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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). FGFR2-IIIb (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*$^{-/-}$ and *Fgfr2b*$^{-/-}$ embryos.

MATERIALS AND METHODS

In situ hybridization

Radioactive and whole-mount in situ hybridization protocols were based on previously described methods (Winnier et al., 1995). The following mouse cDNAs were used as templates for the synthesis of digoxigenin or ^{35}S-labeled riboprobes: a 360 bp *Lef1* probe provided by Dr Grosschedl, a *Fgfr2-TK* and *IIIb* probes previously described (De Moerlooze et al., 2000), a 584 bp *Fgf10* cDNA (Bellusci et al., 1997), a 1.5 kbp *Bmp4* probe (Winnier 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 and transferred in a solution of sodium cacodylate 0.1 M pH7.6/OsO_4 0.1% for 1 hour at room temperature. After a 5 minute wash in distilled water, the embryos were dehydrated in graded ethanols (70% to 100%) and then in amyl acetate (30% to 100%). They were critical-point dried in liquid carbon dioxyde, mounted on aluminum stubs and coated with gold.

Mutant embryos

Fgf10$^{-/-}$ and *Fgfr2b*$^{-/-}$ 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*$^{-/-}$ 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*$^{-/-}$ 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 CO_2 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*$^{-/-}$ (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

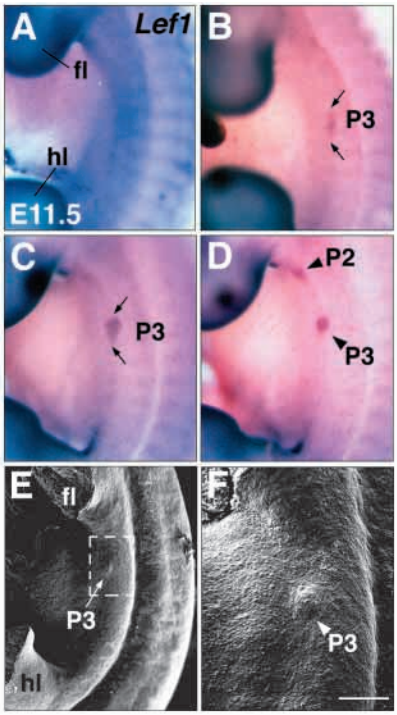

Fig. 1. *Lef1* expression in the mammary placode 3 between E11.5 and E11.75. Whole-mount in situ hybridization on embryos between E11.5 and E11.75 with dig-labeled antisense riboprobe for *Lef1*. (A) *Lef1* is not expressed at significant level at E11.5 between the forelimb and hindlimb. (B) First evidence 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.

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). This was confirmed by radioactive in situ hybridization at E11.5 (Fig. 3G). However, by E15.5 a *Fgf10* signal was observed in the mammary fat pad precursor localized around the mammary bud (Fig. 3H). At E12.5, 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.

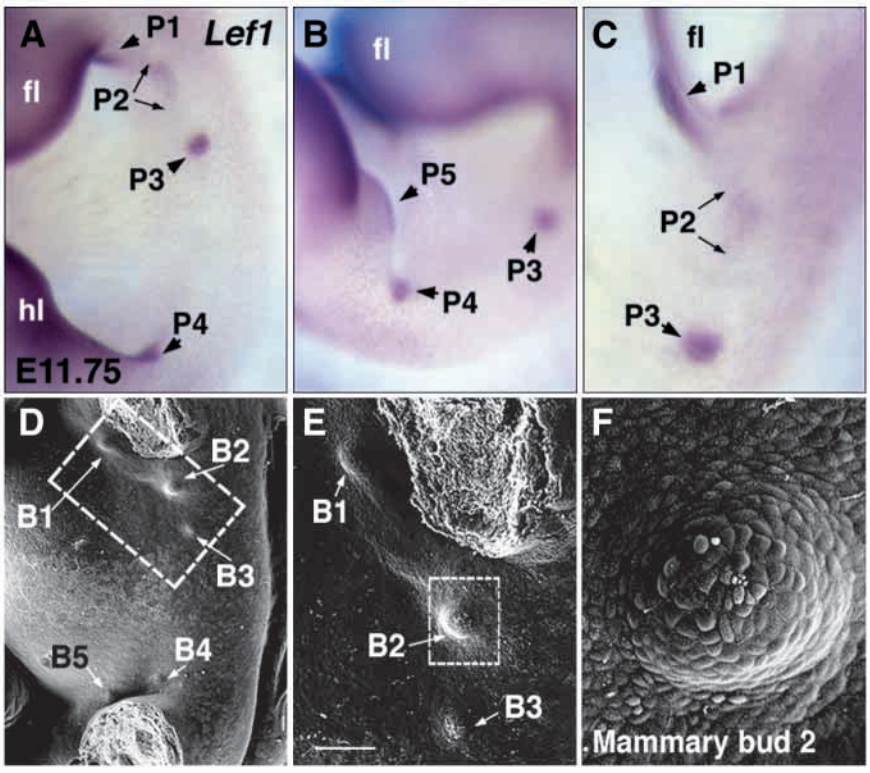

Fig. 2. Mammary placode development is asynchronous. Whole-mount in situ hybridization on embryos at E11.75 with dig-labeled antisense riboprobe for *Lef1*. (A) Lateral view of the embryo showing that placode 3 and placode 4 have formed and are seen as dot-like structures. (B) High magnification of the 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.

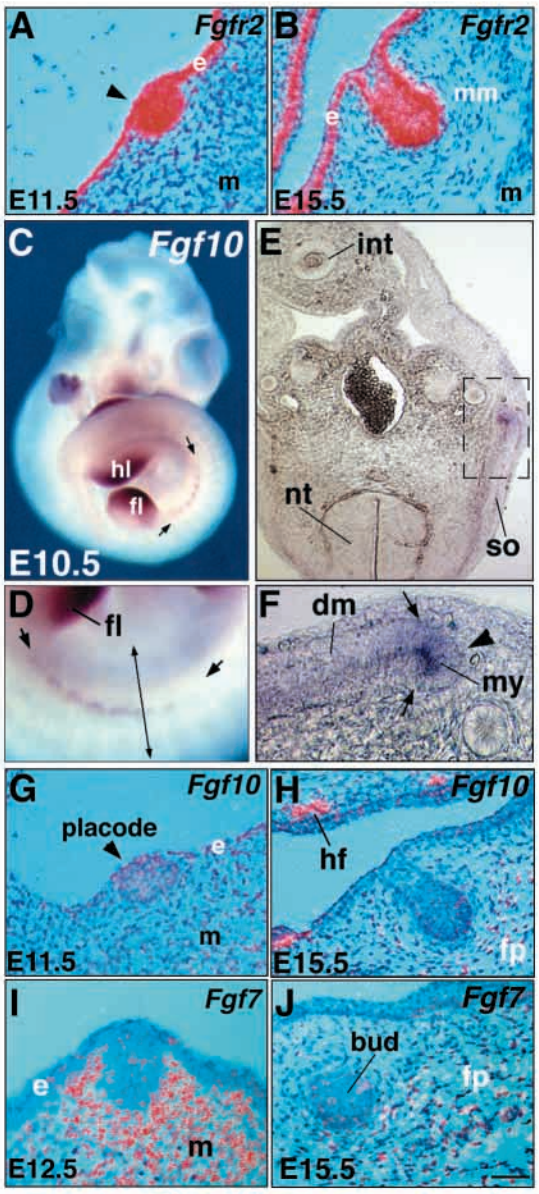

Fig. 3. *Fgfr2*, *Fgf7* and *Fgf10* expression in the mammary buds between E10.5 and E15.5. Section and whole-mount in situ hybridization using ^{35}S-labeled and dig-labeled antisense riboprobes for *Fgfr2*, *Fgf7* and *Fgf10*. (A) *Fgfr2* expression at E11.5 showing a high expression in the epidermis 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.

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. 3I,J).

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

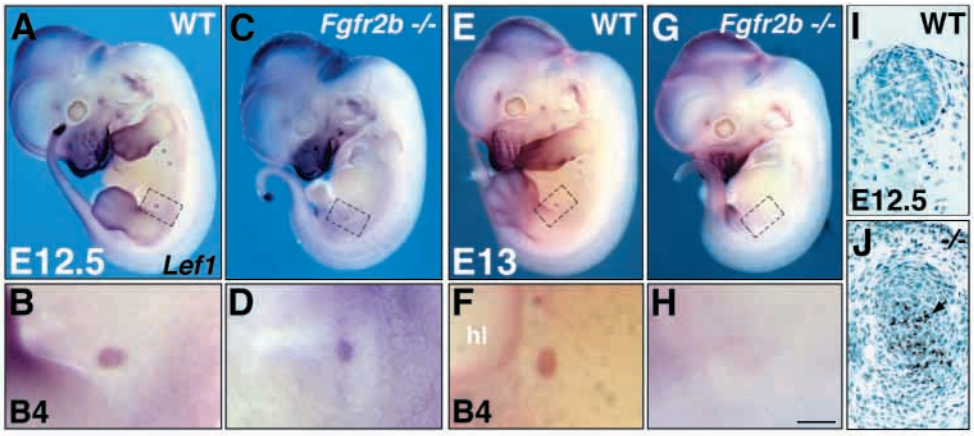

Fig. 4. Mammary buds 1, 2, 3 and 5 are not detected in the *Fgfr2b*$^{-/-}$ embryos. Whole-mount in situ hybridization on *Fgfr2b*$^{-/-}$ and wild-type embryos at E12.5 and E13 with dig-labeled antisense riboprobe for *Lef1* (A-H) and cell death analysis (I,J). B, D, F, H are high magnification views 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) *Lef1* expression at E12.5 in *Fgfr2b*$^{-/-}$ embryo showing that only one mammary bud is detected. (E) *Lef1* expression at E13 in wild-type embryo showing expression in the epithelium of the mammary bud. (F) Mammary bud 4. (G) *Lef1* expression in the *Fgfr2b*$^{-/-}$ embryo is no longer detected in the area corresponding to the mammary bud 4 (dotted box). (I) Section of normal mammary bud at E12.5 showing a complete absence of cells stained by the TUNEL method. (J) *Fgfr2b*$^{-/-}$ mammary bud at E12.5 showing a high number of individual apoptotic cells labeled in the epithelium. Scale bar, A, 875 μm; B, 160 μm; C, 875 μm; D, 160 μm; E, 1220 μm; F, 235 μm; G, 1220 μm; H, 235 μm; I, J, 10 μm.

the *Fgfr2b*$^{-/-}$ embryos, and we therefore refer to it as bud 4 (Fig. 5 and data not shown for *Bmp4*). Sectioning of the E12.5 mutant embryos confirmed the absence of bud 1, 2, 3 and 5 but the presence of bud 4 (Fig. 5H-J). At E13.5, the identification of mammary buds by whole-mount in situ hybridization using a *Lef1* probe was more difficult, as this gene is also expressed in the developing hair follicles (Fig. 6A,C). However, close examination allowed discrimination of the mammary buds as large and round structures from the hair follicles. The presence of a typical mammary bud at this stage was also confirmed by sectioning (data not shown). Therefore we can conclude that one pair of inguinal mammary placodes is induced in the *Fgf10*$^{-/-}$ mutants, but unlike the *Fgfr2b* null mice, the corresponding bud is maintained at E13.5.

Fgf10 expression in the mammary fat pad precursor at E15.5 suggested that it might direct the growth of the mammary epithelium toward the fat pad. As bud 4 is maintained in these mice we tested this idea. Skins containing mammary gland 4 from E18.5 *Fgf10*$^{-/-}$ female embryos (*n*=9) were dissected and examined for the extent of ductal branching after staining. Fig. 6G,H shows that in the *Fgf10*$^{-/-}$ mammary gland, the epithelial bud penetrated the mammary fat pad (*n*=9). In 5 cases it was not branched and in 4 it only formed two short branches at the extremity of the main duct. This result is in contrast to wild-type mammary glands that showed secondary and tertiary branches (Fig. 6E,F). During the dissection we noticed that the white adipose tissue forming the fat pad was very thin in the *Fgf10*$^{-/-}$ embryos compared to the wild type (data not shown), suggesting that the absence of branching in the mutant epithelium may be due to an abnormal recipient fat pad. The competence of *Fgf10*-deficient epithelium to undergo ductal branching was tested by transplantation of mammary bud 4 into a normal fat pad. The dissected glands (epithelium + mesenchyme) were grafted into cleared mammary fat pads of wildtype syngenic C57Bl/6 females. Four weeks post-grafting the wild-type epithelium (*n*=2) was fully competent to invade

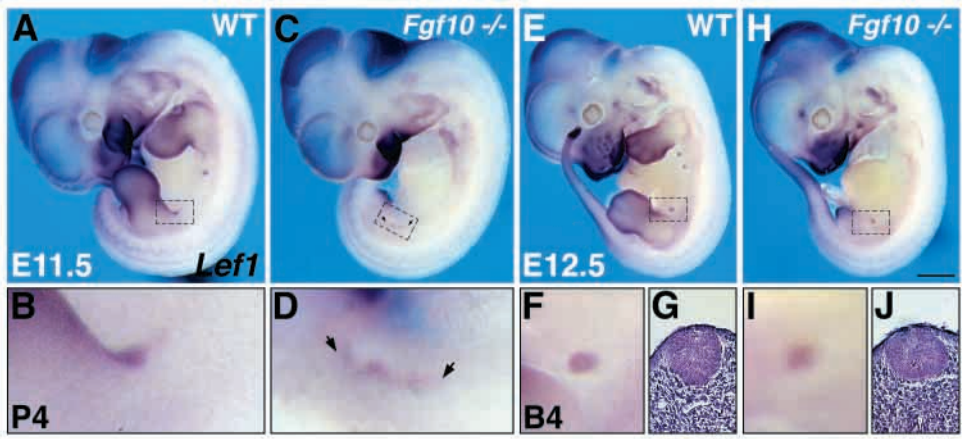

Fig. 5. Mammary buds 1, 2, 3 and 5 are not detected in the *Fgf10*$^{-/-}$ embryos. Whole-mount in situ hybridization on *Fgf10*$^{-/-}$ and wild-type embryos at E11.5 and E12.5 with dig-labeled antisense riboprobe for *Lef1*. B, D, F and I are high magnification views of the boxed areas in A, C, E and 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.

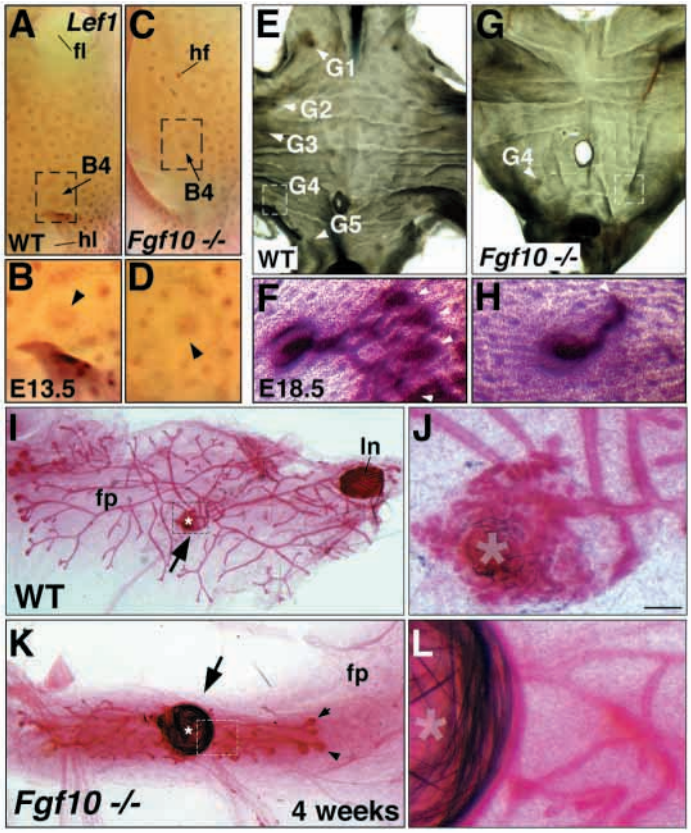

Fig. 6. Mammary bud 4 from *Fgf10*$^{-/-}$ mutant female embryos is maintained and competent to ramify in wild-type stroma. Whole-mount in situ hybridization on *Fgf10*$^{-/-}$ and wild-type embryos at E13.5 with dig-labeled antisense riboprobe for *Lef1* and transplantation of wild-type and mutant mammary glands in cleared fat pads. B and D are higher magnification views of the boxed areas in A and C, respectively. (A) *Lef1* expression is detected in the hair follicles (small dots) and in the mammary buds (dilated dots) in wild-type embryos at E13.5. (B) Note that the mammary bud 4 (black arrowhead) is bigger than the hair follicles. (C) *Lef1* expression on *Fgf10*$^{-/-}$ embryo. (D) Mammary bud 4 is still present at E13.5. (E) Transillumination picture of dissected skin corresponding to wild-type female embryo at E18.5. Note that five pairs of nipples are clearly visible. (F) Carmin Red staining of normal mammary gland 4 showing a ramified structure with numerous end buds (white arrowheads). (G) Transillumination picture of dissected skin corresponding to *Fgf10*$^{-/-}$ female embryos at E18.5. Note that only one pair of nipples is visible. (H) Carmin Red staining of mutant mammary gland 4 showing a single elongated sprout (white arrowhead). (I) Carmin Red staining of cleared fat pads transplanted with wild-type mammary gland 4 and cultured for 4 weeks. The epithelium of wild-type mammary gland 4 (black arrow) has extensively ramified into the cleared fat pad in both directions. (J) High magnification of the transplanted mammary gland (asterisk) showing a nicely ramified epithelium emerging from it. (K) The epithelium of mutant mammary gland 4 (black arrow) has also ramified into the cleared fat pad in both directions. Note the presence of numerous end buds (small black arrows). (L) High magnification of the transplanted mammary gland (asterisk) showing a ramified epithelium emerging from it and hairs (dark stripes) at the graft side. B4, mammary bud 4; fp, fat pad; G1-5, mammary glands; ln, lymph node. Scale bar, A, 800 μm; B, 309 μm; C, 800 μm; D, 309 μm; E, 1800 μm; F, 80 μm; G, 1800 μm; H, 80 μm; I, 2600 μm; J, 315 μm; K, 2100 μm; L, 270 μm.

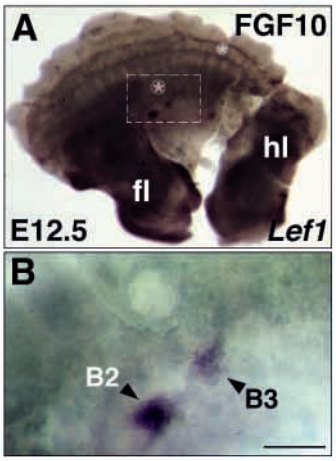

Fig. 7. FGF10-coated beads fail to induce Lef1 expression in the epithelium. Organotypic culture after FGF10-coated bead grafting was carried out for 24 hours and followed by whole-mount in situ hybridization to detect *Lef1* expression. (A) Two FGF10-coated beads (asterisks) were grafted on the flank of E11.5 embryos. One bead was grafted close to the mammary line and the other one was grafted more dorsally. (B) High magnification of the boxed area in A showing endogenous *Lef1* expression in the mammary placode 2 and 3 but no *Lef1* induction in the ectoderm surrounding the bead. fl, forelimb; hl, hindlimb. Scale bar, A, 890 μm; B, 170 μm.

and branch into a wild-type stroma (Fig. 6I,J), 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 *Extratoes* mice, which have a deletion in the *Gli3* gene (Hui and Joyner, 1993). The *Gli3* null embryos exhibit a lack of induction of placode 3 and 5, while the other placodes are induced normally (S. B. and A. de Maximy, unpublished data).

The FGFR2b/FGF10 pathway is involved in the initial phases of development of mammary glands 1, 2, 3 and 5

In contrast to the rabbit, a distinguishable mammary line seen as an elevation on the surface ectoderm has not been observed in the mouse (Bellusci and de Maximy, unpublished data; Balinsky, 1950; Propper, 1978). However, the observation of a line of transient *Fgf10* expression in the dermomyotome between E10.5 and E11, prior to placode formation, may be indicative of such a mammary line. The chemoattractant 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.

FGFR2b 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*$^{-/-}$ fad 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 conlcusion, 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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