Lef1 is required for the transition of Wnt signaling from mesenchymal to epithelial cells in the mouse embryonic mammary gland

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Abstract

Inductive reciprocal signaling between mesenchymal and adjacent epithelia gives rise to skin appendages such as hair follicles and mammary glands. Lef1-mediated canonical Wnt signaling is required for morphogenesis of these skin appendages during embryogenesis. In order to define the role of canonical Wnt signaling during early embryonic mammary gland development, we determined the temporal and spatial changes in Wnt signaling during embryogenesis in wild-type and *Lef1*-deficient embryos harboring a Tcf/Lef1-βgal reporter (TOPGAL) transgene. In contrast to previous studies using TOPGAL mice from a distinct founder, we observe that Wnt signaling acts initially on mesenchymal cells associated with the sequential appearance of mammary placodes. As placode development progresses between 12.5 and 15.5 dpc, Wnt signaling progressively accumulates in the mammary epithelial compartment. By 18.5 dpc, βgal activity is confined to mesenchymal and epithelial cells near the nipple region. In *Lef1*-deficient embryos, the transition of Wnt signaling from mesenchyme to the mammary epithelia is blocked for placodes #1, 4 and 5 despite the expression of *Tcf1* in epithelial cells. These placodes ultimately disappear by 15.5 dpc, while placodes 2 and 3 typically did not form in the absence of *Lef1*. Progressive loss of placodes 1, 4, and 5 is accompanied by increased apoptosis in mesenchymal cells adjacent to the mammary epithelial placodes. While factors important for embryonic mammary gland development, such as *FGF7*, are expressed normally in *Lef1*-deficient animals, one mediator of the Hedgehog (Hh)-signaling pathway is aberrantly expressed. Specifically, *Shh*, *Ihh*, and *Gli2* are expressed in mammary epithelial cells at levels in *Lef1*-deficient animals similar to wild-type littermates. However, the signal for *Ptc-1* is strongly reduced in mesenchymal cells surrounding the mammary placode in Lef1 mutants relative to wild-type embryos. The loss of Ptc-1, both a receptor for and transcriptional target of Hh signaling, suggests that Hh signaling is blocked in *Lef1*-deficient embryos. Thus, these data reveal distinct requirements of different mammary placodes for Lef1-dependent Wnt signaling. They further define dynamic changes in which cells integrate Lef1-dependent Wnt signaling during progression of embryonic mammary gland development.
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Introduction

Embryonic mammary gland development is dependent on reciprocal inductive interactions between the mesenchyme and overlying ectoderm (Cunha, 1994; Pispa and Thesleff, 2003; Robinson et al., 1999; Sakakura et al., 1987). Signals originating in the mesenchyme have been proposed to specify ectodermal cells to form epithelial mammary placodes (Sakakura, 1987). Reciprocal signaling from the placodes to the underlying mesenchyme then directs condensation of fibroblasts which form the mammary mesenchyme. Maintenance of these reciprocal signals stimulates further proliferation and differentiation of the placodes resulting in the development of the primitive mammary ductal tree present at birth.

Murine mammary gland formation initiates near 10.5 dpc with the appearance of the mammary line along the anterior–posterior axis on both sides of the ventral flank of the embryo (Sakakura, 1987; Turner and Gomez, 1933; Veltmaat et al., 2004). These structures give rise to five symmetric pairs of mammary placodes elevated above the surrounding surface

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ectoderm by 11.5 dpc (Mailleux et al., 2002). Rather than developing simultaneously, placodes appear in the order of pair number 3, then 4, followed by 5 and 1, and finally pair number 2 (Mailleux et al., 2002). These epithelial spherical placodes subsequently invaginate into the underlying dermal mesenchyme forming mammary buds (Robinson et al., 1999; Sakakura et al., 1987). In males, the epithelial bud becomes separated from the epidermis under the influence of testosterone by active induction of apoptosis by the mammary mesenchyme (Wysolmerski et al., 1998). In females, at 15.5 dpc, the buds elongate and form a mammary sprout, the distal tip of which breaks through the primary mammary mesenchyme and penetrates a secondary mesenchyme, the underlying fat pad precursor mesenchyme. The mammary mesenchyme induces nipple formation in the overlying epidermis at around 16.5 dpc (Foley et al., 2001). Meanwhile, the epithelial sprout branches into the fat pad precursor mesenchyme, resulting in the formation of a rudimentary ductal tree.

Lymphocyte enhancer factor-1 (Lef1) is one of the earliest known markers defining formation of the mammary placode (Mailleux et al., 2002; van Genderen et al., 1994). Lef1 is an architectural protein chaperoning a number of distinct factors controlling transcription of target genes. In the context of the canonical Wnt-signaling cascade, complexes of Lef1 and the transcriptional repressors, such as Groucho, are replaced by Lef1-β-catenin-containing complexes, resulting in activated transcription of Wnt-target genes (Behrens et al., 1996; Eastman and Grosschedl, 1999).

The canonical Wnt-signaling pathway is important for placode induction and differentiation of mammary epithelium (Hennighausen and Robinson, 2001; Smalley and Dale, 2001). A role for Wnt-induced signaling in mammary bud development is supported by studies of transgenic mouse embryos bearing βgal reporter gene under the control of Lef/Tcf-dependent promoter (Chu et al., 2004; DasGupta and Fuchs, 1999; Veltmaat et al., 2004). During the early stages of mammary development, the mammary line is defined by the expression of *Wnt10b* which becomes localized to the placodes (Veltmaat et al., 2004). *Lef1* is expressed in the epithelial cells of the mammary buds at 12 dpc and is subsequently induced in the condensed mesenchyme surrounding each bud by 15 dpc during embryogenesis (Foley et al., 2001). Induction of *Lef1* expression in the mammary mesenchyme is dependent on paracrine signaling from the mammary epithelium by the secreted protein, PTHLH, and its receptor, PTHR1 (Foley et al., 2001). Defective mammopoeisis arises in *Lef1*-deficient animals; embryos initially exhibit a reduced number of mammary buds and aborted development of those which do form (van Genderen et al., 1994). In contrast, total inhibition of Wnt signaling by targeting Dickkopf-1 to epithelium cues inhibition of mammary bud formation at 11.5 dpc (Andl et al., 2002; Chu et al., 2004).

In order to understand the early genetic events contributing to mammary placode formation, we (i) characterized the expression of the Tcf/Lef1-family proteins, (ii) determined the spatial and temporal induction by canonical Wnt signaling of specific embryonic mammary cells, and (iii) characterized

alterations in Wnt signaling in *Lef1*-deficient embryos. Our data reveal that Wnt signaling first stimulates mesenchymal cells and progressively signals epithelial cells of the developing mammary placode. However, in the absence of *Lef1*, this switch fails to occur, Wnt signaling remaining confined to the mesenchymal cells as mammary placode development is aborted. Furthermore, we present evidence that Lef1-mediated Wnt signaling lies genetically upstream of the Hedgehog-signaling pathway.

Materials and methods

Mice

The generation and genotyping of mutant and transgenic mice were described previously: Lef1 (Galceran et al., 1999; van Genderen et al., 1994); TOPGAL mice (Cheon et al., 2002) were maintained on C57Bl/6 background. These mice contain a *βgalactosidase* gene (βgal) downstream of a c-*fos* minimal promoter and three consensus TCF-binding motifs. TOPGAL/*Lef1*$^{+/-}$ mice were crossed to generate TOPGAL/*Lef1*$^{-/-}$ embryos.

Analysis of βgalactosidase activity

Whole embryos, embryonic ventral skin or adult number 4 inguinal mammary glands were fixed with 4% paraformaldehyde for 1 h, rinsed and washed with Rinse solution A (0.1 M PBS pH 7.4, 2 mM MgCl2, 5 mM EGTA) for 30 min, then rinsed and washed with Rinse solution B (0.1 M PBS, 2 mM MgCl2, 0.01% sodium deoxycholate, 0.02% Tween) for 5 min and then transferred into Tissue Stain Base solution (Specialty Media) with Xgal substrate at a final concentration of 2 mg/ml. Staining was performed in the dark at 37°C overnight. Samples were photographed and/or paraffin-embedded, sectioned, and counterstained with eosin. Mammary gland whole mounts were counterstained with 0.2% carmine, 0.5% aluminum potassium sulfate.

Whole-mount in situ hybridization (ISH) and immunohistochemistry (IHC)

Embryos were fixed in 4% paraformaldehyde (PFA) overnight at 4°C. For whole mounts, embryos were dehydrated serially to 100% methanol, treated for 5 h with 1:5 hydrogen peroxide in methanol and rehydrated to PBST at room temperature. Whole-mount in situ hybridizations were performed with digoxigenin-labeled antisense riboprobes as described previously (Motoyama et al., 1998a). The cDNA for probes for *Lef1* (Hudson et al., 1998), *Tcf1* (Korinek et al., 1998b), *Tcf3* (image clone 444295), *Tcf4* (image clone 774951), *Gli2* and *Gli3* (Mo et al., 1997), *Ptc1* (Motoyama et al., 1998a).

Immunohistochemical analysis of embryo sections was performed according to the instructions provided in the R&D HRP-AEC immunostaining kits, with the following amendments. Antigen retrieval was performed using 10 M sodium citrate solution, pH 6, preheated for 3 min, and then sections were microwaved for 10 min. Primary antibodies for androgen receptor (rabbit polyclonal, Dr. Gail Prins, The University of Illinois, Chicago, Illinois), BrdU (Becton Dickson), β-catenin (610154, BD Biosciences Pharmingen), or Ptc-1 (G-19; Santa Cruz) were incubated overnight at 4°C. All sections were counterstained with hematoxylin (Vector). Whole-mount images were taken by using a Nikon DXM 1200 digital camera driven by Nikon ACT-1 imaging software. Sections were photographed using a Q Imaging Fast 1394 cooled CCD camera and compiled using Simple PCI software (Nikon).

BrdU and TUNEL labeling

For BrdU-labeling experiments, pregnant mice were injected with 0.1 mg BrdU/g body weight, and embryos were collected after 2 h and processed as described (Motoyama et al., 1998b). For apoptosis studies, an ApoAlert DNA fragmentation assay kit (Clonetech) was used according to the manufacturer's directions.

Results

Canonical Wnt signaling is active during mammary gland development

Members of the Lef1/Tcf-family proteins integrate developmental signals from the canonical Wnt-signaling pathway. We first assessed by whole-mount in situ hybridization the expression pattern of these factors during early embryonic mammary gland development (Fig. 1). At 12.5 dpc, during the early stages of mammary placode development, both *Tcf1* and *Tcf3* are expressed in the mammary epithelium, while *Tcf4* is expressed in the surrounding mesenchyme. In addition to the *Lef1/Tcfs*, their co-factor *β-catenin* is also upregulated in the mammary region, as described previously (Chu et al., 2004 and Fig. 1E).

In order to determine which cells are influenced by the canonical Wnt-signaling pathway during mammary gland development, we employed previously described animals harboring a transgene responsive to canonical Wnt signaling (Cheon et al., 2002). The TOPGAL transgene encodes a concatemer of three Tcf/Lef1 binding sites upstream of the minimal c-*fos* promoter and a *β*galactosidase (*β*gal) reporter gene. Fig. 2 illustrates the dynamic changes in Wnt signaling during mammary gland morphogenesis. In TOPGAL embryos, βgal activity is apparent in the AER at 11.0 dpc. Shortly afterwards (11.25–12 dpc), a streak of βgal-positive cells is apparent adjacent to the developing forelimb bud, slightly posterior to its proximal aspect. This streak continues to expand around the limb bud, coincident with the appearance of βgal expression around the first mammary placode to form, placode 3 (Fig. 2E). At this stage, βgal expression is confined to the mesenchymal cells surrounding the epithelial placode (Fig. 3A). Strong expression is evident several hours later in all placodes (placodes 1 and 5 behind the limb bud) with the similar intense mesenchymal staining. Canonical Wnt signaling in the mesenchymal cells is supported by the presence of β-catenin in the nuclei of cells surrounding the epithelial placode (Fig. 3E). As placode development continues (13.5 dpc onwards), βgal activity appears progressively more intense within the epithelial cells during mammary bud development (Figs. 2H–J and Fig. 3B). By 14.5 dpc (Fig. 2I), strong βgal activity is detected in both the epithelial and mesenchymal cells of the developing mammary buds. Strong expression persists at 15.5 dpc (Figs. 2J and 3C).

Between 16.5 dpc and birth, morphogenesis of the mammary bud gives rise to a rudimentary ductal tree. By 18.5 dpc, βgal activity is confined to a discrete region at the origin of this primitive mammary gland. Whole-mount analysis (Figs. 2K–L) and section (Fig. 3D) revealed that mesenchymal cells surrounding the nipple region and the primary duct as well as epithelial cells of the primary ducts proximal to the developing nipple region appear βgal-positive. During postnatal development, βgal activity was not detected during adolescent development (data not shown) but became apparent during mid-pregnancy (12 dpc) and is prominent by 15.5 dpc within the epithelial cells of the developing alveoli (Fig. 2 M). βgal activity persisted in these structures until birth (data not shown). Taken together, these data reveal dynamic alterations in cells affected by canonical Wnt signaling at distinct stages of mammary gland development. Importantly, both mesenchymal and epithelial cells are involved in signaling through this pathway. Mesenchymal cells signal first during the early stages of placode development, while signaling in epithelial cells progressively increases as the mammary bud develops further.

Lef1 is not required for induction of mammary placodes 1, 4, and 5

Previous studies demonstrated that the transcriptional mediator of canonical Wnt signaling, Lef1, is necessary for development of skin appendages, including the mammary gland (Foley et al., 2001; Mailleux et al., 2002; van Genderen et al., 1994). In order to define the basis for the mammary gland defect associated with loss of *Lef1*, we characterized the morphology and histology of mammary placodes in *Lef1*-deficient embryos harboring the TOPGAL reporter transgene. Histological analysis of serial sections from *Lef1*$^{-/-}$ embryos at 13.5 dpc (Fig. 4C) and 15.5 dpc (Fig. 4G) demonstrates that the mutant placodes are smaller and fail to invaginate into the dermis, remaining elevated above the surface of the embryo (Figs. 4A and E, respectively). Relative to wild-type mammary buds, the mesenchyme of the dermis is populated with fewer cells but clearly express mesenchymal markers specific for the mammary
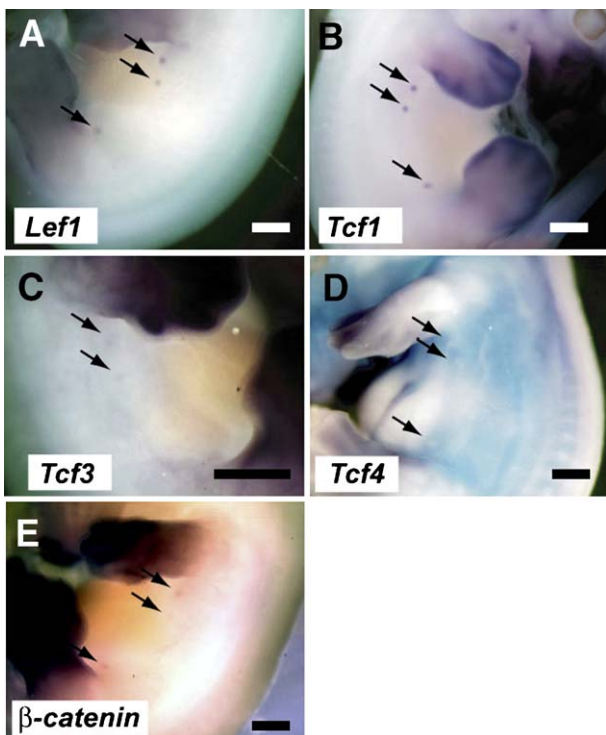

Fig. 1. Expression of nuclear effectors of the canonical Wnt-signaling pathway in the embryonic mammary gland. Whole-mount in situ hybridization analysis demonstrates the expression of *Lef1* (A), *Tcf1* (B), *Tcf3* (C), *Tcf4* (D) and (E) *β-catenin* at 12.5 dpc in developing mammary placodes. *Lef1*, *Tcf1*, and *Tcf3* are localized to the epithelial placode, while *Tcf4* is localized to the surrounding mesenchyme. Scale bar = 500 μm.
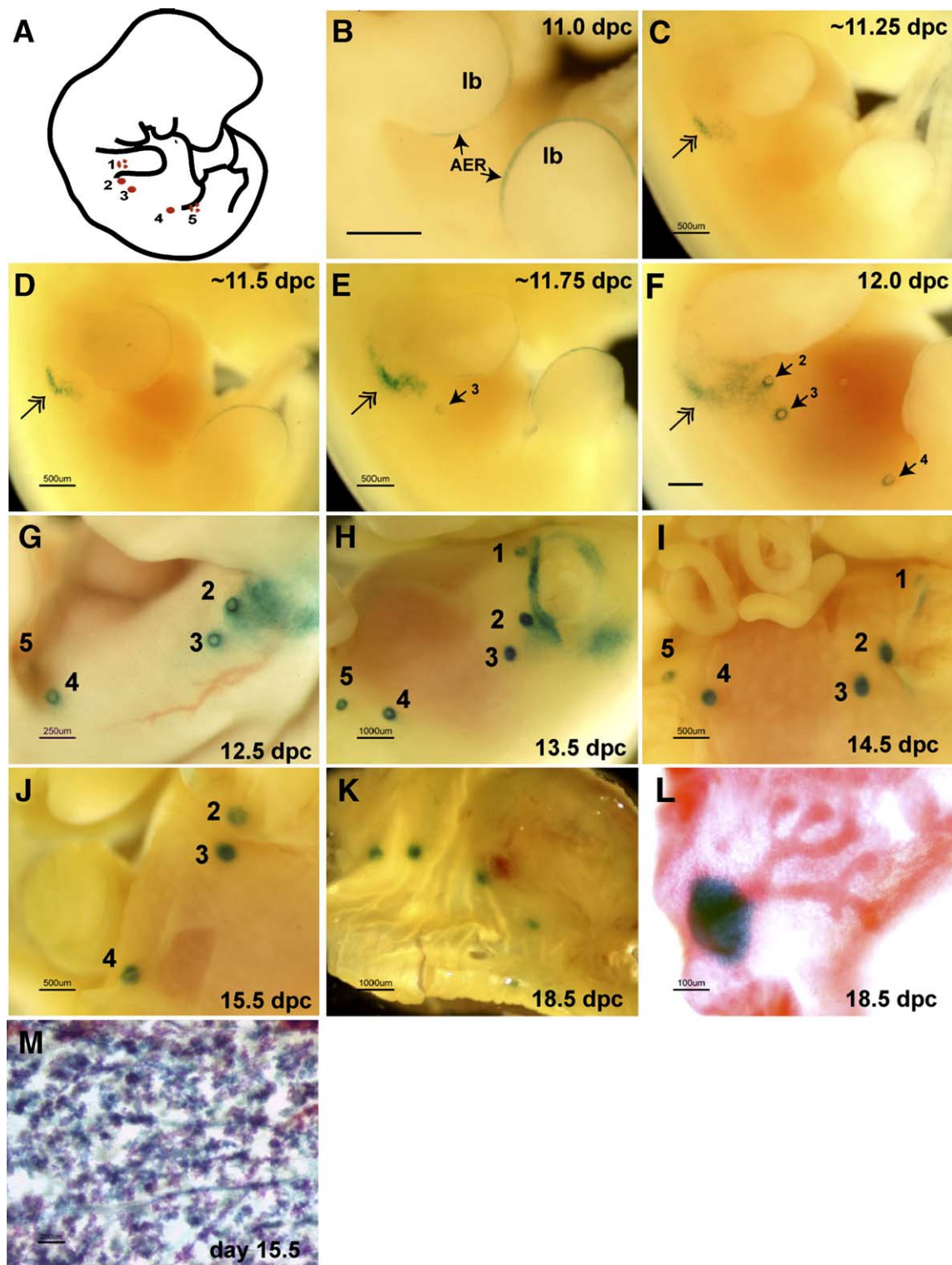
Fig. 2. Wnt-signaling activity during mammary gland development. TOPGAL expression demonstrates epithelial and mesenchymal Wnt activity during mammary gland development. (A) Mammary placodes arise asynchronously, with placode pair number 3 detected first, followed by number 4, then numbers 1 and 5, and finally pair number 2 (Veltmaat et al., 2003). Whole-mount X-gal stained TOPGAL embryos from 11 dpc to 15.5 dpc (B–J). Cells expressing TOPGAL appear blue. TOPGAL-positive cells first appear in a ring of cells around mammary placode 3 at 11.75 dpc (E). By 12 dpc (F), a ring of blue-stained cells surround all mammary placodes. Note increasing TOPGAL activity in the mesenchyme surrounding the forelimb bud in region of placodes 2 and 3 (double arrowheads). Blue-stained cells are seen as a ring around (G) 12.5 dpc placodes. By 13.5 dpc, blue stained cells are visible within the epithelial cells of the bud and remain until 15.5 dpc (H–J). X-gal stained whole-mount of 18.5 dpc ventral skin showing localized staining around nipple region (K). Whole-mount of X-gal and carmine aluminum (red)-stained 18.5 dpc mammary gland (L). Whole-mount of X-gal and carmine aluminum (red)-stained mammary gland from TOPGAL transgenic mouse on day 15 of pregnancy (M). Scale bar in panels B–K—500 μm; scale bar in panel L—100 μm; scale bar in panel M—25 μm.
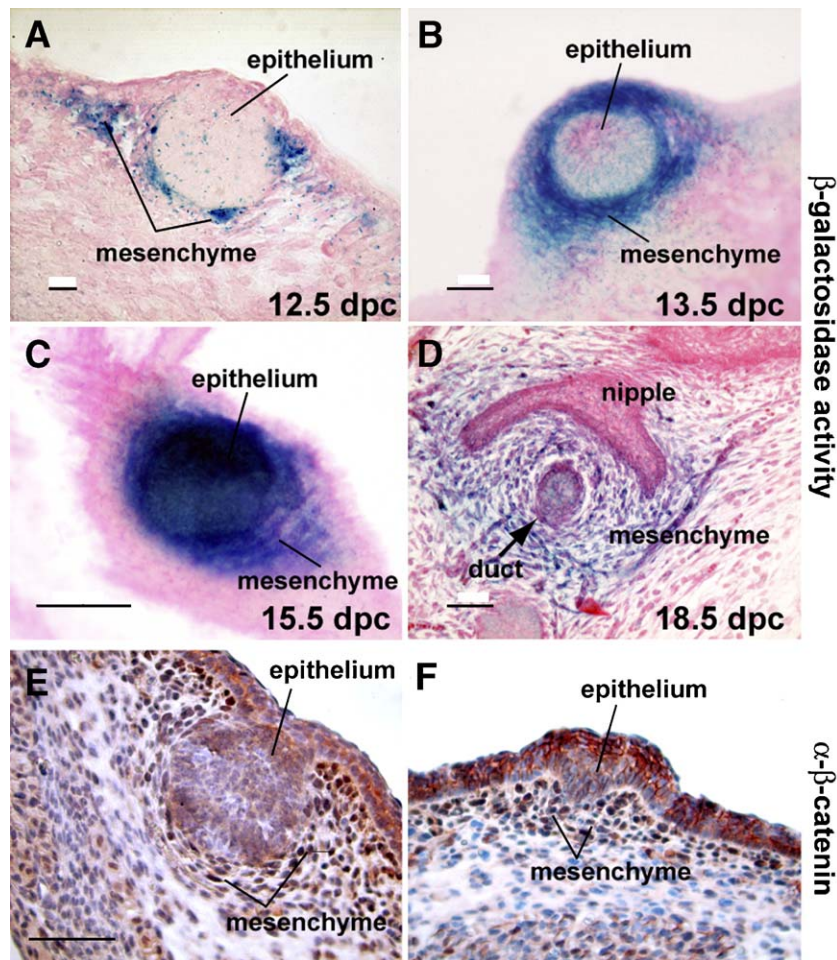
Fig. 3. Changes in Wnt-signaling activity during embryonic mammary gland development. Cross-sections through mammary bud 4 from 12.5–15.5 dpc embryos. At 12.5 dpc (A), mesenchymal cells stained blue with low levels of TOPGAL expression in the epithelial bud. From 13.5 dpc (B) to 15.5 dpc (C), epithelial cells show an increase in transgene expression. Mesenchymal cells continue to express the transgene. (D) At 18.5 dpc, TOPGAL expression is evident in mammary mesenchyme surrounding the nipple region and in the primary duct. Immunohistochemical analysis demonstrates β-catenin expression is apparent in the nucleus of mesenchymal cells of the mammary bud at 15.5 dpc in wild-type (E) and $Lef1^{-/-}$ (F) embryos. In wild-type embryos, weak cytoplasmic and nuclear expression is evident. In contrast, strong cell membrane localization is apparent in $Lef1^{-/-}$ embryos. Scale bar in panels A–D—50 μm; scale bar in panels E–F 25 μm.

gland, such as androgen receptor (Figs. 4D and H). Analysis of serial sections of embryonic skin from *Lef1*-deficient female embryos beyond 15.5 dpc failed to detect any mammary rudiments (data not shown).

While mammary placodes are not observed in $Lef1^{-/-}$ embryos older than 15.5 dpc (van Genderen et al., 1994 and data not shown), development of a variable number of placodes is initiated in these embryos. At 12.5 dpc, a single auxiliary bud (placode 1) and both inguinal buds (placodes 4 and 5) are detected in the majority of $Lef1^{-/-}$ embryos, whereas five pairs of mammary placodes are clearly visible in wild-type embryos (Figs. 5A–B, placodes 1 and 5 not shown). Control TOPGAL and/or TOPGAL/$Lef1^{+/-}$ embryos show βgal reporter activity in the mammary mesenchyme underlying the developing mammary placodes at 12.5 dpc as expected. In TOPGAL/$Lef1^{-/-}$ embryos, βgal activity is evident in the mammary mesenchymal cells surrounding epithelial placodes 1, 4, and 5 (Fig. 5B). However, in contrast to wild-type embryos, localized TOPGAL activity in the region of placodes 2 and 3 was either completely or almost completely absent at

12.5 dpc. As development progresses from 13.5 dpc onward, Wnt signaling localizes to the mammary epithelium in wild-type embryos (Figs. 5C and E). In contrast, Wnt signaling remains restricted to the mesenchyme of $Lef1^{-/-}$ mammary placodes 1, 4, and 5 at 13.5 dpc. βgal expression is also detected in a broad band between the fore and hind limbs, a zone of signaling not evident in wild-type embryos (Fig. 5D). By 14.5 dpc, when βgal expression levels are strong in both the epithelial and mesenchymal cells of wild-type mammary buds (Figs. 2I, 3C and 5E), βgal remains restricted primarily to mesenchymal cells of the $Lef1^{-/-}$ placodes (Fig. 5F). In addition, βgal expression is no longer visible along the flank between the fore limbs and hind limbs as seen at 13.5 dpc in $Lef1^{-/-}$ embryos. Supporting the persistence of βgal expression in mesenchymal cells of the placodes in $Lef1^{-/-}$ embryos is the location of β-catenin protein. Specifically, in cells of the mesenchyme, where βgal activity is observed, β-catenin is expressed in the nucleus whereas cell membrane staining in the epithelial cells of wild-type (Fig. 3E) and mutant placodes is detected (Fig. 3F).
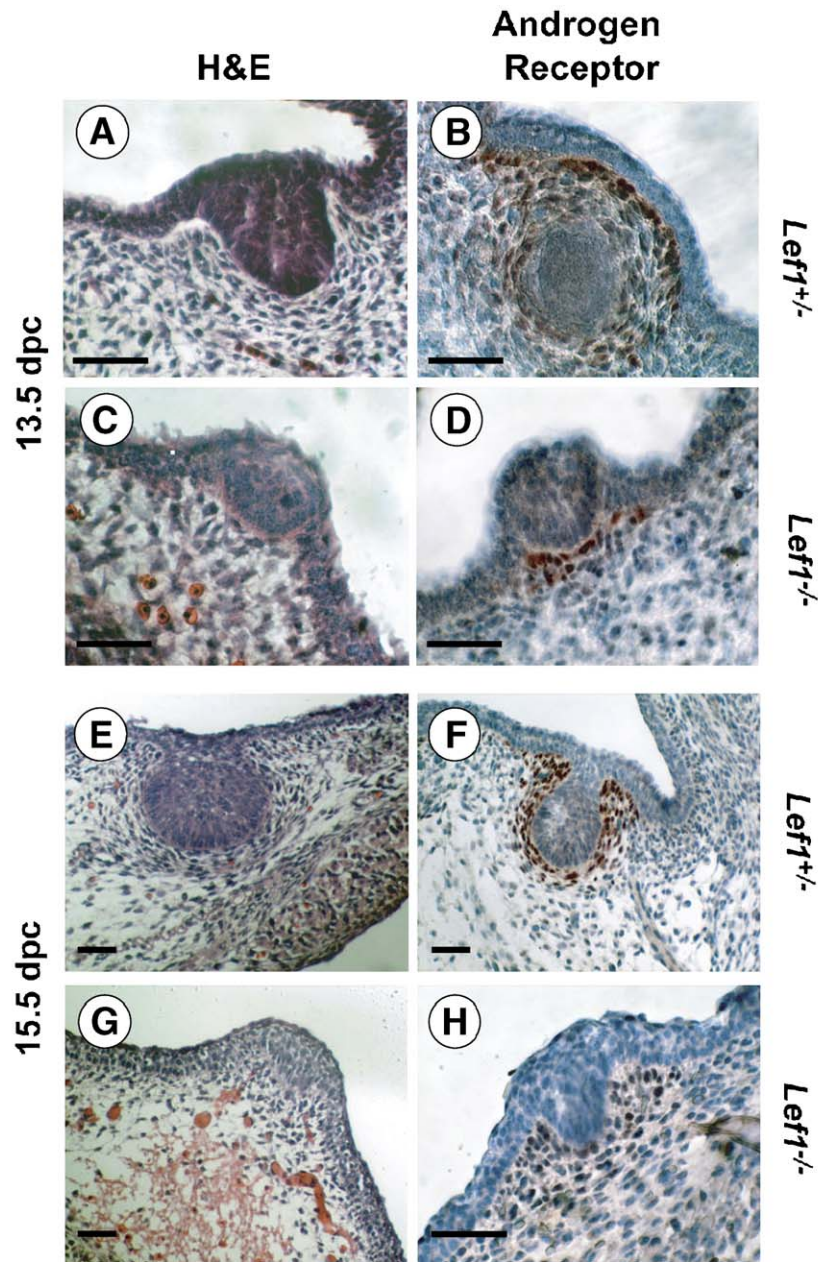
Fig. 4. Arrested mammary gland formation in *Lef1*-null embryos. Histological analysis of wild-type (A) 13.5 dpc and (E) 15.5 dpc mammary buds, and *Lef1*-deficient (C) 13.5 dpc and (G) 15.5 dpc mammary buds. Mammary mesenchymal-specific expression of androgen receptor in control (B and F) and *Lef1$^{-/-}$* (D and H) embryos. Histological sections A, C, E, and G were stained with hematoxylin and eosin. Note the control mammary bud penetrates the dermis and is surrounded by several layers of mammary mesenchymal cells. The mutant bud fails to invaginate further and is surrounded by fewer mesenchymal cells. Scale bar—50 μm.

Thus, despite nuclear localization of β-catenin and active Wnt signaling in the mesenchyme, the absence of *Lef1* is required for the transition of Wnt signaling to the adjacent epithelial placodes. Lack of this transition precedes the disappearance of mammary placodes 1, 4, and 5.

Lef1 is an important mesenchymal survival factor

Arrested development of *Lef1*-deficient placodes and their subsequent regression suggests that defects in cell proliferation and/or apoptosis may result due to the loss of Lef1 activity. We characterized cell proliferation, determined by BrdU incorpo-

ration, and apoptosis, determined in TUNEL assays, in mammary buds or placodes of wild-type and *Lef1$^{-/-}$* mice. In *Lef1*-null mammary placodes, active cell proliferation is apparent at 13.5 dpc (data not shown) and 14.5 dpc, by bromodeoxyuridine (BrdU) incorporation (Figs. 6A–B). Both the epithelial and mesenchymal compartments of the mammary buds stain-positive relative to wild-type embryos. Therefore, arrested placode development does not appear to be due to defective proliferation of cells within the placode itself.

To further explore the cellular mechanism responsible for the failure of mammary gland development, we investigated whether Lef1 is critical for survival of epithelial and/or
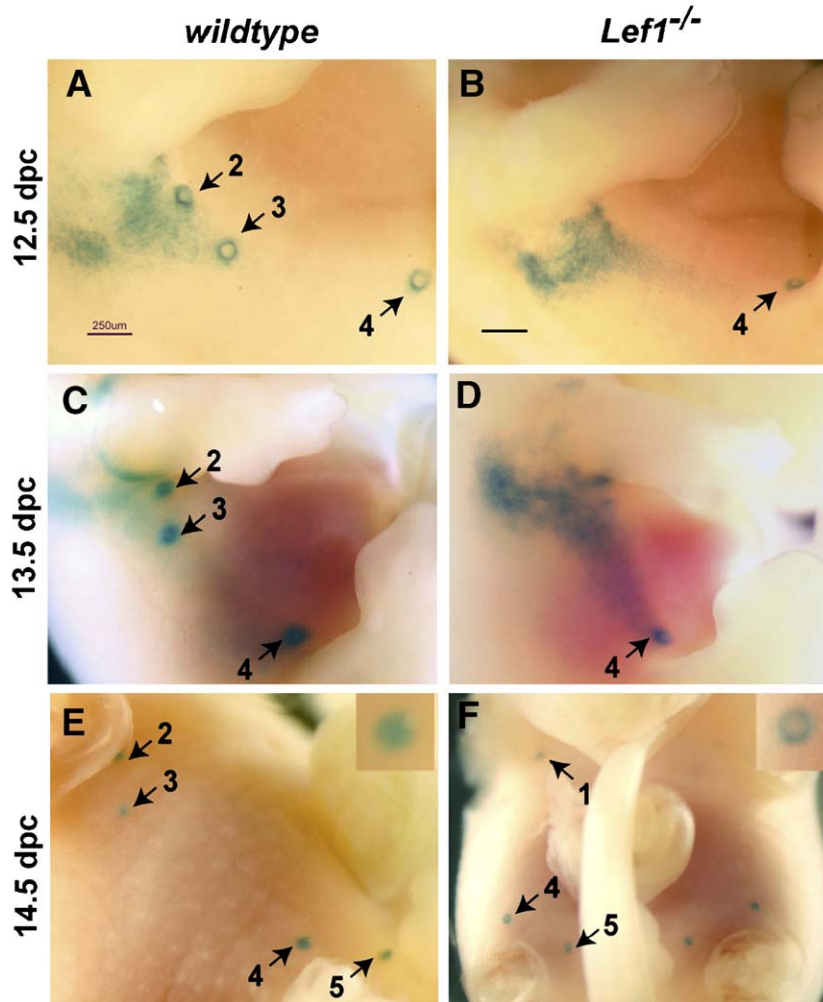
Fig. 5. Deregulated epithelial Wnt signaling in *Lef1*$^{-/-}$ embryos. TOPGAL expression in wild-type (A, C, and E) and *Lef1*$^{-/-}$ (B, D, and F) embryos between 12 dpc and 14.5 dpc. Cells expressing the TOPGAL transgene appear blue. At 12.5 dpc, TOPGAL expression in *Lef1*$^{-/-}$ embryos (B) reveals formation of placodes 1, 4 and 5 as compared to 5 pairs in control embryos (A) (pairs 1 and 5 not shown). Strong TOPGAL expression is detected in mesenchymal cells surrounding the placodes. By 13.5 dpc (C and D), epithelial cells of the bud also show X-gal staining. Note the strong X-gal staining in *Lef1*$^{-/-}$ embryos along the flank between the forelimb and placode 4 not seen in wild-type embryos. At 14.5 dpc, TOPGAL expression is strong in both the epithelial and mesenchymal cells of the mammary bud in wild-type embryos (E). However, in *Lef1*$^{-/-}$ buds, TOPGAL expression is mainly in mesenchymal cells of the bud (H). Insets in E and F are magnification of bud 4. Scale bar—250 μm.

mesenchymal cells of the mammary bud by comparing apoptotic activity between *Lef1*$^{-/-}$ and control mammary buds (Figs. 6C–G). In *Lef1*$^{-/-}$ embryos, TUNEL analysis demonstrates significant staining of mesenchymal cells underlying the mutant placodes, while no detectable cell death is observed in female control embryos at 14.5 dpc. Apoptosis appears in the mesenchymal cells of the dermis underlying the mutant placodes at 14.5 dpc. By 15.5 dpc, TUNEL staining is indistinguishable between wild-type and *Lef1*-deficient embryos at a point when the mutant placodes have begun to disappear.

Hedgehog-signaling pathway is disrupted in the absence of Lef1

Analysis of tooth and hair development in *Lef1*-deficient embryos revealed that Hedgehog (Hh) signaling is lost (DasGupta and Fuchs, 1999; Gat et al., 1998; Niemann et al.,

2002; Zhou et al., 1995). Although the role of the Hh signaling in mammary gland development remains uncharacterized, members of the Hh-signaling pathway have been implicated in the regulation of mammary gland morphogenesis (Lewis et al., 1999; Michno et al., 2003). We determined, therefore, whether the loss of *Lef1* altered the expression pattern of members of the Hh-signaling pathway (Figs. 7 and 8). Despite being expressed at relatively low levels, whole mount analysis for *Shh* and *Ihh* showed no difference in their levels of expression in *Lef1*-deficient mammary placodes compared to wild-type embryos (data not shown; see also Michno et al., 2003). We determined that another soluble factor shown previously to be important in mammary gland development, *FGF7*, was expressed in the mammary placodes of *Lef1*-deficient mice similar to their wild-type counterparts (Figs. 7A–B). Furthermore, alterations in the expression the downstream mediator of Hh signaling, *Gli2*, appear not to be significantly
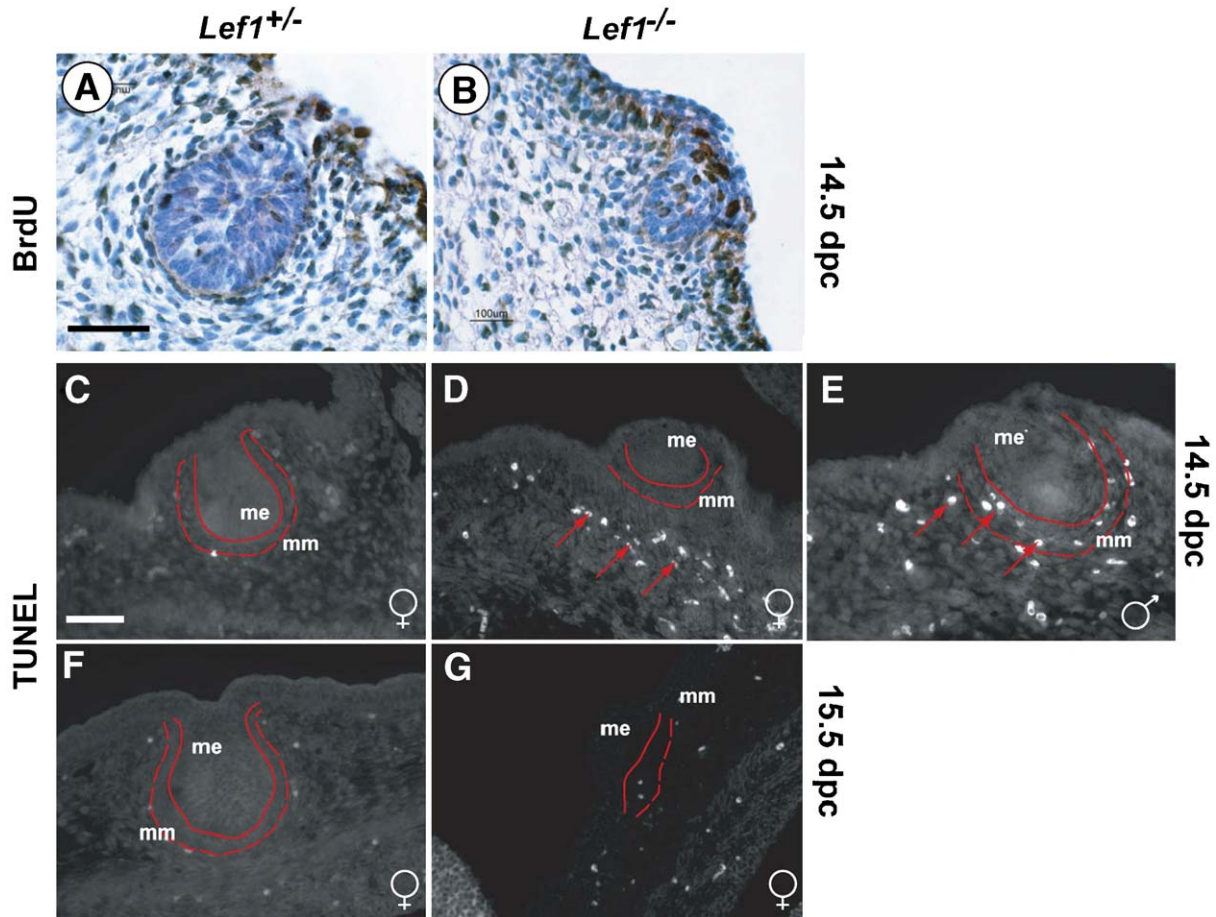
Fig. 6. Increased apoptosis of mammary mesenchymal cells in the absence of *Lef1*. BrdU incorporation was used to determine cell proliferation in (A) control and (B) *Lef1*-defcient embryos at 14.5 dpc. Epithelial cells in both buds stain positive for BrdU incorporation. However, the mesenchymal cells in *Lef1*-null embryos show a complete absence of cells stained with anti-BrdU. TUNEL assays performed on sections of mammary placodes taken from control (C, E (female) and F (male)) and *Lef1* knockout embryos (D and G) at 14.5 dpc and 15.5 dpc, respectively. During 14.5 dpc, mutant buds are positive for apoptosis in dermal mesenchymal cells underlying the mammary bud. Control females are negative, while control males are positive (indicative of male mammary regression). By 15.5 dpc, the mesenchyme underlying mutant buds is no longer positive for apoptosis. Scale bar—50 μm.

altered. So as seen in the whole mount analysis (Figs. 7C–D) and, independently, on sections of 14.5 dpc embryos (Figs. 7E–F), *Gli2* is detected in epithelial cells of both wild-type mammary buds and mutant placodes. In contrast to these factors, which are expressed principally in the mammary epithelia, Ptc-1 expression appears strongly reduced in the mesenchyme of *Lef1*-deficient mice (Fig. 8). In situ analysis of sections (Figs. 8A–B) shows a strong reduction in the apparent level of *Ptc-1* message in *Lef1*-deficient placodes, whereas robust expression is seen in wild-type littermates. Further characterization of the level of expression was performed by immunohistochemical detection of Ptc-1 protein (Figs. 8C–D). While some expression of Ptc-1 is seen in the epithelial cells of both wild-type and mutant mammary buds or placodes, respectively, signal for Ptc-1 protein in the surrounding mesenchyme in *Lef1*-deficient embryos approaches background levels, while strong expression is evident in the mesenchyme of wild-type embryos. Since *Ptc1* is both a mediator and target of the Hh-signaling pathway, these basal levels of *Ptc1* expression are consistent with a loss of Hh signaling in mammary glands of *Lef1*-deficient embryos.

Taken together, these data reveal dynamic alterations in Wnt signaling between mesenchymal and epithelial aspects of the embryonic mammary placode. These changes depend specifically on the expression of *Lef1* and, in its absence, result in apoptosis of the mammary mesenchyme and regression of mammary epithelial placodes.

Discussion

Lef1 is required for embryonic mammary gland development, although details of the effectors and downstream events it coordinates in the mammary gland remain undefined. Expression of Lef1 is dynamic during mammary gland morphogenesis being initially restricted to the epithelial compartment of the mammary placode. Subsequently, it is also detected in the primary mesenchymal cells underlying the mammary bud (Foley et al., 2001; Mailleux et al., 2002); K. Boras, unpublished observation). Lack of *Lef1* expression reveals a central role for this mediator of canonical Wnt signaling in both early specification of the placodes (placodes 2 and 3) and progression of their development (placodes 1, 4, and 5).
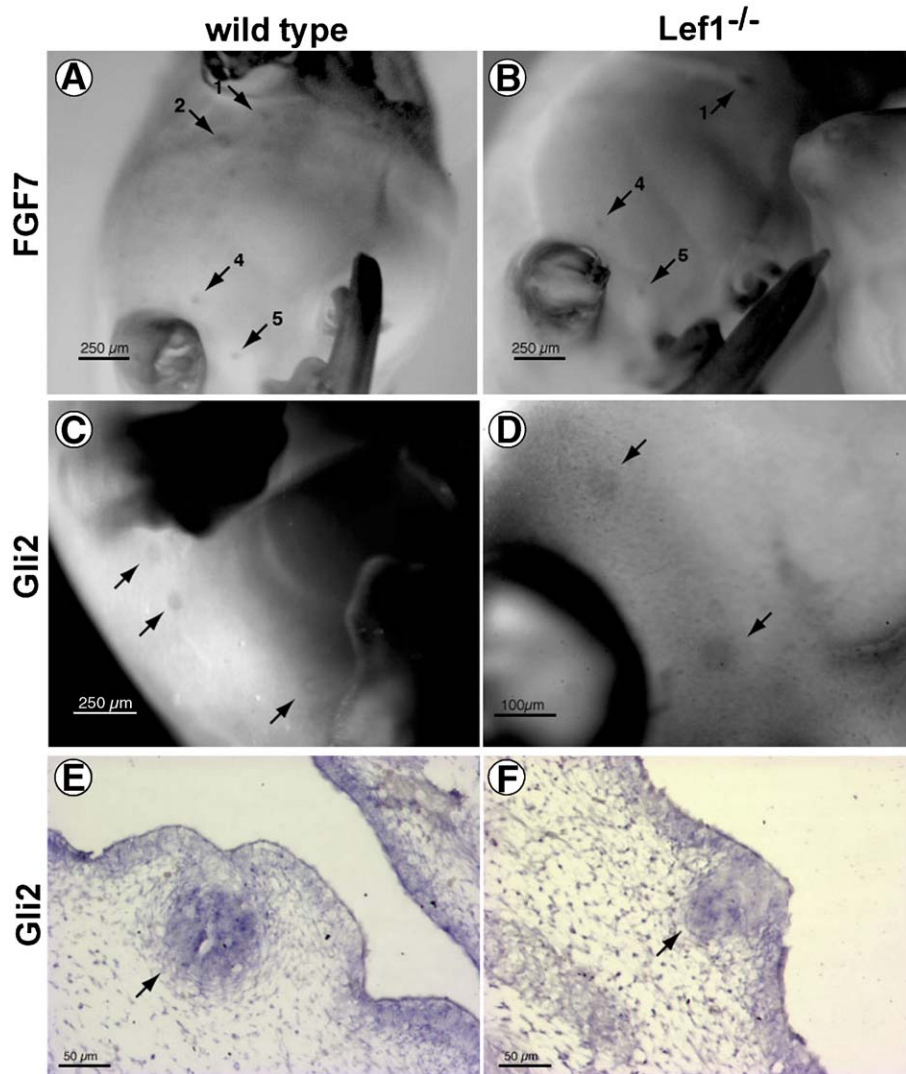
Fig. 7. *FGF7* and *Gli2* are expressed in Lef1-deficient mammary placodes. At 14.5 dpc, transcripts for *FGF7* (A and B) and *Gli2* (C–F) are detected in wild-type (A, C, and E) and *Lef1*-deficient (B, D, and F) embryos. Both *FGF7* and *Gli2* are expressed in the mammary placodes of mutant embryos at levels indistinguishable from that of wild-type mammary buds. Scale bar in panels A–C—250 μm; scale bar in panel D—100 μm; scale bar in panels E–F—50 μm.

Based on gene function studies in the embryonic mammary gland, we have been able to establish a potential hierarchy in mediating early embryonic mammary gland development (Fig. 9). Specifically, development of the embryonic mammary gland is dependent on reciprocal epithelial–mesenchymal signaling. Our data reveal that Wnt-signaling activity involves both mesenchymal and epithelial aspects of the developing embryonic mammary gland. *Lef1*, *Tcf1*, and *Tcf3* are expressed in the epithelial placode and bud, whereas *Tcf4* is detected in the adjacent mesenchyme, consistent with recent studies detecting *Tcf1*, *Tcf3*, and Tcf*4* in the mammary buds by RT-PCR (Chu et al., 2004). Furthermore, β-catenin expression is localized to the cell membrane of the epithelial placode and to the nucleus of cells in the adjacent mesenchyme when βgal activity in TOPGAL mice is restricted to the mesenchyme. During 14.5 dpc and 15.5 dpc, when Wnt activity is apparent in the mesenchyme and the epithelium, β-catenin is localized to nucleus in both cell types.

These data suggest that mesenchymal cells are the first to integrate Wnt signals during early stages of mammary gland development. Progression of development is accompanied by induction of Wnt signaling in the epithelial compartment of the developing mammary bud. In the absence of *Lef1*, Wnt signaling remains confined to the mesenchyme, and development is halted at the placode stage. These data suggest that epithelial-specific Lef1 activity may be required during mammary placode maturation at the point where the placode descends into the dermis, although the observed mesenchymal-specific cell death in Lef1-deficient mammary placodes does not allow us to preclude a role for Lef1 in the mesenchyme. Tissue recombination studies in whisker follicles and tooth in *Lef1*-deficient mutants demonstrated previously compartment-specific Lef1-dependent events in their development (Kratoch-wil et al., 1996). Whisker follicle development requires, for example, both epithelial and mesenchymal Lef1 activity, whereas tooth development is dependent on epithelial-specific
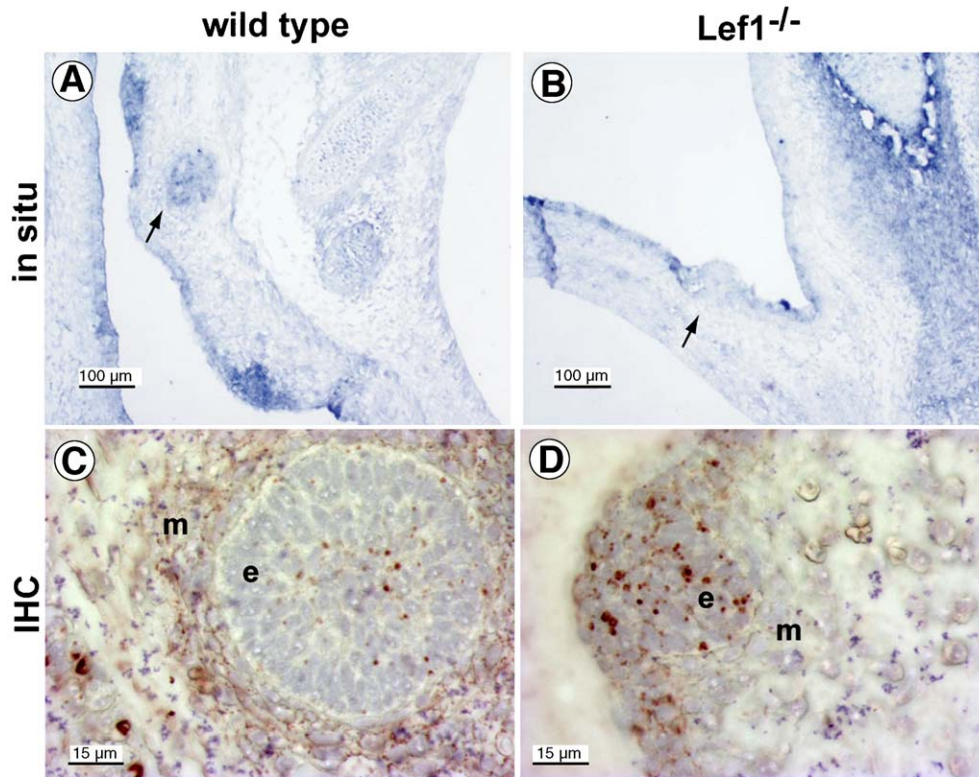
Fig. 8. Loss of expression of Ptc-1 in *Lef1*-deficient mammary placodes. Expression of Ptc-1 in wild type (A and C) and *Lef1*-deficient (B and D) was determined by in situ detection of *Ptc-1* message (A and B) or *Ptc-1* protein (C and D). Expression for *Ptc-1* message is strongly reduced in mutant embryos. Immunohistochemical analysis of Ptc-1 reveals further that Ptc-1 expression is close to background in the mesenchyme of *Lef1*-deficient mammary placodes while strong expression in the mesenchyme is evident in wild-type mammary mesenchyme (e—mammary epithelia; m—mammary mesenchyme). Scale bar in panels A and B—100 μm; scale bar in panels C and D—15 μm.

Lef1 activity. Similar recombination studies will be required to dissect the requirements for Lef1 in specific tissue compartments of the developing mammary gland.

Our data indicate that, while other members of the Tcf/Lef1-family are involved in specific stages of early mammary gland development, they do not exhibit completely overlapping activities. In both wild-type and *Lef1*-deficient embryos, mesenchymal Wnt signaling is apparent, suggesting that Tcf4 may mediate the canonical Wnt pathway for placodes 1, 4, and 5 at this stage. Subsequently, epithelial
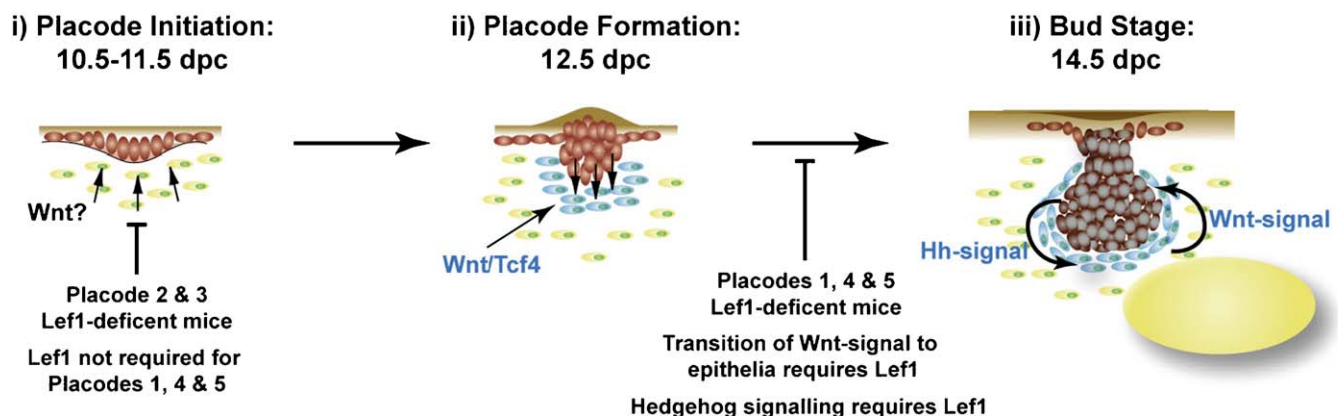

Fig. 9. Model of functional roles of genes during mammary gland development. (i) Placode initiation (11.5 dpc): induction of different placodes has distinct requirements for Lef1. For placodes 2 and 3 in the mouse, placode initiation depends on Lef1 while for placodes 1, 4, and 5, Lef1 expression is not required (ii) placode stage (12.5 dpc): formation of individual mammary epithelial placodes is associated with Wnt signaling in the adjacent mesenchyme. Here, the Wnt signal may specifically be mediated by the mesenchymally expressed Tcf4. In the absence of Lef1, development of placodes 1, 4, and 5 arrests. Failure to progress to the bud stage is associated with a failure of Wnt signaling to switch from being exclusively mesenchymal to both the epithelial and mesenchymal compartments. Furthermore, activation of the Hh-signaling pathway is blocked at this stage in the absence of Lef1. (iii) Bud stage (14.5 dpc): Epithelial-specific Wnt-signaling activity requires Lef1 expression. This requirement is not compensated for by the expression of other Tcfs in mammary epithelial or mesenchymal cells.

Wnt signaling requires Lef1 activity but may also involve Tcf1 and Tcf3, the latter two also expressed in the epithelial placode. However, we detected *Tcf1* in the epithelial cells of *Lef1*-deficient placodes (data not shown). It is clear, therefore, that Tcf1 does not act redundantly with Lef1 at this stage of mammary gland development. Indeed, the presence of *Tcf1* in *Lef1*-deficient placodes supports the notion that it may regulate canonical Wnt-signaling gene targets which are distinct from those regulated by Lef1. *Tcf1*-, *Tcf3*-, and *Tcf4*-deficient embryos have not been reported to exhibit mammary gland defects during embryogenesis (Korinek et al., 1998a; Merrill et al., 2004; Verbeek et al., 1995). Thus, at some stages of development, redundant activities of the Tcf/Lef1 family compensating for the lack of specific Tcf/Lef1 family members may occur. A more detailed study of the expression pattern and activities of the Tcf/Lef1 family proteins during specific stages of mammary gland development will be required to determine their precise roles in this tissue.

During embryonic mammary gland formation, placode pairs initiate in specified positions and order. Expression of *Lef1* in wild-type embryos demonstrated placodes initiate in the order of pair 3, then 4, followed by 5 and 1 and finally pair 2 (Mailleux et al., 2002 and data not shown). While loss of *Lef1* in mice results in a complete arrest of mammary gland development (van Genderen et al., 1994), we demonstrated that $Lef1^{-/-}$ embryos display defects in placode initiation and in placode progression. Morphological and histological analysis of *Lef1*-deficient embryos reveals that the initiation of the five placode pairs is variable. Placode pairs 1, 4, and 5 always initiate, while placodes 2 and 3 are only occasionally observed and always as individual placodes rather than in pairs. Interestingly, the first (placode 3) and last (placode 2) placode pairs to develop are absent in $Lef1^{-/-}$ embryos. Although these placodes are spatially localized to one another, their formation is temporally distant. Based on gene targeting studies, individual placodes appear to develop independently of one another or via different cellular/molecular mechanisms (Davenport et al., 2003; Mailleux et al., 2002; Schimmang et al., 1992). It is possible that development of each placode may have different genetic requirements. The mechanism of temporal and spatial localization of placode pairs is uncharacterized and mechanistically unexplored (reviewed in Veltmaat et al., 2003).

While our results suggest defective initiation of placodes 2 and 3, the defect for placodes 1, 4, and 5 in Lef1-deficient animals is placode progression. All placodes initiating in these animals arrest early in development (around 13.5 dpc). Both the epithelium (p63, K14, E-cadherin-positive, K. Boras, unpublished observation) and the mesenchyme (androgen receptor, see Fig. 4) express cell-specific markers demonstrating that specification of the mammary placode is normal. These data suggest that epithelial–mesenchymal interactions required for placode induction and mammary mesenchymal condensation are intact in the absence of Lef1. However, by 14.5 dpc, Wnt activity in the mammary epithelium of $Lef1^{-/-}$ placodes is blocked. We propose, therefore, that this signal is needed to respond to and integrate the adjacent underlying mesenchymal

signals. The epithelium either fails to respond to the received mesenchymal signal and/or to instruct the adjacent mesenchymal cells to develop further.

We suggest that one result of deregulated reciprocal signaling is the loss of a survival factor instructing the mesenchyme. In $Lef1^{-/-}$ embryos, we detect TUNEL-positive cells in the underlying dermis adjacent to the mutant buds. Also known as the secondary mesenchyme, these cells give rise to the fat pad precursor. It is unclear, however, if apoptosis in these cells contributes to the failure of mammary gland development and subsequent regression. The mechanism underlying the condensation of the fat pad precursor is unclear, although it occurs as the bud approaches this tissue suggesting regulation by reciprocal tissue interactions. Gene targeting studies indicate differentiation of this mesenchyme is required for bud development (Satokata et al., 2000). For example, targeted deletion of *Msx2* (Satokata et al., 2000) or *Fgf10* (Mailleux et al., 2002; Sakaue et al., 2002), genes expressed during the development of the fat pad precursor, result in mammary bud arrest before sprout elongation (15 dpc).

Our data characterizing the expression of factors in the Hh-signaling pathway suggest that loss of Hh signaling may contribute to the failure of the mammary placodes in *Lef1*-deficient animals to progress. The role of Hh signaling during embryonic mammary gland development is not well characterized. Analysis of embryonic mammary gland development determined that *Shh* and *Ihh* are expressed in the mammary placode (Gallego et al., 2002; Michno et al., 2003). Furthermore, members of the Hh-signaling pathway including *Gli2*, *Gli3*, and *Ptc1* are expressed in the mammary placode (Veltmaat et al., 2003; Figs. 7, 8 and data not shown). Loss individually of *Shh* or *Ihh* expression has no apparent effect on mammary gland development in embryos due, apparently to their redundant activities (Gallego et al., 2002; Michno et al., 2003). Embryonic defects in mammary glands have not been noted for either *Gli1* or *Gli2*, although in embryos homozygous for the $Gli3^{xt}$ (extra toes) allele, mammary placodes 3 and 5 are not induced (A. DeMaximy, J. Veltmaat and S. Bellusci, unpublished results cited in Lewis and Veltmaat, 2004; Mailleux et al., 2002). As expression of *Shh* in both hair and tooth development has been shown to require Lef1 expression (Gat et al., 1998; Kratochwil et al., 2002; Zhou et al., 1995), loss of *Lef1* in the embryonic mammary gland is consistent with deregulation of the Hh pathway in this skin appendage. We observed that the transcriptional regulators of Hh signaling, *Gli2*, are aberrantly expressed in $Lef1^{-/-}$ placodes and that the expression of the Hh-ligand receptor, *Ptc1*, is strongly reduced. As a target of Hh signaling, low levels of *Ptc1* expression are suggestive that the Hh pathway is inactive. Since expression of *Shh* and *Ihh* are detected in $Lef1^{-/-}$ placodes (data not shown), we propose that *Ptc1* may be a downstream target of Lef1/Wnt-signaling pathway.

Our results detecting Wnt signaling in cells of the developing embryonic mammary gland contradict those previously reported (Chu et al., 2004). This latter group employed animals (designated here as TOPGAL-F) harboring a Tcf/Lef1-βgal reporter transgene which appears to be identical to the one used

to generate the animals we employed (designated here as TOPGAL-C (Cheon et al., 2002; Staal et al., 2001)). In TOPGAL-F animals, Wnt signaling was detected exclusively in epithelial cells of the developing placode; no βgal expression was observed in the adjacent mesenchymal cells nor were alterations in Wnt signaling reported. For both TOPGAL-F and TOPGAL-C animals, no βgal activity was observed for either mouse in the mammary gland during puberty, and βgal activity was lost specifically in the mammary placode at 16.5 dpc where development halts just prior to sprout formation. However, 2 days later during embryogenesis, divergent Wnt signaling arises again; epithelial staining detected in the primitive ductal tree in TOPGAL-F embryos, while we observed in the TOPGAL-C embryos restricted βgal expression in the mesenchyme and primary ducts at the nipple region. Other differences in expression were also observed for all skin appendages. Specifically, we did not detect βgal activity in the developing whiskers and hair follicles, a pattern distinct from that of the TOPGAL-F mouse. Given the expression pattern we observed for TOPGAL-C animals in our study, we sought to ensure that an additional marker could support the pattern of Wnt-signaling activity. The presence of nuclear β-catenin is required for canonical Wnt signaling. Thus, we determined that when mesenchymal-specific activity of βgal is detected, β-catenin is present in the nucleus of mammary mesenchymal cells. Likewise, when Wnt signaling switches to the epithelial placode, β-catenin staining at cell junctions is difficult to detect while intracellular staining is more prominent. However, in the absence of *Lef1*, β-catenin strongly stains the cell junctions in the epithelial placode, consistent with the lack of Wnt signaling observed. We propose, therefore, that the different transgenic TOPGAL animals read out signaling of distinct Wnt ligands through specific Frizzled receptors. Indeed, we suggest that these and other TOPGAL founders may be invaluable tools in revealing specific Wnt/Frizzled signaling cascades in tissues whose development is influenced by this complex family of developmental factors.

Acknowledgments

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