Little is known about the regulation of cell fate decisions that lead to the formation of five pairs of mammary placodes in the surface ectoderm of the mouse embryo. We have previously shown that fibroblast growth factor 10 (FGF10) is required for the formation of mammary placodes 1, 2, 3 and 5. Here, we have found that Fgf10 is expressed only in the somites underlying placodes 2 and 3, in gradients across and within these somites. To test whether somitic FGF10 is required for the formation of these two placodes, we analyzed a number of mutants with different perturbations of somitic Fgf10 gradients for the presence of WNT signals and ectodermal multilayering, markers for mammary line and placode formation. The mammary line is displaced dorsally, and formation of placode 3 is impaired in Pax3ILZ/ILZ mutants, which do not form ventral somitic buds. Mammary line formation is impaired and placode 3 is absent in Gli3+/- and hypomorphic Fgf10 mutants, in which the somitic Fgf10 gradient is shortened dorsally and less overall Fgf10 is expressed, respectively. Recombinant FGF10 rescued mammogenesis in Fgf10−/− and Gli3+/-;Fgf10 mutants, which we correlated increasing levels of somitic FGF10 with progressive maturation of the surface ectoderm, and show that full expression of somitic Fgf10, co-regulated by GLI3, is required for the anteroposterior pattern in which the flank ectoderm acquires a mammary epithelial identity. We propose that the intra-somitic Fgf10 gradient, together with ventral elongation of the somites, determines the correct dorsoventral position of mammary epithelium along the flank.

KEY WORDS: Ectodermal patterning, Mammary gland, Somites, Placode individuality, FGF10/FGFR2B, GLI3, PAX3, WNT signaling, Mouse

INTRODUCTION

Mammogenesis in the mouse starts around embryonic day 10.5 (E10.5) with the formation of three separate streaks on both flanks. These streaks consist of multilayered surface ectoderm, specifically expressing Wnt10b (Veltmaat et al., 2004) and engaged in canonical WNT signaling (Chu et al., 2004). The first streak forms the mammary line between the forelimb and hindlimb, and mammary placode 3 develops first on this line at E11.0-E11.5 (Mailleux et al., 2002; Eblaghie et al., 2004; Veltmaat et al., 2004). Meanwhile, the inguinal and axillary streaks arise, from which placodes 5 and 1, respectively, form. Placodes 4 and 2 emerge where the mammary line abuts these respective streaks (Veltmaat et al., 2004).

Few genes are known to be involved in early mammogenesis (Mustonen et al., 2003; Chu et al., 2004; Mustonen et al., 2004; Howard et al., 2005; Jerome-Majewska et al., 2005). Most of them are expressed in the ectoderm, yet factors from the underlying mesenchyme initiate mammogenesis (Veltmaat et al., 2003). We have previously shown that FGF10 signaling via the FGFR isof orm 2b (FGFR2B) is required for the formation of mammary placodes 1, 2, 3 and 5. We proposed the ventral (hypaxial) dermomyotome of the somites as the source of FGF10 (Mailleux et al., 2002). Hypaxial FGF10 may be a mesenchymal initiator of mammogenesis, as it could reach the ectodermal FGFR2B either via diffusion through the thin lateral plate mesoderm or via delamination of hypaxial cells.

The emergence of the mammary line as fragments overlying the hypaxial somitic buds further suggested an involvement of hypaxial somitic signals in induction (Veltmaat et al., 2004). However, only the thoracic somites 11-24 between the forelimb and hindlimb possess hypaxial buds (Eloy-Trinquet, 2000). Therefore, we now hypothesize that hypaxial signals are important only for the formation of the mammary line on which mammary placodes 2, 3 and 4 form (Fig. 1), whereas other sources of signals may be decisive for the formation of the streaks on which placodes 1 and 5 form. As placode 4 forms in the absence of FGF10/FGFR2B signaling, the requirement for hypaxial FGF10 would thus be restricted to the formation of placodes 2 and 3 only.

To test this hypothesis, we examined mammary development in PAX3 null mutants that lack the hypaxial somitic buds (Relaix et al., 2003); in GLI3 null and Fgf10 hypomorphic embryos, both expressing reduced levels of somitic Fgf10; in Fgf10 and Fgfr2b null embryos; and by applying recombinant FGF10. We identified a signaling cascade involving somitic GLI3 upstream of FGF10 within the somites, which in turn activates ectodermal FGFR2B, leading to Wnt10b expression and canonical WNT signaling. This cascade is required for the induction and correct positioning of the
mammary line on the flank, and indispensable for the formation of placode 3. We propose that correct patterning is achieved through a combination of somitic elongation and somitic Fgf10 gradients.

RESULTS

The absence of hypaxial somitic buds correlates with impaired initiation of mammogenesis

To test whether hypaxial signals are required for the initiation of mammogenesis, we analyzed mammary line formation in Pax3<sup>3LZ/3LZ−</sup>-null mutant embryos. These mutants lack the hypaxial buds of somites 11-24, as shown by the shortened domain of somitic lacZ expression replacing Pax3 expression (Fig. 2A-D) (Relaix et al., 2003). Somites 11-24 are located between the forelimb and hindlimb, underlying the mammary line and placodes 2, 3 and 4 (Fig. 1, Fig. 2A).

Whole-mount in situ hybridization shows that Wnt10b is expressed at reduced levels and in a narrower line on the flank of Pax3<sup>3LZ/3LZ−</sup> embryos compared with control littermates (Fig. 2E-H). Placode 3 forms a day late, while the other placodes emerge in time (Fig. 2I,J, and not shown). Furthermore, Wnt10b expression is not attenuated between the placodes, and seems to be located slightly more dorsal in Pax3<sup>3LZ/3LZ−</sup> mutants. Transverse sections demonstrate that the mammary line is located at the level of the notochord in Pax3<sup>3LZ/3LZ−</sup> mutants, but more ventrally in control embryos (lines in Fig. 2K-N). The narrower width of the mammary line in Pax3<sup>3LZ/3LZ−</sup> mutants, as inferred from Wnt10b expression (Fig. 2F), correlates with a narrower band of multilayered ectoderm (between asterisks in Fig. 2O,P), which moreover contains one cell layer less than in control embryos (Fig. 2O,P). This phenotype strongly suggests that hypaxial signals are required for the correct temporal formation and dorsoventral position of the mammary line and placode 3, but surprisingly, not for the formation of placodes 2 and 4, and as expected, not for placodes 1 and 5.

Our previous data had suggested that at E10.5, the hypaxial buds produce the FGF10 required for mammary placode formation (Mailleux et al., 2002). However, by E11.25 (43 somites), Fgf10 expression extends throughout the entire thoracic somites 12-18 spanning the region underlying mammary placodes 2 and 3, while expression is highest in the hypaxial buds (Fig. 2Q). This domain of highest Fgf10 expression is absent in the somites of Pax3<sup>3LZ/3LZ−</sup> mutant embryos, while the level of Fgf10 expression in the central part of the somites seems unaffected (Fig. 2R). As mammary placode 3 is formed in Pax3<sup>3LZ/3LZ−</sup> mutants, and Pax3 is neither expressed in the surface ectoderm nor in the underlying mesenchyme (Fig. 2A,C) (Relaix et al., 2003), we now hypothesize that both hypaxial and central somitic Fgf10 are required for the formation of placode 3, and perhaps for the mammary line prior to placode formation.

Defective initiation in mammogenesis in Fgf10<sup>Δ10</sup> and Fgfr2b<sup>Δ11</sup> mutants

To test this hypothesis, we examined ectodermal maturation in detail and determined when the defect in mammogenesis becomes first apparent in Fgf10<sup>Δ10</sup> and Fgfr2b<sup>Δ11</sup> embryos. Normally, the surface ectoderm starts out as a single layer, the stratum germinativum, of squamous cells (Fig. 3A,B). At the Wolfian ridge (i.e. the flank), these cells become cuboidal and covered by a layer of squamous periderm by E8 (Fig. 3C) (Sengel, 1976; Stephens, 1982). By E11.5 (45 somites) the cells of the stratum germinativum become cylindrical at the flank while remaining squamous ventral and dorsal to the flank (Sengel, 1976) (Fig. 3B). Subsequently, one or two non-stratified layers of cylindrical cells form the stratum intermediate between the stratum germinativum and the periderm (Sengel, 1976). We show that this multilayering occurs first ventrally on the flank, and co-localizes with Wnt10b expression (Fig. 3D,G), indicative for the formation of the mammary line and the individual placodes. In

Fig. 1. The position of mammary rudiments relative to the somites. E11 embryo with the somites (numbered) indicated as grey contours. Only the thoracic somites 11-24 possess a ventrally elongated hypaxial bud, underlying the region where mammary placodes 2, 3 and 4 develop (black dots). Mammary placodes 1 and 5 are covered by the forelimb and hindlimb, respectively, and are not indicated.

MATERIALS AND METHODS

Mice

Fgf10<sup>+/−</sup> (Sekine et al., 1999) and Gli3<sup>−/−</sup> mice [Jackson Laboratories, stock 00026; for genotyping see Maynard et al. (Maynard et al., 2002)] were maintained on a C57BL/6 background; Fgfr2b<sup>+/−</sup> mice (De Moerlooze et al., 2000) were maintained on a mixed C57BL/6×BK129 background; TOPGAL mice (DasGupta and Fuchs, 1999) were maintained on a CD1 background and backcrossed at least three generations with the mutants above on their respective backgrounds; Pax5<sup>−/−</sup> mice (Relaix et al., 2003) were maintained on a mixed C57BL/6×BCA background; and Fgf10<sup>−/−</sup> (here Fgf10<sup>Δ10</sup>) mice (Kelly et al., 2001) were maintained on a mixed agouti background. The latter were mated with Fgf10<sup>+/−</sup> mice to obtain Fgf10<sup>+/−</sup> normal hypomorphic embryos. Noon of the day of a vaginal plug was considered E0.5. Mutant embryos were stage matched with their littermate controls on the basis of somite counts.

X-gal staining

Pax3<sup>3LZ/3LZ−</sup>, TOPGAL and Fgf10<sup>Δ10</sup> embryos were fixed in 4% PFA/D-PBS and stained with 1 mg X-gal/ml dimethylformamide in 5 mM K<sub>4</sub>Fe(CN)<sub>6</sub>/5 mM K<sub>4</sub>Fe(CN)<sub>6</sub>4H<sub>2</sub>O/2 mM MgCl<sub>2</sub> in D-PBS pH 7.4 to reveal β-galactosidase (lacZ) activity.

RNA in situ hybridization

Whole-mount in situ hybridization was performed as described (Veltmaat et al., 2004) with digoxigenin-labeled riboprobes of Fgf10 (Bellusci et al., 1997), Wnt10b or Lef1. Paraffin sections (10 μm) were hybridized with [35S]UTP labeled riboprobes of Fgr1b, Fgfr1c, Fgfr2b, Fgfr2c, Fgfr3b, Fgfr3c (Kettunen et al., 1998) and Fgfr4 (Partanen et al., 1991) and analyzed as described (Rice et al., 2000).

Explant culture and application of recombinant FGF10

Eviscerated embryonic flanks were cultured as described for tooth anlagen (Kratochwil et al., 2002). Heparin-acrylamide beads (Sigma) were incubated in D-PBS containing 100 ng BSA/μl with or without 100 ng rFGF10 (R&D Systems)/μl, and implanted underneath the surface ectoderm, at the hypaxial bud of somite 14-15 or 17-18. After 2-3 days of culture, explants were processed for whole-mount in situ hybridization or X-gal staining.

Histology

Specimens fixed in 60% methanol/30% CHCl<sub>3</sub>/10% HAc or 4% PFA/D-PBS were embedded in paraffin wax, sectioned (6 μm) and stained with Hematoxylin/Eosin or Nuclear Fast Red (Lab Vision Corporation), respectively.
E11.5-E11.75 (44-47 somites) Fgf10−/− and Fgfr2b−/− embryos, the cells of the stratum germinativum of the flank are cuboidal instead of cylindrical (Fig. 3E,F), and formation of the periderm is impaired. Furthermore, the stratum intermedium and Wnt10b expression are absent (Fig. 3E,F,H,I), except in the area of placode 4. There, a small streak of weak Wnt10b expression coincides with the formation of a stratum intermedium composed of cuboidal instead of cylindrical cells (not shown). As the role of WNT10B in mammary line formation is unknown, but canonical WNT signaling is required (Chu et al., 2004), we crossed Fgf10+/− and Fgfr2b+/− mice with mice carrying the TOPGAL transgenic reporter for canonical WNT signaling (DasGupta and Fuchs, 1999), used here as a functional marker for mammary line and placode formation (Fig. 3Q,R). Like Wnt10b expression, TOPGAL expression is weak, and restricted to the area of placode 4 in E11.5 (46 somites) Fgf10−/− and Fgfr2b−/− mutants (Fig. 3J-L). Accordingly, the expression pattern of Lef1, another early marker for mammogenesis (Mailleux et al., 2002) demonstrates that all buds except 4 are absent at E12.5 (Fig. 3M-O).

We conclude that ectodermal maturation preceding mammary line formation requires FGF10/FGFR2B signaling, except in the region of placode 4. If FGFR2B is activated by somitic FGF10, then central somitic Fgf10 expression appears sufficient for initiation, as seen in Pax3ILZ/ILZ mutants. Additional hypaxial Fgf10 expression is required for the normal formation of the stratum intermedium, a hallmark of the mammary line, and for the correct temporal formation of placode 3.

Expression patterns support a role for hypaxial and central somitic Fgf10 in mammogenesis

To further determine the precise Fgf10 expression domain responsible for the formation of the mammary line, we analyzed Fgf10 expression from E7.5 onwards. Whole-mount in situ hybridization reveals Fgf10 expression only in the head region until E9 (not shown). At E9.5-10 (26-30 somites), expression is also detectable in the heart, the lateral plate mesoderm and limb bud mesenchyme (Fig. 4A,B). Around E10.25, Fgf10 expression is further detectable in the hypaxial bud of thoracic somites 11-13 at the position of the prospective placode 2, whereas it is no longer detectable in the lateral plate mesenchyme of the flank (not shown).
By E10.5, hypaxial Fgf10 expression has descended to thoracic somites 17-18 (Mailleux et al., 2002), which lie just posterior to the prospective mammary placode 3 (Eblaghie et al., 2004) (J.M.V., unpublished). Using the more sensitive radioactive in situ hybridization, we detected Fgf10 mRNA in the hypaxial buds and at a lower level throughout the somites from E10.5 onwards, shown for E10.75 (37 somites) (Fig. 4C), but not in the dermal mesenchyme and surface ectoderm. By E11.5, when placode 3 has formed, Fgf10 expression has increased throughout the somites, and is still relatively high in the hypaxial buds (Fig. 4D). A radioactive signal above background levels in the dermal mesenchyme suggests that Fgf10-expressing cells delaminate from the somites. While the mammary placodes transform into buds, somitic Fgf10 expression becomes progressively reduced [data not shown at E12.0 (50 somites) and E12.5 (54 somites)]. These data support a role for hypaxial and central somitic Fgf10 expression from around E10.5 onwards in the induction of a mammary cell fate.

Fgfr2b mRNA was detected in the surface ectoderm at E9.5 (26s) and during the onset of mammary line formation at E11.0 (41s), but not in the somites and dermal mesenchyme (Fig. 4E-H). No expression of any other Fgfr isoform was detected in the somites, dermal mesenchyme or ectoderm at these stages (not shown). Notably, Fgfr2b expression is relatively high in the surface ectoderm overlying the hypaxial buds at E11.5-E12.25 (45-50 somites) (Fig. 4G,H), corresponding to the area of the mammary line. These expression data support the hypothesis that somitic FGF10 acts via ectodermal FGFR2B in mammary line formation, prior to placode formation. However, it is still possible that Fgf10 expression in the lateral plate mesoderm at E9.5-10 makes the ectoderm receptive to later somitic signals involved in mammogenesis.

**Recombinant FGF10 rescues placode formation in Fgf10−/− embryos**

To address the latter possibility, we dissected the flanks including a small piece of ventral skin of E11.5 and E12.5 wild-type and Fgf10−/− embryos. These explants were completely eviscerated, to comprise only the skin and somites, and included the limbs in the case of a wild type. The flanks were cultured for 2 or 3 days with a bead soaked in BSA or recombinant FGF10 (rFGF10) implanted on the hypaxial buds of somites 14-15 or 17-18, just anterior or posterior to the position where placode 3 normally develops.

In wild-type flanks without bead or with a BSA bead (Fig. 4I), we either found no, one, two or occasionally all three buds (2, 3 and 4) expected between the limbs (Table 1), as assessed by Wnt10b expression and histology (Fig. 4L-N). Although rFGF10 failed to induce extra placodes in wild-type flanks, the rescue of bud formation in Fgf10−/−
explants of fairly advanced stages (E11.5 and even E12.5) indicates that the initiation of mammogenesis does not depend on Fgf10 expression prior to somitic Fgf10.

Impaired mammogenesis correlates with reduced somitic Fgf10 expression in Gli3Xt-J/Xt-J embryos

The Gli3Xt-J mutation represents a functional null allele for the transcription factor GLI3 (Maynard et al., 2002). Gli3Xt-J/Xt-J embryos lack mammary bud 3 at E13.5 (Johnson, 1967). In the wild-type mouse embryo, Gli3 is expressed in the somites (McDermott et al., 2005), and GLI and FGF family members genetically interact in organogenesis (Brewster et al., 2000; Aoto et al., 2002; te Welscher et al., 2002; Kuschel et al., 2003). Therefore, we examined whether the bud defect is preceded by impaired initiation of mammogenesis in Gli3Xt-J/Xt-J embryos, and whether initiation of mammogenesis required an interaction between somitic Fgf10 and Gli3.

Wnt10b and TOPGAL expression are reduced at the level of the mammary line, and not elevated at the position of placode 3 in Gli3Xt-J/Xt-J embryos (Fig. 5A-D). Accordingly, Lef1 is expressed at the position of mammary buds 2 and 4, but not of bud 3 by E12.5 (Fig. 5E,F). The cells of the stratum germinativum are cylindrical, slightly enlarged along the width of the mammary line and, as in Pax3ILZ/ILZ mutants and wild-type embryos, covered with periderm. However, the stratum intermedium is absent (Fig. 5G,H), in accordance with the reduced expression levels of Wnt10b and TOPGAL, and absence of Lef1 expression. Thus, mammary line formation is indeed impaired in Gli3Xt-J/Xt-J embryos. In contrast to the delayed formation of placode 3 in Pax3ILZ/ILZ mutants, gland 3 was not found at all in histological sections or skin preparations of Gli3Xt-J/Xt-J embryos between E12.5 and term (not shown).

In situ hybridization revealed high Gli3 expression in all thoracic somites at E10.0-E11.0 (whole-mount data not shown; Fig. 5I). Gli3 is less expressed in the dermal mesenchyme, and not detected in the surface ectoderm. Gli3 expression is normal in the somites of E10.5-E11.5 Fgf10–/– embryos (Fig. 5J,K). Conversely, Fgf10 expression is not detected in the central somitic domain, and reduced in the hypaxial somitic domain of E10.5-E11.5 Gli3Xt-J/Xt-J embryos. Moreover, Fgf10 expression extends posteriorly to only the level of somite 16 instead of 18 in Gli3Xt-J/Xt-J embryos (Fig. 5L,M).
Furthermore, hypaxial Fgf10 expression is highest in somites 15-16, underlying mammary placode 3 in control embryos. Whole-mount in situ hybridization for Wnt10b reveals impaired mammary line formation (between arrows), the presence of placodes 2 and 4 (numbered), but not placode 3 in an E11.5 (44s) Gl3Xt-J/Xt-J embryo. X-gal staining for TOPGAL expression indicates that WNT signaling in an E11.5 Gl3Xt-J/Xt-J embryo is reduced in a pattern similar to Wnt10b expression. Whole-mount in situ hybridization for Lef1 reveals the absence of bud 3 in an E12.5 Gl3Xt-J/Xt-J embryo. Hematoxylin/Eosin-stained sections through the plane indicated in Fig. 2E reveals the absence of the stratum intermedium at the mammary line of an E11.5 (45 somites) Gl3Xt-J/Xt-J mutant. Radiolabeled in situ hybridization for Gli3 (red) on a section of an E11.0 (41 somite) wild-type embryo (l) through the plane indicated in Fig. 2E, and whole-mount in situ hybridization for Gli3 on a control (j) and Fgf10–/– (k) embryo shows unchanged somitic Gli3 expression in the Fgf10–/– mutant. Whole-mount in situ hybridization for Fgf10 reveals that somitic Fgf10 expression (between black arrowheads) is restricted to the hypaxial domain in the Gl3Xt-J/Xt-J embryo. Whole-mount in situ hybridization for Wnt10b on flanks of Gl3Xt-J/Xt-J embryos, cultured with an implanted bead (circle) soaked in BSA (n) or rFGF10 (o). Arrows indicate the mammary placodes or the expansion of a Wnt10b expression domain around the bead in O. Nuclear Fast Red stained section through the planes indicated by lines through the flank in L. Broken black lines outline the mammary epithelium. Abbreviations: s, somite stage; p, periderm; sg, stratum germinativum; si, stratum intermedium; pl #3, mammary placode 3; nt, neural tube; st, stomach; fl, forelimb; hl, hindlimb; rFGF10, recombinant FGF10. Scale bars: 10 μm in G-H,P-Q; 100 μm in A-F,I-M; 1 mm in N,O. Panel I is not to scale.

Implantation of an rFGF10 bead in eleven cultured Gl3Xt-J/Xt-J flanks resulted once in an extended streak of Wnt10b expression and thickened ectoderm (Fig. 5O,P; Table 1). Given the low frequency of normal placode development in control explants (8/70, see Table 1), this extension may indicate a rescue of formation of the mammary line and placode 3. We conclude that Gli3 expression is required to maintain high levels of Fgf10 expression along the dorsoventral gradient within somites 12-18, and the anteroposterior Fgf10 gradient across these somites, which is in turn required for the complete formation of the mammary line and placode 3.

**Hypomorphic Fgf10 mutants lack placode 3**

To test whether reduced Fgf10 expression is indeed responsible for the mammary phenotype in Gl3Xt-J/Xt-J embryos, we generated embryos carrying one Fgf10– allele and one Fgf10mlcv allele (Kelly et al., 2001), which expresses reduced levels of Fgf10 (Mailleux et al., 2005). This allelic combination results in a hypomorphic Fgf10 phenotype in the embryonic lung (Mailleux et al., 2005), limbs (arrows in Fig. 6D,F) and gut (F.G.S., J. L. Curtis, J. M. V., P. M. Del Moral, T. Fairbanks, D. Warburton, K. Wang, R. C. Burns and S. B., unpublished). The mammary line does form in these embryos, and so do mammary placodes 2 and 4. However, we could neither detect
placode 3 in E11.5 and E12.5 hypomorphs by Wnt10b and Lef1 expression (Fig. 6A-H), nor in histological sections of embryos until term (not shown). Along the mammary line, cells of the stratum germinativum are still cuboidal at E11.0 (40 somites) instead of cylindrical and covered by periderm (not shown). By E11.5 (45 somites) cells of the stratum germinativum are also enlarged cylindrical in hypomorphs, and covered by periderm as in control embryos, but fail to generate a stratum intermedium (Fig.

DISCUSSION

Anterior thoracic somitic Fgf10 expression is required for the initiation of a mammary cell fate

Our study concerned the role of somitic signals, in particular of FGF10, in the initiation of mammary development. With the Pax3 mutant data, we showed that in the absence of hypaxial signals, placode 3 develops late and remains hypoplastic, while the other placodes develop apparently normal. This differential requirement of placodes for hypaxial somitic signals reflects their differential requirement for total somitic FGF10 (see Table 2). Mammary placode 3 is most sensitive to reduced somitic FGF10 expression, as found in Gli3Xt-J/Xt-J and Fgf10 hypomorphic mutants, in accordance with the highest hypaxial Fgf10 expression found in somites 15 and 16 underlying this placode in wild-type embryos; placodes 1, 2 and 5, though requiring FGF10/FGFR2B signaling, develop in the presence of reduced somitic FGF10 expression in Pax3–/–/–, Gli3Xt-J/Xt-J and Fgf10 hypomorphic mutants; placode 4 does not require FGF10 or FGFR2B at all for its formation. With the Pax3 mutant data, we also correlated the position of the ventral edge of the thoracic somites with the position of the mammary line on the dorsoventral axis of the flank. We now conclude that hypaxial somitic FGF10 is required for the correct dorsoventral positioning of the mammary line, and additional central somitic FGF10 is required for the formation of placode 3 on the anteroposterior aspect of this line. We present here the first evidence that slight modulations in levels and localization of gene expression, regulating interactions between the somites and the flank surface ectoderm, determine epidermal versus mammary cell fate decisions in, and thus patterning of, this ectoderm.

Identification of a GLI3-FGF10/FGFR2B-WNT signaling cascade, required for induction of the mammary cell fate at the position of mammary placode 3

Fgf10 expression is reduced in the somites in Gli3Xt-J/Xt-J mutants, indicating that GLI3 acts upstream of Fgf10 (Fig. 7A). We have observed the same genetic interaction in lambdoid suture formation (R.R., E. Connor, J.M.V., E. Lana-Elola, S.B. and D.P.R., unpublished). To our knowledge, this is the first time that any GLI function has been placed upstream of FGF10. GLI3 is a transcriptional activator or repressor dependent on the level of Hedgehog signaling (Lewis and Veltmaat, 2004; Stamatakis et al., 2005). GLI3 exerts an activator function in the epaxial-medial domain of the somites, and a repressor function in the lateral domain of the somites (McDermott et al., 2005). GLI3 may therefore directly or indirectly activate Fgf10 transcription in somitic subdomains. No GLI-binding sequence has been found within 6.6 kb upstream of the Fgf10-coding sequence (Ohuchi et al., 2005). However, sequences further upstream, e.g. as far as 114 kb (Kelly et al., 2001; Mailleux et al., 2005), may contain regulatory elements including GLI3-binding sites. Interestingly, null mutants for sonic hedgehog form all mammary placodes (Gallego et al., 2002; Michno et al., 2003), indicating that sonic hedgehog is not required for GLI3-mediated activation of Fgf10 transcription.
We also show that Wnt10b expression and canonical WNT signaling are activated downstream of FGF10/FGFR2B signaling in the surface ectoderm (Fig. 7A), and thus identified a genetic cascade from GLI3 via FGF10/FGFR2B to Wnt10b expression, and to canonical WNT signaling required for the induction of a mammary cell fate in the ectoderm. Wnt10b expression precedes TOPGAL expression, and coincides with the acquisition of the enlarged cylindrical cell shape (Fig. 5H, Fig. 6J). Both Wnt10b and TOPGAL expression increase with localized multilayering of the flank ectoderm. Multilayering is initially restricted to the mammary line. Previously, the enlargement of cells of the single-layered ectoderm was suggested to indicate mammary line formation (Turner and Gomez, 1933; Sakakura, 1987). Although enlargement of cylindrical cells coincides with low levels of Wnt10b expression, it is not sufficient for the formation of a mammary placode, as seen in Gli3Xt-J/Xt-J and hypomorphic Fgf10 mutants. Therefore, we postulate that multilayering is the first histological manifestation of a mammary cell fate.

A model combining ventral elongation of the thoracic somites with gradients of somitic Fgf10 expression in the patterning of mammary epithelium in the interlimb region

Based on the similarity in mammary phenotype of Fgf10−/− and Fgfr2b−/− mutants (Table 2), and on the complementary expression patterns of Fgf10 and Fgfr2b in wild-type embryos, we conclude that somitic FGF10 binds to and activates ectodermal FGFR2B, leading to a mammary cell fate in the ectoderm. An analysis of transverse sections at the level of somites 14–18 of progressively older wild-type embryos revealed that the prospective hypaxial buds of the thoracic somites are located dorsal to the flank at E10.0 (30 somites). The somites extend ventrally, growing into the lateral plate mesoderm of the flank between E10.5 and E11.5 (35–45 somites). Elongating more rapidly than the dorsoventral axis of the body, they reach the ventral side of the flank by E12.0 (50 somites). We observed that the ectodermal cell morphology changes from cuboidal to cylindrical along the dorsoventral axis of the flank during somitic elongation. Subsequently, the cylindrical cells enlarge, express Wnt10b (Fig. 5B,H) and give rise to the stratum intermediate. This enlargement and multilayering occurs first at the ventral position of the flank between E11 and E11.5, where it indicates mammary line formation. Somitic elongation also coincides with the onset of somitic Fgf10 expression (pink in Fig. 7B, based on Fig. 4A-D and data not shown). Therefore, somitic FGF10 may mediate ectodermal maturation even before the differentiation into mammary epithelium. The cuboidal morphology of the ectodermal cells in Fgf10−/− and Fgfr2b−/− mutants (Fig. 3E,F) demonstrates that FGF10/FGFR2B signaling is not required for the maturation of squamous ectoderm cells into cuboidal cells. However, it is required for the progression to a cylindrical ectodermal cell shape. In Gli3Xt-J/Xt-J and hypomorphic Fgf10 mutants, the somites elongate normally, but express reduced levels of Fgf10. This correlates with the acquisition of an enlarged cylindrical cell morphology and low levels of Wnt10b expression, yet failure to form the stratum intermediate by E11.5 (Fig. 5H, 6G).

Table 1. Recombinant FGF10 rescues placode formation in vitro

<table>
<thead>
<tr>
<th>Genotype and treatment</th>
<th>Number of glands</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>

Total number of explants: 104

Rows 1 lists the genotypes of the flanks cultured with either no bead implanted, or with a bead soaked in BSA or rFGF10. Column 1 lists the total number of developing glands in these flanks as observed by Wnt10b or TOPGAL expression. The body of the table lists the number of flanks in which gland formation was analyzed. *rFGF10 induced a supernumerary rudiment in ~30% (7/23) of Gli3Xt-J/- flanks and 9% (1/11) of Gli3Xt-J/- flanks, but never in flanks of control embryos.

Abbreviations: n.d., not determined; rFGF10, recombinant FGF10.

For each mutant used in this study (column 1), the Fgf10 expression pattern in the somites (column 2) and the histology of the mammary line at around E11.5 (column 3) are described. The formation (+) or the absence (−) of each of the five placode pairs is also indicated (columns 4-8).

Abbreviations: s.g., stratum germinativum; s.i., stratum intermedium.

Table 2. Correlation between somitic Fgf10 expression and mammary development

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Histology of mammary line at E11.5 (±45 seconds)</th>
<th>Development of mammary placodes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>Cylindrical s.g., cylindrical s.i., covered with periderm</td>
<td>+ + + + +</td>
</tr>
<tr>
<td>Pax3ILZ/ILZ</td>
<td>Cylindrical s.g., reduced cylindrical s.i., covered with periderm</td>
<td>+ + Late + +</td>
</tr>
<tr>
<td>Fgf10−/−</td>
<td>Cuboidal s.g., no s.i., periderm impaired</td>
<td>− − − + −</td>
</tr>
<tr>
<td>Fgfr2b−/−</td>
<td>As wild type (absent receptor for somitic FGF10)</td>
<td>− − − + −</td>
</tr>
<tr>
<td>Gli3Xt-J/Xt-J</td>
<td>Cylindrical s.g., no s.i., covered with periderm</td>
<td>+ + − − +</td>
</tr>
<tr>
<td>Fgf10−/− hypomorphic</td>
<td>As Gli3Xt-J/Xt-J</td>
<td>+ + − − +</td>
</tr>
</tbody>
</table>

For each mutant used in this study (column 1), the Fgf10 expression pattern in the somites (column 2) and the histology of the mammary line at around E11.5 (column 3) are described. The formation (+) or the absence (−) of each of the five placode pairs is also indicated (columns 4-8).

Abbreviations: s.g., stratum germinativum; s.i., stratum intermedium.
Therefore, we conclude that progressive maturation of the ectoderm requires increasing amounts of somitic FGF10 expression with the acquisition of a mammary cell fate at the position of mammary placode 3. In these mutants, the stratum intermedium is being formed at E11.5 (Fig. 2P), suggesting that total somitic FGF10 expression is higher than in \textit{Gli3}^{ILZ/ILZ} mutants. The absence of the ventral bud of the somites correlates with a more dorsal location of the mammary line in \textit{Pax3}^{ILZ/ILZ} mutants. As the line is located above the ventral-most edge of the somite, we conclude that the position of the mammary line is determined by the ventral edge of the thoracic somites, or ventral-most delivery point of somitic FGF10, rather than being predetermined in the ectoderm and waiting for FGF10 signals. In summary, we propose that the intra-somitic Fgf10 expression gradient and dynamics, combined with the rapid hypaxial elongation of the thoracic somites, determines the dorsoventral position of the mammary line and the progressive differentiation towards a mammary cell fate at the position of placode 3 on the anteroposterior aspect of this line.

Delaminating epaxial and central somitic dermomyotomal cells form the dorsal dermis before E11 in the mouse (Houzelstein et al., 2000). Some of these cells may mix with the flank mesenchyme at the dorsolateral boundary, as shown in chick (Olivera-Martinez et al., 2000). The absence of the ventral bud of the somites in \textit{Pax3}^{ILZ/ILZ} mutants form placode 3, albeit delayed and hypoplastic. The profile and dynamics of somitic Fgf10 expression may explain this phenotype: in wild-type embryos, Fgf10 is expressed from E10.5 onwards, at a higher level in the hypaxial bud than in the central somitic compartment. Expression increases throughout the entire somite between E10.5 and E11.5, during which time the mammary line and placode 3 are formed. It decreases but remains present towards E12.5 (Fig. 4C,D and Fig. 7B). This prolonged Fgf10 expression may compensate for the absence of hypaxial Fgf10 in \textit{Pax3}^{ILZ/ILZ} mutants, and allow the ectoderm to complete its maturation and differentiation into mammary epithelium. By contrast, central somitic Fgf10 expression in \textit{Gli3}^{ILZ/ILZ} and Fgf10 hypomorphic mutants is too low to provide a similar compensatory mechanism. This suggests that the total amount of FGF10 signaling via FGFR2B, accumulated during a prolonged period of time rather than during a short moment, determines the formation of mammary epithelium.

The absence of the ventral bud of the somites correlates with a more dorsal location of the mammary line in \textit{Pax3}^{ILZ/ILZ} mutants. As the line is located above the ventral-most edge of the somite, we conclude that the position of the mammary line is determined by the ventral edge of the thoracic somites, or ventral-most delivery point of somitic FGF10, rather than being predetermined in the ectoderm and waiting for FGF10 signals. In summary, we propose that the intra-somitic Fgf10 expression gradient and dynamics, combined with the rapid hypaxial elongation of the thoracic somites, determines the dorsoventral position of the mammary line and the progressive differentiation towards a mammary cell fate at the position of placode 3 on the anteroposterior aspect of this line.

Delaminating epaxial and central somitic dermomyotomal cells form the dorsal dermis before E11 in the mouse (Houzelstein et al., 2000). Some of these cells may mix with the flank mesenchyme at the dorsolateral boundary, as shown in chick (Olivera-Martinez et al., 2000).
al., 2000; Nowicki et al., 2003). Although it remains to be elucidated whether somitic FGF10 reaches the overlying ectoderm by a similar delamination of dermal precursors, or by diffusion, or by both mechanisms, we provide evidence that somitic FGF10 is required for the earliest differentiation steps of the ectoderm, and subsequently for the formation of mammary epithelium. To our knowledge, this is the first evidence that the hypaxial and central domain of the somites are implicated in the differentiation and patterning of the flank ectoderm. The failure of cells of the stratum germinativum to become cylindrical and generate a stratum intermedium in E11.5 Fgfr2b−/− and Fgf10+/− mutants may underlie the hypoplasia of the stratum granulosum [derived from the stratum germinativum and intermedium (Sengel, 1976)] observed in these mutants at birth, and the impaired hair follicle formation in Fgfr2b−/− but not in Fgf10+/− mutants (Suzuki et al., 2000; Petiot et al., 2003).

Does FGF10 regulate epithelial migration during the initiation of mammogenesis?

The mammary line and placodes show an increased cell density compared with the surrounding ectoderm. As this density seems to be established without locally increased cell proliferation, it has been suggested that cells migrate towards and along the mammary line (Balinsky, 1949-1950). Although cells have never been shown experimentally to migrate along the mammary line, the observation of elongated, fibroblast-like morphology of cells along the mammary line may indeed suggest migratory behavior of these cells (Propper, 1978; Chu et al., 2004). This view may be supported by the fragmented Wnt10b expression domain on the flank, fusing into one continuous mammary line (Veltmaat et al., 2003), and by the transition of a small stripe of Left1 expression into a dot via a comet-shaped intermediate at the level of placode 3 (Maileux et al., 2002).

It now raises the interesting issue of whether FGF10 mediates cell migration during mammary line and placode formation, as it does in eyelid development (Tao et al., 2005) and lung development (Bellusci et al., 1997; Park et al., 1998). In this scenario, cells along the flank would be pulled in a ventral direction along with the elongating hypaxial buds. As Fg10 expression is highest in the hypaxial buds of somites 15 and 16, Wnt10b-expressing cells along the mammary line would be recruited to the position above these somites to form placode 3. Such recruitment is supported by the condensation of initially three fragments of high Wnt10b expression somites 15, 16 and 17, to one continuous domain above somite 15 (Veltmaat et al., 2004). The shorter somites in Pax3ILZ/ILZ mutants would draw ectodermal cells across a narrower, more dorsal domain, resulting in a more dorsal position of the mammary line. In combination with reduced production of total somitic FGF10, fewer ectodermal cells would be recruited, leading to a narrower line and delayed multilayering. Subsequently, the absence of the high hypaxial Fg10 expression of somites 15 and 16 would then explain why Wnt10b expression remains present for a prolonged period along the mammary line, instead of coalescing at the position of placode 3.

Does mammogenesis depend on a unique molecular network for the induction of each pair of placodes?

Little is known about the initiation of mammogenesis, and less is known about the sources of molecules used in the formation of the five individual placode pairs. We show here that placode 3 is most sensitive to a reduction of somitic Fgf10. Similarly, placode 3 is more sensitive than placodes 2 and 4 to loss of Gli3 (this report) or neuregulin 3 (Howard et al., 2005). Gland 3 is also the gland most frequently absent in wild-type mice (Little and McDonald, 1945). Furthermore, increased signaling through EDA/EDAR leads to formation of supernumerary placodes only between placode 3 and 4 (Mustonen et al., 2004), while Left1-null and Thbx2/3 double heterozygous mutants have a more severe placode induction or maintenance defect in the thoracic region than in the inguinal region (van Genderen et al., 1994; Jerome-Majewska et al., 2005). We can therefore conclude that different molecular networks regulate mammogenesis at different levels along the anteroposterior axis, and different developmental thresholds exist for these networks along this axis. This may explain the differences in number and position of glands along the mammary line in different species, and the incidence of supernumerary nipples and breasts found in 2-5% of the human population (Schmidt, 1998; Gross, 2000). In particular, with our findings that somitic signals are required for placode formation, we may begin to understand the etiology of Poland’s syndrome (Poland, 1841), characterized by compound hypoplasia of the breast and the somite-derived pectoral muscles and thoracic skeletal structures.

References


Somitic signals determine mammary cell fate


Kratcochwil, K., Galceran, J., Tontsch, S., Roth, W. and Grosschedl, R. (2002). FGFR4, a direct target of LEF1 and Wnt signaling, can rescue the arrest of tooth organogenesis in Leff1(-/-) mice. Genes Dev. 16, 3173-3185.


