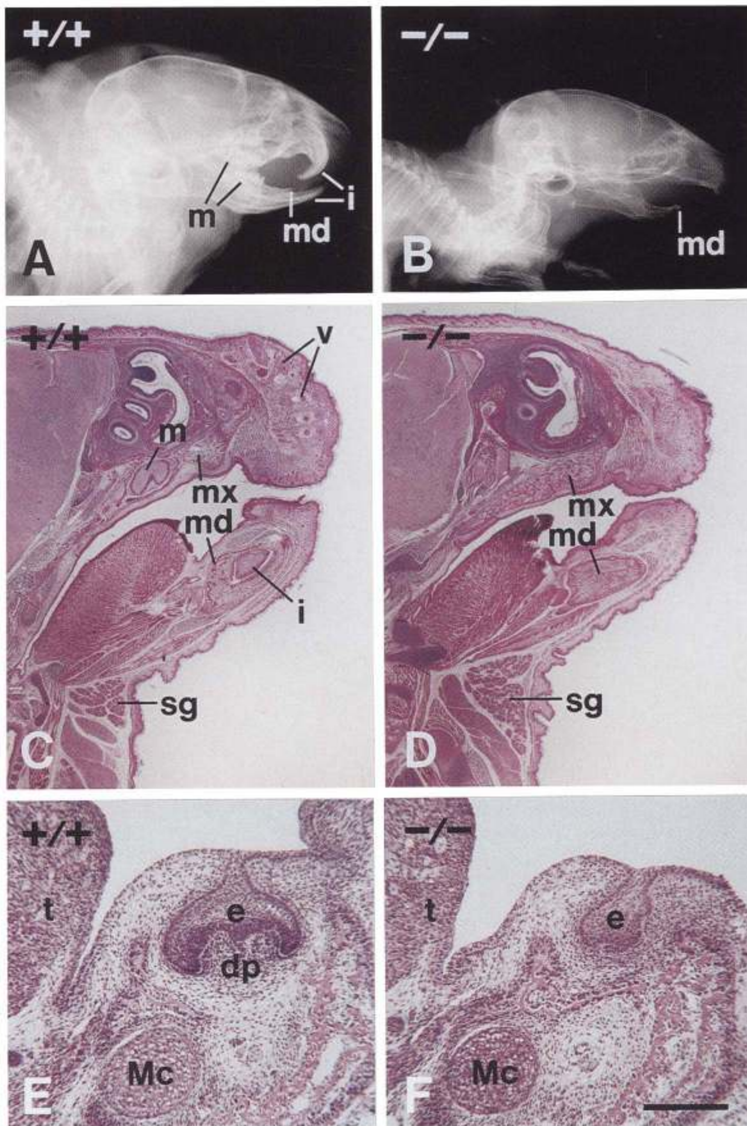

Figure 5. Arrested tooth development in *LEF-1* −/− mice. (*A*,*B*) Radiographic analysis of wild-type (*A*) and homozygous mutant (*B*) mice at day 18 after birth. No differences in the mandibular bone (md) can be detected, whereas the homozygous mutant mouse (−/−) lacks both incisor (i) and molar (m) teeth. (*C*,*D*) Sagittal section of 3-day-old mice stained with hematoxylin–eosin. In the mutant mouse (*D*) no vibrissae (v) and teeth can be detected. (mx) Maxillar bone; (md) mandibular bone; (sg) salivary gland. (*E*,*F*) Tooth germ morphology in E15 embryos. Mandibles were sectioned coronally and stained with hematoxylin–eosin. The molar tooth germ in the wild-type mouse embryo (*E*) is at late cap stage of development. The tooth germ in the mutant embryo (*F*) is arrested at the bud stage of development. (e) Dental epithelium; (dp) dental papilla mesenchyme; (Mc) Meckel's cartilage; (t) tongue. Bar, 200 μm.

which the TMN is developing (Easter et al. 1993). TMN neurons are similar to other primary sensory neuronal populations in both their function to convey sensory information from the periphery to the central nervous system and in their neural crest origins (Davies 1988). However, in contrast to other sensory neurons, TMN neurons are located within the midbrain instead of within a peripheral ganglion. Thus, the TMN contains the only neurons within the brain that are derived from the neural crest (Narayanan and Narayanan 1978). Because of this unique location and the high levels of *LEF-1* expression in the developing TMN, we examined this nucleus in the brains of 10-day-old postnatal animals. The characteristic morphology of fully differentiated TMN neurons allowed their identification in Nissl-stained sections of the brain of wild-type mice (Fig. 7A). In contrast, we could not detect TMN neurons in complete series of sagittal sections through the brain of *LEF-1* −/− mice (Fig. 7B). Absence of TMN neurons in *LEF-1*-deficient mice was confirmed by immunohistochemistry using antibodies directed against $p75^{LNGFR}$, a component of the low-affinity receptor for nerve growth factor present in TMN neurons (Fig. 7C,D). Primary sensory neurons and glial cells in the trigeminal ganglion, however, appeared to be normal in wild-type and mutant mice (Fig. 7E,F). Likewise, all other neuronal populations in the peripheral nervous system developed normally in the *LEF-1*-deficient mice (data not shown). Thus, only a specific population of neurons derived from the neural crest is affected by mutation of the *LEF-1* gene.

No other defects were observed in the brain of *LEF-1*-deficient mice except for the absence of "barrels" in the somatosensory cortex (data not shown). Barrels are morphologically distinguishable multineuronal units that develop in response to the sensory information conveyed from the whiskers to the cerebral cortex (Woolsey 1990).

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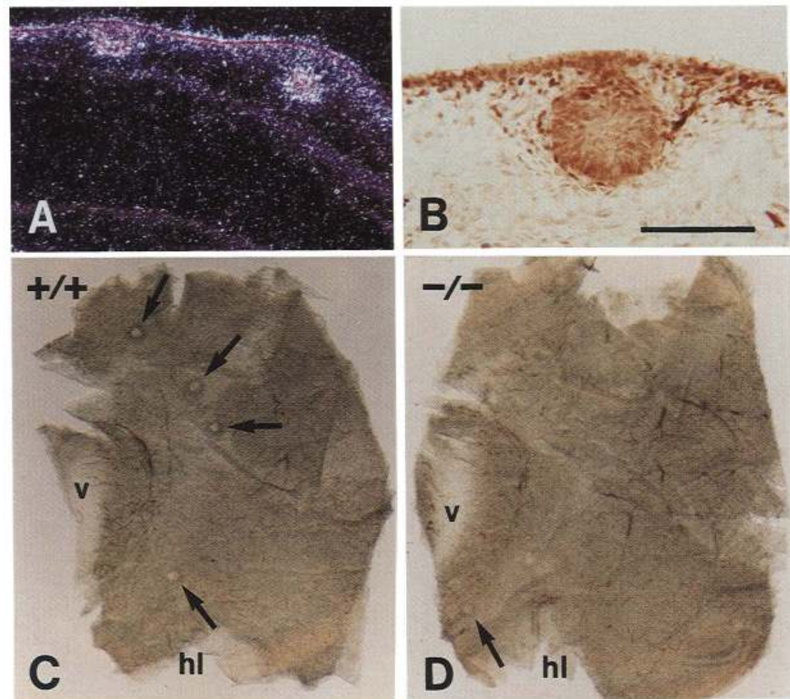

Figure 6. Expression of *LEF-1* in mammary glands and phenotype in *LEF-1*-deficient mice. (*A*) In situ hybridization of the ventral skin of an E13.5 mouse with a ^{35}S-labeled *LEF-1* antisense probe. Expression can be detected in the epidermis and in two mammary buds. (*B*) Localization of LEF-1 protein in a mammary bud and surrounding epidermis at E13.5 by immunohistochemistry. Bar, 100 μm. (*C*) Whole mount of the left ventral skin of a wild-type E13.5 mouse embryo. Three thoracic and one of the two inguinal mammary buds are visible (arrows). (*D*) In a corresponding *LEF-1*-deficient specimen, one rudimentary inguinal mammary bud is visible. (v) Ventral; (hl) hind leg.

Discussion

Our analysis of mice carrying a mutation in the *LEF-1* gene indicates that this gene is essential for the development of several structures and organs. In addition, the penetrance of the defects appears to be complete. All 20 mutant mice examined lacked teeth, mammary glands, hair follicles, and whiskers and all 6 mutant mice analyzed at the histological level showed the lack of the TMN. Moreover, mice that were heterozygous for the mutation of the *LEF-1* gene did not show any of the phenotypes that the homozygous mutant mice displayed. The distribution of defects in the mutant mice, however, appears to be significantly more limited than might have been anticipated from the expression pattern of *LEF-1* during embryogenesis. In particular, thymocytes and peripheral T cells that normally express *LEF-1* were detected in the mutant neonatal mice in numbers and distributions indistinguishable from those found in wild-type sibling mice (data not shown). However, maturation of the immune system, which is precluded by the postnatal lethality of the homozygous mutant mice, may be necessary to detect the function of *LEF-1* in lymphoid organs. Likewise, no obvious defects were detected in the pituitary gland, inner ear, kidney, and the genital eminence, which all express *LEF-1* during embryogenesis (data not shown).

The apparent normal development of thymus and other organs that express *LEF-1* raises the possibility of a redundant genetic function in *LEF-1* −/− mice. A gene closely related to *LEF-1*, termed *TCF-1*, has been identified that encodes a protein with a virtually identical DNA-binding domain (Oosterwegel et al. 1991; van de Wetering et al. 1991). *TCF-1* has a developmental expression pattern that overlaps extensively but not completely with that of *LEF-1* (Oosterwegel et al. 1993). Both genes were found to be coexpressed in the thymus, lung, kidney, and tooth germs, whereas unique *LEF-1* expression was detected in the brain and inner ear. However, a simple genetic redundancy is unlikely because the mutant phenotypes in the *LEF-1*-deficient mice do not correlate precisely with the expression pattern of *LEF-1* and *TCF-1*. For example, development of tooth germs that normally express both genes is arrested in *LEF-1*-deficient mice, whereas no obvious phenotype was detected in the inner ear despite exclusive expression of *LEF-1*. Two other candidate genes, termed *TCF-3* and *TCF-4*, which contain HMG boxes virtually identical to those in *LEF-1* and *TCF-1*, have also been identified (Castrop et al. 1992). It is possible that these genes might compensate for the absence of *LEF-1* in organs not affected by the mutation, although we cannot rule out that expression of *LEF-1* is dispensable in these organs. Alternatively, *LEF-1*-expressing organs that apparently are unaffected by the mutation may contain lower levels of LEF-1 protein as compared to organs whose development is impaired.

The defects in *LEF-1* −/− mice and the pattern of *LEF-1* expression during normal embryogenesis raise the possibility that *LEF-1* plays a regulatory role in inductive tissue interactions and particularly in the development of organs that depend on invagination of ectodermally derived epithelium into subjacent mesenchyme. Formation of teeth and mammary glands are prototypes for such processes and have been studied in detail (Kollar 1983; Kratochwil 1986). Multiple observations have sug-

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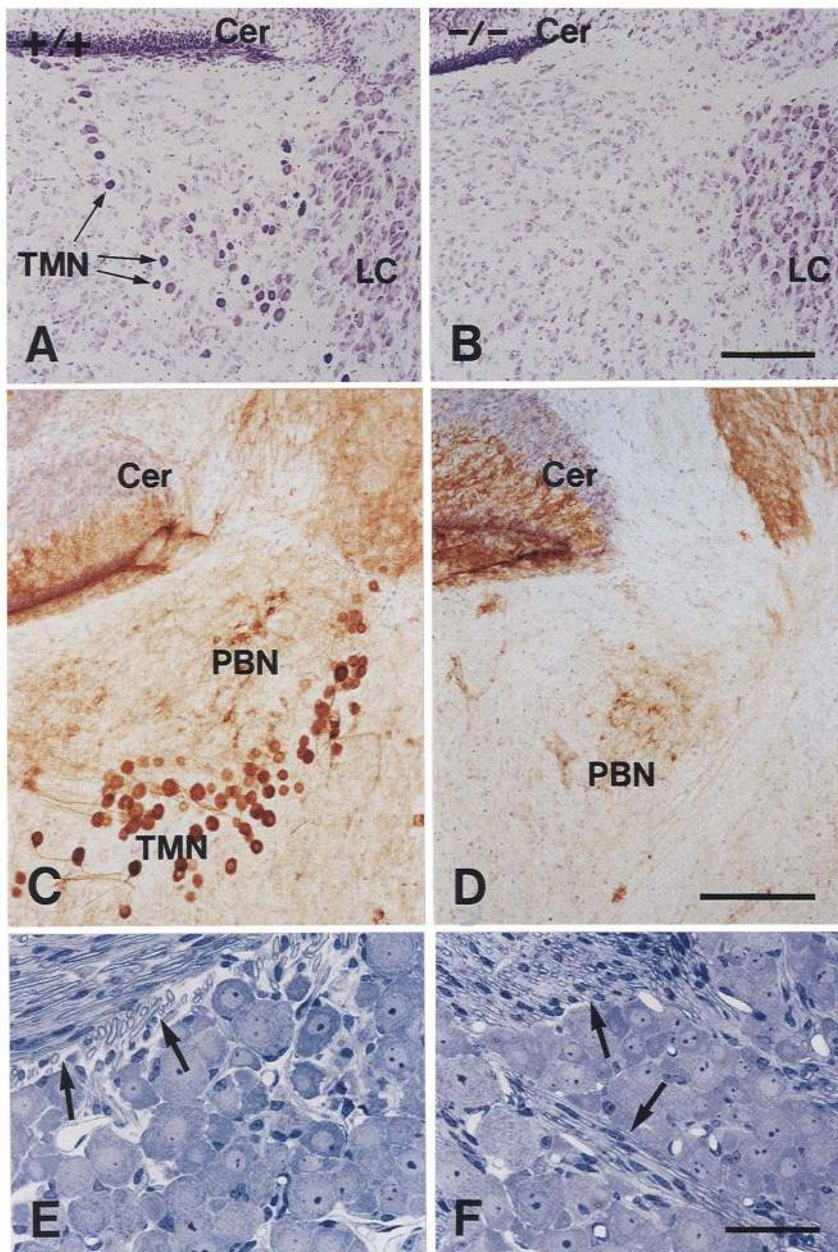

Figure 7. Sensory neurons of the trigeminal system of 10-day-old postnatal wild-type (+/+) and mutant (−/−) mice. (*A,B*) Nissl-stained sections through the midbrain of wild-type (*A*) and mutant (*B*) mice showing the complete absence of neurons belonging to the TMN. These neurons derive from the neural crest and express high levels of LEF-1. The locus coeruleus (LC) and cerebellum (Cer) are indicated. Bar, 200 μm. (*C,D*) Sections through the same brain region were stained with antibodies to $p75^{LNGFR}$, the 75-kD component of the low-affinity nerve growth factor receptor that is a marker for TMN neurons. The parabrachial nucleus (PBN), another immunoreactive nucleus, is indicated. Bar, 200 μm. (*E,F*) Sections through the trigeminal ganglion of wild-type (*E*) and mutant (*F*) mice, stained with toluidine blue, showing normal peripheral trigeminal neurons, which are also derived from the neural crest. The arrows point to myelinated axons, indicative of the presence of neural crest-derived glial (Schwann) cells. Bar, 50 μm.

gested that tooth development is dependent on reciprocal signals between oral epithelium and cranial neural crest-derived mesenchymal cells (Thesleff and Hurmerinta 1981; Kollar 1983; Nichols 1986; Lumsden 1988). Tissue recombination studies have demonstrated that only specific regions of oral epithelium can induce tooth development by interacting with competent mesenchyme derived from either the head or the trunk of early embryos (Lumsden 1988). Moreover, the developmental potential to induce ectopic partner tissue to form a tooth and the expression of various marker genes shifts between epithelial and mesenchymal tissues during organogenesis (Mina and Kollar 1987; MacKenzie 1991; Vainio et al. 1991, 1993; Jowett et al. 1993). The possibility of a functional role of *LEF-1* in such epithelial–mesenchymal interactions is suggested both by the changing spatial and temporal pattern of *LEF-1* expression during normal embryogenesis and by the arrest of tooth development in *LEF-1*-deficient mice. Interestingly, this developmental arrest occurs precisely at a stage that appears to be completely dependent on the interaction between epithelium and mesenchyme. However, the deficit may become morphologically evident at a stage subsequent to that of *LEF-1* function.

Recent analysis of mice deficient in the homeo domain protein Msx-1 indicated a defect in tooth development very similar to that observed in the *LEF-1* −/− mice. Tooth formation in *Msx-1* −/− mice is initiated

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but does not progress beyond the bud stage at E13 (Satokata and Maas 1994). This similarity of phenotype raises the possibility that *LEF-1* and *Msx-1* are either part of a regulatory hierarchy in which one factor regulates the expression of the other or, alternatively, that these two genes cooperate with one another to regulate a particular developmental process. Despite the striking similarity in the arrest of early tooth development in *LEF-1*- and *Msx-1*-deficient mice, no other overlapping phenotypes are evident. In particular, the characteristic craniofacial abnormalities observed in the *Msx-1*-deficient mice, which include a cleft palate and altered mandibular bones, do not occur in the *LEF-1*-deficient mice. Moreover, despite similar expression patterns for *Msx-1* and *LEF-1* in the ear and the hair follicles during normal embryogenesis (Jones et al. 1991; Oosterwegel et al. 1993), mutation of either gene does not produce similar defects in these organs. In the *Msx-1* −/− mice, one of the components of the middle ear is malformed (Satokata and Maas 1994), whereas it is normal in the *LEF-1*-deficient mice (data not shown). Likewise, development of hair follicles is arrested in *LEF-1* −/− mice, whereas no mutant phenotype has been reported for the *Msx-1*-deficient mice. Therefore, we favor the interpretation that LEF-1 and Msx-1 regulate different processes underlying inductive tissue interactions.

The pleiotropic nature of the deficiencies in the *LEF-1* −/− mice raises the question as to whether the formation of the various affected organs and neuronal structures share a common developmental process that is dependent on LEF-1. Although teeth and the TMN, which are absent from the *LEF-1*-deficient mice, are comprised of neural crest derivatives, the mutation of the *LEF-1* gene does not affect the formation of other structures derived from head neural crest, including the visceral skeleton (Meckel's cartilage, maxillary and mandibular bone). Moreover, the trunk neural crest-derived melanocytes, the peripheral nervous system, and the adrenal medulla are present normally in the mutant mice (data not shown). Neural crest cells are characterized by their extensive migration during early embryogenesis (Le Douarin 1982; Jaenisch 1985; Bronner-Fraser 1993). Therefore, the observed deficits in tooth and TMN formation, in principle, could be explained by a failure of neural crest cells to migrate to certain target sites, or by deficiencies in their interactions with other tissues. We consider the former possibility unlikely because preliminary data indicate that the *LEF-1*-expressing cells in the midbrain and tooth germ can be detected at their normal location in the mutant embryos.

TMN neurons share both function and origin with other primary sensory neurons, such as those located in the trigeminal ganglion or in spinal dorsal root ganglia. The TMN is involved in jaw reflexes and chewing (Luschei and Goldberg 1981) and, therefore, the absence of the TMN in *LEF-1*-deficient mice may contribute to their postnatal lethality. In contrast to other neural crest-derived neurons that are found in the periphery, the TMN neurons are located within the central nervous system in the midbrain. The possible functional or developmental significance of the unusual positioning of these neurons is unknown. It is likely that the interactions between these neurons and their cellular environment are unique among sensory neurons. These interactions may be important for the successful survival of a fraction of the TMN population during the period of extensive cell death observed during late embryogenesis (Rogers and Cowan 1973; Alley et al. 1974). This would be consistent with a complete absence of the TMN in the *LEF-1*-deficient mice despite the apparently normal presence of these cells in the developing brain at early stages. According to this view, the formation of the mesencephalic nucleus of the trigeminal nerve could share with formation of other organs the requirement for interactions between cells of different developmental origin. Alternatively, *LEF-1* could play different roles in the formation of the various structures that are affected by the mutation.

The genetic targets for LEF-1 are still obscure. Although in lymphocytes the minimal TCRα enhancer has been identified as a biochemical target for LEF-1 (Travis et al. 1991; Waterman et al. 1991), expression and rearrangement of the endogenous *TCRα* gene is not affected by the mutation in the *LEF-1* gene (data not shown). This observation can be explained formally by a functional redundancy of *LEF-1* with *TCF-1* in T lymphocytes or, alternatively, by the presence of a second enhancer/locus control region downstream of the previously described enhancer in the endogenous *TCRα* locus (Diaz et al. 1994). Likewise, the developmental signal that turns on the expression of *LEF-1* is unknown. Bone morphogenetic protein-4 (BMP-4) has been proposed as an important signal mediating induction between epithelial and mesenchymal tissues (Lyons et al. 1991). In particular, recent experiments with organ cultures of early tooth germs have shown that BMP-4 can mimic the action of dental epithelium in inducing the expression of *Msx-1* in the dental mesenchyme (Vainio et al. 1993). However, the developmental expression pattern of *BMP-4* overlaps only partially with that of *LEF-1* during embryogenesis (Jones et al. 1991; Oosterwegel et al. 1993), suggesting that either *LEF-1* is regulated by a distinct signal, or that *BMP-4* may regulate expression of *LEF-1* only in some organs.

In conclusion, our experiments identified *LEF-1* as an essential component of the development of various organs that are dependent on reciprocal interactions between mesenchyme and ectodermal epithelia. Molecular analysis of the deficiencies in the *LEF-1* −/− mice may elucidate the mechanisms underlying inductive tissue interactions that control the determination and differentiation of cells during organogenesis.

Materials and methods

Construction of the LEF-1 *targeting vector and cell culture*

We screened a mouse genomic library from 129/Sv mice with a *LEF-1* cDNA probe encompassing sequences that encode the DNA-binding domain of LEF-1. One phage was isolated that included the second exon of the HMG domain (nucleotides

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1899–2119 of the *LEF-1* cDNA). An 8-kb genomic *LEF-1* fragment was excised with *Sal*I and *Xho*I and subcloned into a Bluescript vector (Stratagene) that lacked the *Sma*I (*Xma*I) site. Insertion of a filled-in *Eco*RI–*Bam*HI fragment containing the *PGK-neo* gene into the filled-in *Xma*I site of the 8-kb *LEF-1* subclone generated an insertion type *LEF-1* targeting vector. Finally, an *Eco*RI–*Hin*dIII fragment containing the *PGK–tk* gene was inserted into the targeting vector to generate the final gene construct used for homogenous recombination in ES cells.

For electroporation of embryonic stem cells, we linearized the targeting vector with *Cla*I. Electroporation of ES cells and selection of G418- and FIAU- (a kind gift from Bristol-Myers Squibb) resistant colonies were performed as described by Ramírez-Solís et al. (1993), with the exception that G418-resistant primary embryonic fibroblasts were used as feeders. For DNA blot analysis, genomic DNA was prepared as described by Ramírez-Solís et al. (1993).

DNA blot analysis

Genomic DNA was isolated from the livers of E16 embryos and 20 μg of each sample was digested with *Eco*RI and *Bam*HI. The fragments were separated on a 0.9% agarose gel and transferred to GeneScreen Plus (DuPont). The probe, a 180-bp *Sty*I–*Xho*I fragment from the *LEF-1* locus, was labeled with $[\alpha\text{-}^{32}P]$dCTP by random priming (Boehringer Mannheim) and hybridized overnight at 42°C. Blots were washed to a final stringency of 0.1× SSC, 0.1% SDS at 65°C, and exposed to film overnight.

Immunoblot analysis

Pre-B cell lines were generated from bone marrow by transformation with Abelson MuLV. Nuclear extract from 5×10^7 cells of each pre-B cell line were separated on a 10% SDS–polyacrylamide gel and transferred to nitrocellulose. The blot was incubated with a rabbit anti-murine LEF-1 antibody (Travis et al. 1991) and developed with an alkaline phosphatase-conjugated secondary antibody (Boehringer Mannheim).

In situ hybridizations

Embryos were fixed in 4% (wt/vol) paraformaldehyde in phosphate-buffered saline (PBS) for 24 hr at 4°C, immersed in 20% (wt/vol) sucrose/PBS for 24 hr at 4°C, frozen in powdered dry ice, and then stored at −80°C until cryostat sectioning. Serial sections of 14- to 16-μm thickness were thaw-mounted onto microscope slides (Superfrost Plus, Fisher) and stored at −80°C until use.

RNA expression vectors containing 0.36-kb protein-coding region of the *LEF-1* cDNA (nucleotides 1158–1517; Travis et al. 1991) were used to generate ^{35}S-labeled sense and antisense RNA probes as described by Tecott et al. (1993). Embryo sections were hybridized, washed, and developed essentially as described by Tecott et al. (1993). Hybridizations were carried out at 52°C in 50% (vol/vol) formamide, 0.3 M NaCl, 20 mM Tris-HCl (pH 8.0), 5 mM EDTA, 1× Denhardt's solution, 10 mM NaH_2PO_4, 10 mM dithiothreitol, 0.5 mg/ml of yeast tRNA, 10% (wt/vol) dextran sulfate. After hybridization, sections were rinsed in 2× SSC, 10 mM 2-mercaptoethanol, 1 mM EDTA at room temperature and then incubated in RNase (30 mg/ml), 0.5 M NaCl, 10 mM Tris-HCl (pH 8.0), for 40 min at 37°C. A high-stringency wash was then performed in 0.1× SSC, 10 mM 2-mercaptoethanol, 1 mM EDTA for 2 hr at 55°C. The slides were rinsed in 0.5× SSC, dehydrated through a graded ethanol series containing 0.3 M ammonium acetate, air dried, and dipped in Kodak NTB2 emulsion, diluted 1:1 with H_2O. The emulsion was developed in Kodak D19 developer, and sections were counterstained with cresyl violet.

Histological procedures

All postnatal animals were perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2–7.4). Embryos were dissected out and fixed by immersion in the same fixative solution. Fixed newborn mice and embryos were dehydrated in ethanol, embedded in paraffin, sectioned at 7 μm, and stained with hematoxylin–eosin for conventional analysis. Brains from older animals (P9–P10) were also embedded and paraffin sections were Nissl stained using 0.1% cresyl violet. Some tissues were postfixed with 2% osmium tetroxide in phosphate buffer, dehydrated, and embedded in araldite resin (Durcupan, Fluka). One-micron-thick sections were obtained in a Reichert Ultracut E and stained with 1% toluidine blue.

For immunocytochemistry using antibodies against $p75^{LNGFR}$, brains of P9–P10 mice were dissected out from perfused animals and left in the same fixative overnight. Sagittal sections at 60 μm were obtained with a vibratome and collected in TBS [10 mM Tris (pH 7.5), 150 mM NaCl]. Sections were incubated in 3% hydrogen peroxide and 10% methanol, to quench endogenous peroxidase activity, blocked for 2 hr in a TBS solution containing 0.4% Triton X-100, 1% glycine, 3% bovine serum albumin, and 10% normal goat serum, and left overnight at 4°C in the same blocking solution containing polyclonal antibodies against $p75^{LNGFR}$ at 1 μg/ml. The ABC method (ABC Elite kit, Vector Labs) was used for immunodetection. Briefly, after washing the sections several times with blocking solution, they were incubated for 1 hr in biotinylated secondary anti-rabbit antibodies at 1:200, washed several times, and incubated for an additional hour in avidin-biotin-peroxidase complex, 1:300. Peroxidase was then reacted with 0.05% diaminobenzidine tetrahydrochloride and 0.003% hydrogen peroxide in 0.1 M Tris buffer (pH 7.5).

Melanocytes were stained with a rat monoclonal antibody against c-kit (ACKZ; Nishikawa et al. 1991). Pieces of skin from the back of newborn mice were dissected, fresh frozen in OCT medium (Tissue Tek) using dry ice, sectioned at 10 μm in a cryostat, and thaw mounted on poly-L-lysine coated slides. Sections were fixed with cold acetone for 1 min and probed with c-kit antibody as described above.

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