Role of FGF10/FGFR2b signaling during mammary gland development in the mouse embryo

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SUMMARY

The mouse develops five pairs of mammary glands that arise during mid-gestation from five pairs of placodes of ectodermal origin. We have investigated the molecular mechanisms of mammary placode development using Lef1 as a marker for the epithelial component of the placode, and mice deficient for Fgf10 or Fgfr2b, both of which fail to develop normal mammary glands. Mammary placode induction involves two different signaling pathways, a FGF10/FGFR2b-dependent pathway for placodes 1, 2, 3 and 5 and a FGF10/FGFR2b-independent pathway for placode 4. Our results also suggest that FGF signaling is involved in the maintenance of mammary bud 4, and that Fgf10 deficient epithelium can undergo branching morphogenesis into the mammary fat pad precursor.

Key words: Fgf10, Fgfr2b, Lef1, Mammary placode development, Mouse, Cell signaling

INTRODUCTION

Mammary gland formation in the mouse initiates around E10 on the surface ectoderm of both lateral flanks of the embryo (Turner and Gomez, 1933). By E11-E12 five mammary placodes are detected as thickenings of the ectoderm. The placodes develop into bud-like structures that are located at precise points along the antero-posterior axis of the embryo. The position of the mammary buds, three thoracic and two inguinal, are reproducible between embryos suggesting a tight genetic control of their induction. Previous reports suggest that mammary placodes form by the migration of ectodermal or epidermal cells, rather than by cell proliferation, along a putative line running in an anterior to posterior direction just dorsal to the limb buds (Balinsky, 1950; Propper, 1978).

Little is known about the genes that regulate the initial phases of mammary placode development. The transcription factor Lef1, an effector of WNT/β-catenin signaling, is the earliest known marker of mammary placode formation. Its inactivation in vivo leads to embryos with only a single pair of inguinal placodes (van Genderen et al., 1994). The development of mammary buds, in both female and male mouse embryos, is dependent upon signaling by parathyroid hormone related-protein (PTHRP) through its receptor (PTHR1), which are expressed in the epithelium and condensed mammary mesenchyme respectively. In the absence of PTHrP, or its receptor, mammary buds fail to elongate and branch into the primitive fat pad of female embryos, or undergo the expected androgen-mediated apoptosis in males (Wysolmerski et al., 1998; Dunbar et al., 1999).

The fibroblast growth factor (FGF) family comprises at least 22 members, many of which have been implicated in multiple aspects of vertebrate development [for review see Ornitz and Itoh (Ornitz and Itoh, 2001)]. In particular, FGF10 has been associated with instructive mesenchymal-epithelial interactions, such as those that occur during branching morphogenesis. For example, in the developing lung, Fgf10 is expressed in the distal mesenchyme at sites where prospective epithelial buds will appear. Moreover, its dynamic pattern of expression and its ability to induce epithelial expansion and budding in organ cultures have led to the hypothesis that FGF10 governs the directional outgrowth of lung buds during branching morphogenesis (Bellusci et al., 1997). Furthermore, FGF10 was shown to be a potent chemoattractant for the distal lung epithelium (Park et al., 1998; Weaver et al., 2000). Consistent with these observations, mice deficient for Fgf10 show multiple organ defects including lung agenesis (Min et al., 1998; Sekine et al., 1999; Ohuchi et al., 2000).

The mammalian Fgf receptor family comprises four genes (Fgfr1 to Fgfr4), which encode at least seven prototype receptors. Fgfr1, 2 and 3 encode two receptor isoforms (termed IIIb or IIIc) that are generated by alternative splicing, and each
bind a specific repertoire of FGF ligands (Ornitz et al., 1996). FGF2/IIb (FGFR2b) is found mainly in epithelia and binds four known ligands (FGF1, FGF3, FGF7 and FGF10) which are primarily expressed in mesenchymal cells. While mice null for the Fgfr2 gene die early during embryogenesis, those that are null for the Fgfr2b isoform, but retain Fgfr2c, survive to birth (Arman et al., 1998; Xu et al., 1998; De Moerlooze et al., 2000; Revest et al., 2001). Mice deficient for Fgfr2b show agenesis and dysgenesis of multiple organs indicating that signaling through this receptor is critical for mesenchymal-epithelial interactions during early organogenesis.

Mammary gland development has been studied extensively in the post-natal animal, but less is known about the embryonic stages. We have investigated the initial phases of mammary placode development, and demonstrate using molecular markers and scanning electron microscopy that the placodes form asynchronously. Placode 3 is the first to appear, followed by placode 4, then placodes 1 and 5 and finally placode 2. The role of FGF10/FGFR2b signaling in the epithelial/mesenchymal interactions that characterize embryonic mammary gland development is demonstrated through the analysis of the mammary gland phenotypes of Fgf10<sup>−/−</sup> and Fgfr2b<sup>−/−</sup> embryos.

MATERIALS AND METHODS

In situ hybridization

Radioactive and whole-mount in situ hybridization protocols were based on previously described methods (Winnter et al., 1995). The following mouse cDNAs were used as templates for the synthesis of digoxigenin or 35S-labeled riboprobes: a 360 bp Lef1 probe provided by Dr Grosschedl, a Fgfr2-TK and IIb probes previously described (De Moerlooze et al., 2000), a 584 bp Fgf10 cDNA (Bellusci et al., 1997), a 1.5 kbp Bmp4 probe (Winnter et al., 1995) and a 622 bp Fgf7 cDNA (kindly provided by Dr Mason).

Scanning electron microscopy

E11.5 and E12.5 mouse embryos (C57BL/6) were extracted quickly from the uteri, washed 6 times in filtered PBS and fixed in a solution of sodium cacodylate 0.1 M pH 7.6/glutaraldehyde 2% at room temperature for 1 hour and then overnight at 4°C. They were washed three times in 0.2 M sodium cacodylate for 1 hour at room temperature for 1 hour and then overnight at 4°C. After a 5 minute wash in distilled water, the embryos were dehydrated in graded ethanol (70% to 100%) and then in amyl acetate (30% to 100%). They were critical-point dried in liquid carbon dioxide, mounted on aluminum stubs and coated with gold.

Mutant embryos

Fgf10<sup>−/−</sup> and Fgfr2b<sup>−/−</sup> embryos were generated as previously described (Sekine et al., 1999; De Moerlooze et al., 2000) and were on the C57BL/6 background. C57BL/6 or wild-type littermates mice were used as control embryos at different stages of development. The number of Fgf10<sup>−/−</sup> embryos used in this study at the different stages were as follows: E11.5 (n=5), E12.5 (n=2), E13.5 (n=2), E14.5 (n=3 females), E18.5 (n=9 females). The number of Fgfr2b<sup>−/−</sup> embryos used in this study were as follows: E11.5 (n=3), E12.5 (n=11), E13 (n=6), E14.5 (n=1 female), E15.5 (n=2 females), E16.5 (n=2 females).

Organotypic culture

Embryos were removed at E10.5 and E11.5. At E11.5, the heads were surgically removed and the remaining body of the embryos cut into halves along the dorsal-ventral axis. Embryos were placed on Nucleopore filters, which were then laid on the surface of 500 μl F12: DMEM medium containing 50 Units/mg penicillin and streptomycin, 1% glutamine and 10% heat-inactivated fetal calf serum in NUNCLON dishes [technique adapted from Lebeche et al. (Lebeche et al., 1999)]. We investigated the local effects of FGF10 on these cultures by implanting heparin beads (Sigma) impregnated with human recombinant FGF10 (Research and Development) (100 μg/ml) in the flanks of the embryos in the area of mammary placode formation. The embryos were usually incubated for 24 hours at 37°C under CO2 and then fixed for 2 hours in 4% PFA and processed for whole-mount in situ hybridization. BSA-impregnated beads were used as controls.

Mammary gland transplantation

The mammary gland 4 from Fgf10<sup>−/−</sup> (n=3) and wild-type fetuses (n=3) at E18.5 were freshly dissected and transplanted into cleared mammary fat pads of syngenic mice (Medina, 1996). In these experiments, 21-24 days old females were used as transplant recipients. The endogenous mammary epithelium was surgically removed from the fourth inguinal glands to provide a cleared mammary fat pad. Mutant and wild-type mammary glands were transplanted separately into contralateral glands of each recipient (n=3) to ensure an identical host environment. After 4 weeks, the mice were sacrificed and the fourth inguinal mammary glands dissected. The epithelium was stained using Carmin Red as previously described (Faraldo et al., 1998).

Analysis of cell death

Apoptotic cells were detected by the incorporation of terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) using the ApopTagRPlus In Situ Apoptosis Detection Kit (Oncor, USA) as recommended by the manufacturer.

RESULTS

Lef1 is dynamically expressed during mammary placode formation

Previous reports have shown that Lef1 is expressed initially in the epithelium of the mammary placode at E11-E12 (van Genderen et al., 1994; Foley et al., 2001). A detailed temporal analysis using whole-mount in situ hybridization, shows that Lef1 is expressed in the emerging placodes in a dynamic fashion between E11.5 and E11.75 (Fig. 1). Early E11.5 embryos show no Lef1 expression between the fore- and hindlimbs (Fig. 1A), but at a slightly later stage, Lef1 expression appears as a short line (Fig. 1B) which progressively changes through a comet-shape (Fig. 1C) to a disc (Fig. 1D). To gain more insight into the surface features of the mammary placodes, the embryos were visualized by scanning electron microscopy (SEM). At E11.5, placode 3 can be seen as a knob-like structure elevated above the surrounding epidermis (Fig. 1E,F).

Placode formation is asynchronous

Using the shape dynamics of Lef1 expression in the forming placode, together with SEM, the timing of mammary placode formation was determined. Placode 3 is the first to form as shown in Fig. 1, this is followed by placode 4, then placodes 1 and 5 and finally placode 2 (Fig. 2A-C). Analysis by SEM of the E12.5 embryo shows that at this stage of development, five mammary buds are localized on the flank of the embryo that showed distinctive features. At E12.5, the first mammary bud to appear (number 3) was far less elevated above the
epidermis than the newly formed mammary bud 2 (Fig. 1E,F and Fig. 2D-F). It appears that when the mammary buds first form they are elevated above the forming epidermis before subsiding.

**Fgfr2 and Fgf10 expression in the embryonic mammary gland**

The phenotypic similarities between *Fgf10* and *Fgfr2b* null mice indicate that FGF10 is the major ligand for FGFR2b. Hybridization of E11.5 and E15.5 embryo sections with a probe that recognizes *Fgfr2b* and *Fgfr2c* revealed a high level of *Fgfr2* in the epithelium of the mammary bud (Fig. 3A,B). Confirmation that *Fgfr2b* was the isoform present was shown with IIIb- and IIIc-specific probes (data not shown). Using whole-mount in situ hybridization, a transient expression of *Fgf10* was seen between E10.5 and E11 as a fine segmented line extending between the fore- and hindlimb (Fig. 3C,D). This domain of expression corresponds to the territory of the mammary line that is seen as a ridge in other mammals. Vibratome sections at E10.5 showed that this expression corresponds to the most ventral epithelial part of the dermamyotome (Fig. 3E,F). At E11.5, when mammary bud 3 has formed, no significant signal was detected (data not shown). However, by E15.5 a *Fgf10* signal was observed in the mammary fat pad precursor localized around the mammary bud (Fig. 3G). Using whole-mount sections through mammary placodes were analyzed with radioactive antisense probes to the known ligands of FGFR2b, namely *Fgf1, Fgf3, Fgf7* and *Fgf10*. Expression of *Fgf1, Fgf3* or *Fgf10* was not detected at this developmental stage.

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**Fig. 1.** *Lef1* expression in the mammary placode 3 between E11.5 and E11.75. Whole-mount in situ hybridization on embryos between E11.5 and E11.75 with dig-labeled antisense riboprobe for *Lef1*. (A) *Lef1* is not expressed at significant level at E11.5 between the forelimb and hindlimb. (B) First evidence of *Lef1* expression in the forming mammary placode (P3) occurs as a discrete line (between the 2 black arrows). (C and D) At E11.75, *Lef1* is now expressed as a comet-like shape with the tail of the comet being localized caudally (between the 2 arrows) and then as a round like structure (P3). (E) Scanning electron microscopy of E11.5 embryo showing placode 3 as a knob of cells slightly elevated above the surface of the epidermis. (F) High magnification of placode 3. P: mammary placode. Scale bar, A–D, 785 μm; E, 980 μm; F, 390 μm.

**Fig. 2.** Mammary placode development is asynchronous. Whole-mount in situ hybridization on embryos at E11.75 with dig-labeled antisense riboprobe for *Lef1*. (A) Lateral view of the embryo showing that placode 3 and placode 4 have formed and are seen as dot-like structures. (B) High magnification of the hindlimb area showing placode 4 as a dot-like structure and placode 5 as a line. (C) High magnification of the forelimb area showing placode 3 as a dot-like structure, placode 1 as a line and placode 2 as a diffuse line. (D–F) Scanning electron microscopy of the flank of a E12.5 wild-type embryo showing 5 mammary buds localized at precise points along the anteroposterior axis (3 thoracic and 2 inguinal). (E) High magnification of the box shown in D. Note that mammary bud 2, the last one to form, is the bud that is the most elevated above the surface of the epidermis. (F) High magnification of the mammary bud 2 showing individual cells at the surface of the knob-like structure. B1–5, mammary buds; P1–5, mammary placodes. Scale bar, A, 950 μm; B, 630 μm; C, 470 μm; D, 440 μm; E, 180 μm; F, 30 μm.
However, Fgf7 was detected in the mesenchyme surrounding the mammary bud at E12.5, but by E15.5 its expression had decreased and extended into the adjacent fat pad precursor (Fig. 3LJ).

**Fgfr2b−/− embryos transiently develop mammary bud 4**

Using whole-mount in situ hybridization with a Lef1 probe, the appearance of mammary placodes was monitored in Fgfr2b−/− embryos taken between E11.5 and E14.5. At E11.5 a characteristic placode 3 was clearly detected in wild-type or heterozygous embryos from the same litter, but not in the homozygous mutants (data not shown). The absence of placode 3 was also confirmed by histological analysis of serial sections. At E12.5, five pairs of mammary buds were clearly observed in the control embryos using the Lef1 probe as a marker (Fig. 4A). By contrast, only a single bud was located in the inguinal region of the homozygous mutant (Fig. 4C). Mammary bud identity was confirmed at the molecular level using a Bmp4 probe (Bmp4 is expressed in the condensed mesenchyme) (Phippard et al., 1996) and by sectioning and histological analysis (data not shown). Although the absence of limbs in the Fgfr2b−/− embryo makes it difficult to discriminate between the two inguinal buds, its relative position suggested it was number 4. We will therefore refer to this bud in mutant mice as bud 4. At E13, Lef1 expression was no longer detected in the inguinal region indicating the disappearance of bud 4 (compare Fig. 4G and 4E). A TUNEL assay was used to detect apoptotic cells, and this showed that the epithelium of the mutant mammary bud at E12.5 undergoes extensive apoptosis (Fig. 4I,J). The absence of bud 4 at E13 and E14.5 was also confirmed by histological analysis of serial sections (data not shown). In addition, an examination of embryonic skin from wild-type (n=2) and Fgfr2b null (n=2) female embryos at E16.5 failed to detect mammary bud development in the mutants, while five pairs were clearly seen in the wild-type skins (data not shown). In conclusion, placode 4 is the only placode to form in the Fgfr2b−/− embryos. A bud arising from this placode forms transiently at E11.5 and is then lost within a day through apoptosis. Hence, signaling through FGFR2b by one or more of its ligands is necessary to maintain mammary bud 4 after E12.5 and to induce the other mammary placodes.

**Mammary placode 4, but not placodes 1, 2, 3 and 5, is induced and maintained in Fgf10−/− embryos**

FGFR2b is the main receptor for FGF10 as evidenced by the remarkable similarity of phenotypes exhibited by embryos where these genes have been inactivated (De Moerlooze et al., 2000; Ohuchi et al., 2000). To examine the effect of Fgf10 abrogation on mammary placode induction and maintenance, we used whole-mount in situ hybridization with Lef1 and Bmp4 probes on Fgf10−/− embryos. At E11.5 and E12.5 a single inguinal bud was detected in a similar position to that seen in

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**Fig. 3. Fgfr2, Fgf7 and Fgf10 expression in the mammary buds between E10.5 and E15.5.** Section and whole-mount in situ hybridization using 35S-labeled and dig-labeled antisense riboprobes for Fgfr2, Fgf7 and Fgf10. (A) Fgfr2 expression at E11.5 showing a high expression in the epidermis and in the epithelium of the mammary bud (arrowhead). (B) Fgfr2 expression at E15.5 showing a high expression in the basal layer of the epidermis and in the epithelium of the mammary bud. (C) Fgf10 expression at E10.5. Note the expression of Fgf10 between the fore- and hindlimb (between the two black arrows) corresponding to the area were the mammary line is forming. Fgf10 is also detected in the progress zone of the limbs. (D) High magnification of C showing Fgf10 expression as a dotted line (between the 2 arrows). Double headed arrow indicates the position of section in E. (E) Vibratome cross section (40 μm) between the fore- and hindlimb of the embryo shown in (C). Fgf10 is detected in the most ventral part of the somites. (F) High magnification of the boxed area in E showing Fgf10 expression in the epithelial part of the dermamyotome. (G) No expression of Fgf10 is detected in the mammary bud at E11.5. (H) Fgf10 expression at E15.5. Note the expression in the mammary fat pad precursor surrounding the mammary bud and in the mesenchyme of the hair follicles. (I) Fgf7 expression at E12.5. Note the high expression in the mesenchyme surrounding the epithelial bud. (J) Fgf7 expression at E15.5. Note the expression in the fat pad precursor. dm, dermamyotome; e, epithelium; fl, forelimb; fp, fat pad precursor; hf, hair follicles; hl, hindlimb; int, intestine; m, mesenchyme; my, myotome; nt, neural tube; mm, mammary mesenchyme; so, somites. Scale bar, A,B, 8 μm; C, 430 μm; D, 210 μm; E, 130 μm; F, 32 μm; G-J, 10 μm.
Fig. 4. Mammary buds 1, 2, 3 and 5 are not detected in the Fgfr2b−/− embryos. Whole-mount in situ hybridization on Fgfr2b−/− and wild-type embryos at E12.5 and E13 with dig-labeled antisense riboprobe for Lef1 (A-H) and cell death analysis (I,J). B, D, F, H are high magnification views of the boxed areas in A, C, E and G, respectively. (A) Lef1 expression at E12.5 in wild-type embryo showing expression in the epithelium of the mammary bud. Note that only three mammary buds, located between the forelimb and hindlimbs, are visible. Mammary buds 1 and 5 are located behind the limbs. (B) Mammary bud 4. (C) Mammary bud 4. (D) Mammary bud 4. (E) Mammary bud 4. (F) Mammary bud 4. (G) Hematoxylin-eosin staining of mammary bud 4. (H) Hematoxylin and Eosin staining of mutant mammary bud 4 showing a normal structure. Scale bar, A, 670 μm; B, 100 μm; C, 670 μm; D, 100 μm; E, 1220 μm; F, 235 μm; G, 1220 μm; H, 235 μm; I, J, 10 μm.

Fig. 5. Mammary buds 1, 2, 3 and 5 are not detected in the Fgfr2b−/− embryos. Whole-mount in situ hybridization on Fgf10−/− and wild-type embryos at E11.5 and E12.5 with dig-labeled antisense riboprobe for Lef1. B, D, F and I are high magnification views of the boxed areas in A, C, E and H respectively. (A) Lef1 is expressed in the mammary placodes at E11.5. Note that only mammary placodes 3 and 4 are clearly visible. (B) Mammary placode 4. (C) Lef1 expression is not detected in mammary placodes 1, 2, 3 and 5. Note Lef1 expression as a line in the area corresponding to mammary placode 4 (dotted box). (E) Lef1 expression at E12.5 in wild-type embryo showing expression in the epithelium of the mammary bud. Note that only mammary buds 2, 3 and 4 are visible. (F) Mammary bud 4. (G) Hematoxylin-eosin staining of mammary bud 4. (H) Lef1 expression in the Fgf10−/− embryo shows that only mammary bud 4 is detected (dotted box). (J) Hematoxylin and Eosin staining of mutant mammary bud 4 showing a normal structure. Scale bar, A, 670 μm; B, 100 μm; C, 670 μm; D, 100 μm; E, 1050 μm; F, 150 μm; G, 14 μm; H, 1050 μm; I, 150 μm; J, 14 μm.
and branch into a wild-type stroma (Fig. 6J), as was the epithelium (n=1) from Fgf10–/– (Fig. 6K,L). Epithelium from both wildtype and mutant mice grew radially from the implant and showed prominent terminal end buds.

**FGF10 fails to induce Lef1 expression in the epithelium**

The similarity in mammary placode phenotype between Fgf10–/– and Lef1–/– embryos raised the interesting possibility that FGF10 induces Lef1 expression. To test this idea, FGF10-coated beads were grafted onto the flank of E10.5 and E11.5 embryos. After 30 hours of culture the FGF10-coated beads failed to induce Lef1 expression in the surrounding ectoderm of E10.5 or E11.5 embryos (Fig. 7A,B). Importantly, endogenous Lef1 expression corresponding to the normal mammary buds was detected, indicating that endogenous placode formation occurred normally (Fig. 7B). Note that the positions of the beads were dorsal, ventral and coincident with the putative mammary line. The positive control used in this experiment was the induction of Sprouty2 in the lung endoderm by FGF10-coated beads (Mailleux et al., 2001).

**DISCUSSION**

**Lef1 expression in the mammary placode is dynamic and correlates with cell migration**

In mouse and rabbit embryos, proliferation index studies on epidermis and mammary buds have suggested that placode formation primarily involves cell migration, since cells within the buds showed less proliferation than the surrounding epidermis (Balinsky, 1950). This hypothesis received support from SEM studies with E13.5 rabbit embryos that showed
individual cells along a raised mammary line having loose intercellular contacts and exhibiting pseudopodia, a hallmark of cell motility (Propper, 1978).

Until recently, no molecular marker was available to monitor the emergence of mammary epithelial cells during the initial phases of placode development. The transcription factor *Lef1* has been shown to participate in Wnt signaling by complexing with β-catenin to form a transcriptional complex that modulates the expression of WNT-responsive genes (Behrens et al., 1996; Eastman and Grosschedl, 1999). *Lef1* is expressed at the very early stages of placode formation (van Genderen et al., 1994). We show here that *Lef1* has a very dynamic expression pattern that appears to mark the cells that aggregate to form the mammary placode. For each placode, *Lef1* expression goes from a line, to a comet shape and finally to a characteristic disc. These observations would suggest that epithelial cells are recruited locally along a mammary line and migrate to a precise location to form the mammary placode.

**Mammary placode formation is asynchronous**

The timing of placode formation has not been addressed to date, but might be expected to occur sequentially, from anterior (placode 1) to posterior (placode 5) in line with other aspects of mouse development. However, using *Lef1* expression to monitor placode formation, they were found to emerge between E11.5-E11.75 in the order 3, 4, 1 and 5 and then 2. This order of placode appearance is supported by SEM observations that show that they initially form an epidermal mound that subsides and become undetectable by E14.5 (Fig. 1, Fig. 2 and S. B. and A. de Maximy, unpublished data). One explanation for asynchrony is that placode formation is autonomous. This is consistent with placode 4 formation in *Fgf10*-/- and the *Fgfr2b*-/- embryos, although we cannot exclude the idea that the single inguinal placode is actually a fusion of placodes 4 and 5 which fail to separate. The autonomous nature of placode formation is also supported by our recent work on the *Fgf10* expression in the dermomyotome between E10.5 and E11, prior to placode formation, may be indicative of such a mammary line. The chemotactant properties attributed to FGF10 in the migration of lung epithelium during branching morphogenesis, suggests a similar role in directing the migration of the epithelial cells along such a hypothetical mammary line. Alternatively, FGF10 may act to specify ectodermal cells destined to form mammary placodes.

The lack of placodes 1, 2, 3 and 5 in both *Fgf10*-/- and *Fgfr2b*-/- mice was based on the absence of expression of the molecular markers *Lef1* or *Bmp4* as well as by direct histological examination. Apart from placode 4, these findings suggest a model where FGF10 might regulate *Lef1* expression that in turn helps to specify the mammary epithelium. This would be consistent with *Lef1*-/- embryos also having a similar mammary phenotype, with a single inguinal placode. The possibility that *Lef1* regulates Fgf10 seemed unlikely since its expression precedes *Lef1* in mammary placode development. However, FGF10-coated beads did not induce *Lef1* expression when placed in the epidermis close to or within the proposed mammary line, indicating that FGF10 is either not involved in *Lef1* induction, or it is required earlier to help specify the mammary epithelium. It is also possible that the epithelium is only competent to respond to FGF10 for a short period of time, or that other growth factors act in synergy with FGF10 to induce *Lef1* expression.

**FGF2b ligands are involved in mammary bud 4 maintenance**

In the *Fgfr2b*+/- embryos, bud 4 is formed but undergoes apoptosis after E12.5, while in *Fgf10*+/- embryos this bud is maintained. This finding suggests that an additional FGFR2b ligand is involved in the maintenance of the inguinal mammary bud. In situ hybridization analysis for genes encoding known FGFR2b ligands during this stage of development showed that *Fgf7* was the only one detected at E12.5 in the surrounding mesenchyme of the mammary bud (Cunha and Holm, 1996), and therefore may act redundantly with *Fgf10* to maintain placode integrity.

**Fgf10 is not critical for mammary bud 4 epithelium ingrowth into the fat pad precursor**

In female embryos between E12 and E16, the mammary bud shows a low level of proliferation termed the resting phase. At late E16 proliferation increases and the mammary bud elongates to form the mammary sprout. The sprout grows rapidly downward, penetrating the mammary fat pad precursor tissue that underlies the mammary placode (for reviews see Sakakura and Robinson et al. (Sakakura, 1987; Robinson et al., 1999)). As the ductal epithelium penetrates the fat pad it begins to branch. PTHrP is expressed in the mammary epithelium and appears to signal to PTHR1 expressed in the surrounding mesenchyme. Disruption of the *PTHrP* gene leads to an absence of epithelial bud elongation and subsequent ductal branching and to the degeneration of the mammary epithelium (Wysolmerski et al., 1998). As *Fgf10* is expressed in the presumptive fat pad, it is plausible that FGF10 could be a downstream target of the PTHrP/PTHR1 signaling pathway. However, our results indicate that FGF10 was not critical for the growth of the epithelium into the mammary fat pad to form the mammary sprout, although this result only applies for the mammary bud 4. FGF10 could certainly play a role in the directional growth of the other buds. Interestingly, the epithelial sprout of the mutant mammary gland did not ramify extensively after penetrating the fat pad, but this abnormality was not apparent when the *Fgf10*-deficient epithelium was transferred into a wild-type stroma. This suggests that the branching defect is due to a defect in the *Fgf10*+/- fat pad that is unable to support proper branching. Consistent with this finding is a recent work demonstrating that FGF10 has a role in the development of adipose tissue, where it plays a role in the differentiation of the pre-adipocytes into adipocytes (N. Itoh unpublished data).
In conclusion, we have shown that LeF1 expression in the mammary placode is dynamic, that mammary placode formation is asynchronous and involves two different signaling pathways, a FGF10/FGFR2b-dependent pathway for placodes 1, 2, 3 and 5 and a FGF10/FGFR2b-independent pathway for placode 4. Our results also suggest that one or several members of the FGF family are involved in mammary bud 4 maintenance and that Fgf10 expression is not crucial for penetration of the mammary duct of bud 4 into the fat pad precursor.

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