*J. Anat.* (2004) **205**, pp1–13

# Interactions between FGF and Wnt signals and *Tbx3* gene expression in mammary gland initiation in mouse embryos

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## Abstract

Interactions between Wnts, Fgfs and Tbx genes are involved in limb initiation and the same gene families have been implicated in mammary gland development. Here we explore how these genes act together in mammary gland initiation. We compared expression of *Tbx3*, the gene associated with the human condition ulnar–mammary syndrome, expression of the gene encoding the dual-specificity MAPK phosphatase *Pyst1*/MKP3, which is an early response to FGFR1 signalling (as judged by sensitivity to the SU5402 inhibitor), and expression of *Lef1*, encoding a transcription factor mediating Wnt signalling and the earliest gene so far known to be expressed in mammary gland development. We found that *Tbx3* is expressed earlier than *Lef1* and that *Pyst1* is also expressed early but only transiently. Patterns of expression of *Tbx3*, *Pyst1* and *Lef1* in different glands suggest that the order of mammary gland initiation is 3, 4, 1, 2 and 5. Consistent with expression of *Pyst1* in the mammary gland, we detected expression of *Fgfr1b*, *Fgf8* and *Fgf9* in both surface ectoderm and mammary bud epithelium, and *Fgf4* and *Fgf17* in mammary bud epithelium. Beads soaked in FGF-8 applied to the flank of mouse embryos, at a stage just prior to mammary bud initiation, induce expression of *Pyst1* and *Lef1* and maintain *Tbx3* expression in flank tissue surrounding the bead. Grafting beads soaked in the FGFR1 inhibitor, SU5402, abolishes *Tbx3*, *Pyst1* and *Lef1* expression, supporting the idea that FGFR1 signalling is required for early mammary gland initiation. We also showed that blocking Wnt signalling abolishes *Tbx3* expression but not *Pyst1* expression. These data, taken together with previous findings, suggest a model in which *Tbx3* expression is induced and maintained in early gland initiation by both Wnt and Fgf signalling through FGFR1.

**Key words** embryonic mammary gland; Fgfs; T-box; Wnt.

## Introduction

The development of embryonic mammary glands is not only an important process in its own right but is also a classic example of epithelial–mesenchymal interactions. The later stages are also under hormonal action (Hennighausen & Robinson, 2001; Veltmaat et al. 2003). Relatively little is known about how mammary gland development is initiated in correct locations in different mammals, although there are now several mouse mutants in which absence of mammary glands

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Accepted for publication *6 May 2004*

and/or abnormal development of the glands has been reported (van Genderen et al. 1994; Wysolmerski et al. 1998; Lewis et al. 1999; Bocchinfuso et al. 2000; Lewis et al. 2001; Spencer-Dene et al. 2001; Mailleux et al. 2002; Medina et al. 2002; Satokata et al. 2002; Davenport et al. 2003; Pispa et al. 2003). For other organs, such as limbs, it has been shown that signalling networks involving precise interactions between Wnt, Fgf and Tbx genes are required for initiation (Takeuchi et al. 2003). Members of the same families of genes have also been implicated in mouse mammary gland development (reviewed in Veltmaat et al. 2003). Here, we determine how these genes are integrated in mammary gland initiation.

In mice, the development of mammary glands involves a spacing pattern of repeated structures in which five individual mammary glands (designated Mb1 to Mb5, reading anterior–posterior) form along each of two mammary lines on the ventral side of the body (Turner & Gomez, 1933). The early development of mammary glands begins in the ectoderm of the ventral flanks on both sides of the embryo around E10.5. By E11.5–E12.5, five mammary placodes on each side can be detected. These epithelial thickenings then enlarge into bud-like structures. At E13.5, the mammary buds sink into the underlying mesenchyme (Turner & Gomez, 1933; Veltmaat et al. 2003).

*Wnt* genes have been suggested to play key roles in glandular development, including the induction of mouse mammary glands (Gavin & McMahon, 1992; Weber-Hall et al. 1994). Evidence in favour of this suggestion comes from the regulated expression of these genes in mammary development as well as their proliferative effects on mammary epithelial cells when overexpressed (Wong et al. 1994; Bradbury et al. 1995). *Wnt10b* and *Wnt12*, in particular, have been detected in a presumptive mammary line between the fore- and hind limb of E11.5 mouse embryos (Christiansen et al. 1995) and in the mammary placodes at E12.5 (Veltmaat et al. 2003). *Lef1*, a gene encoding a transcription factor involved in the canonical Wnt signalling pathway, is expressed in the ectoderm of presumptive placode regions, first as a short line of expression that then resolves to a dot shape via a comet-like intermediate (van Genderen et al. 1994; Mailleux et al. 2002). Later, *Lef1* is expressed in mammary mesenchyme (Satokata et al. 2002). In mice carrying the TOPGAL reporter for Wnt signaling, blue cells can be detected in an arc between fore- and hindlimb at E10.5 (DasGupta & Fuchs, 1999; reviewed in Veltmaat et al. 2003). However, *Lef1*$^{-/-}$ embryos have mammary placodes (van Genderen et al. 1994) but these fail to develop past the bud stage and eventually disappear. Thus, *Lef1* does not seem to be essential for placode induction but might participate later in cell fate determination.

Several genes encoding Fgf ligands are known to be expressed during early mammary gland development together with the gene that encodes a receptor, to which they are known to bind. *Fgfr2b* has been reported to be expressed in mouse mammary ectoderm at E11.5 and E15.5 (Mailleux et al. 2002), *Fgf7* in mammary mesenchyme at E12.5 and *Fgf10* in mammary placode at E11.5. Mouse embryos lacking *Fgf10* gene function fail to develop mammary placodes 1, 2, 3 and 5, whereas placode 4 is unaffected (Mailleux et al. 2002), suggesting that *Fgf10* signalling is required to initiate development of mammary glands 1–4.

We recently reported that *Pyst1*/MKP3, an immediate early gene encoding a negative regulator of FGF signalling (Eblaghie et al. 2003), is expressed in embryonic mouse mammary glands (Dickinson et al. 2002). In the limb, *Pyst1*/MKP3 is expressed in mesenchyme in response to FGF signalling but is not expressed in ectoderm, suggesting that expression of this gene is mediated via Fgfr1/2c, which is expressed in limb mesenchyme, but not via Fgfr2b, which is expressed in limb ectoderm. The fact that *Pyst1* is expressed in mammary glands suggests that FGF signalling via Fgfr1/2c receptors may play a role in mammary gland initiation.

*Tbx2* and *Tbx3*, members of the T-box family of transcription factors, are also expressed in the early mammary rudiment (Chapman et al. 1996). *Tbx3* mutations in humans (ulnar–mammary syndrome) lead to severe mammary hypoplasia, or sometimes a complete lack of mammary glands (Bamshad et al. 1997). Moreover, *Tbx3* null mice do not form mammary buds and *Wnt10b* and *Lef1* expression is absent (Davenport et al. 2003). This suggests that *Tbx3* is upstream of Wnt signalling in mammary gland initiation.

During limb initiation, *Tbx* genes act in concert with *Wnt* and *Fgf* signals in both mesenchyme and ectoderm (Takeuchi et al. 2003). There is evidence that *Tbx* genes lie upstream of *Wnt* and *Fgf* genes and that these signals in turn maintain *Tbx* gene expression. Consequently, expression of genes encoding *Wnt* and *Fgf* signals and *Tbx3* is mutually sustained. Indeed, *Tbx5* misexpression in the interlimb region in chick embryos induces formation of additional forelimb-like structures with ectopic

expression of *Fgf10* and *Wnt2b* in lateral plate mesoderm (Rallis et al. 2003). Not only is there a Wnt–Fgf autoregulatory loop in the presumptive limb mesenchyme, but a similar loop is also involved in formation and maintenance of the apical ectodermal ridge. In chick embryos, *Wnt2b* can induce *Fgf10* in the limb lateral plate mesoderm, and *Fgf10* can, in turn, induce and maintain expression of *Fgf8* in surface ectoderm (Kengaku et al. 1998) via *Wnt3a* (Kawakami et al. 2001). Thus, an elegant cascade involving alternating Wnt and FGF signalling is established in early limb bud development.

Here we focus on relationships between Wnt and FGF signalling and *Tbx3* in initiation of mammary gland development. We compare early expression patterns of *Lef1*, *Pyst1* and *Tbx3*. We manipulate FGF signalling by either applying FGFs or blocking FGF signalling with an inhibitor (SU5402) and examine the effects on expression of these genes. We also investigate whether Wnts participate in FGF signalling and maintenance of *Tbx3* expression by specifically inhibiting Casein Kinase I, a factor implicated in the canonical Wnt pathway.

## Materials and methods

### Experimental animals

Adult mice were housed in a temperature-controlled room (22 °C) under artificial illumination (lights on from 05:00 h to 17:00 h) and at 55% relative humidity, with access to food and water *ad libitum*. B6D2F1 (C57/B6 females × B6 males matings) mouse embryos were obtained from time-mated pregnant mice. The day a vaginal plug was confirmed was designated embryonic day 0.5 (E0.5).

### *In situ* hybridization

*In situ* hybridization on whole mouse embryos was performed as previously described (Wilkinson & Nieto, 1993; Nieto et al. 1996) and on wax sections using standard protocols. Briefly, embryos were fixed in 4% paraformaldehyde (PFA), embedded in wax and sectioned at 9 μm. Sections were baked at 65 °C, de-waxed in Xylene, rehydrated through a graded series of alcohol washes and post-fixed in 4% PFA. Sections were prehybridized in a humid chamber containing 50% formamide in 2×SSC, at 55 °C, for 30 min. Digoxigenin-labelled RNA probes were prewarmed at 95 °C and hybridized to sections overnight at 75 °C. The following mouse DNA plasmids were used as templates for the synthesis of DIG-labelled RNA probes: a 506-bp *Tbx3* probe previously described (Tumpel et al. 2002); a 1.1-kb *Pyst1/MKP3* probe provided by Dr Steve Keyse; a 360-bp *Lef1* probe (Dr Yi-Ping Chen); *Fgf4* (620 bp), *Fgf7* (227 bp), *Fgf8* (875 bp), *Fgf9* (900 bp), *Fgf10* (584 bp) and *Fgf17* (700 bp) (Dr Gail Martin); *Fgfr1* (133 bp) (Dr David Rice). Frozen sections were prepared for photomicrography according to Stern (1993).

### *In vitro* organ culture

B6D2F1 mouse embryos were isolated at stages just prior to mammary bud formation (approximately E10.75). Individual embryos were dissected into left and right halves using a fine tungsten needle to bissect the neural tube. Flank tissues were placed on Whatman Nuclepore Track-etch filter membranes (BDH), laid on top of stainless steel grids (a kind gift of Professor Rolf Zeller) in sterile multiwell culture dishes and cultured at the air–medium interface with the culture medium replaced every 24 h at 37 °C and 7.5% $CO_2$ for 48 h. The culture medium ($BGJ_b$, Sigma) was supplemented with 20 μg $mL^{-1}$ (v/v) ascorbic acid (Sigma) and 1% (v/v) penicillin/streptomycin. Tissues were then fixed and processed for *in situ* hybridization.

### Bead implantation

Heparin beads (Sigma H-5263) were incubated in 1 mg $mL^{-1}$ FGF8 (R & D Systems) for 1 h at room temperature. PBS-soaked beads were used as controls. AG-1 X2 (Bio-Rad Laboratories) formate-derived beads were incubated in 10 mM SU5402 (a kind gift from Sir Philip Cohen) or 20 mM CKI-7 (Seikagaku, USA) for 1 h at room temperature. DMSO beads were used as controls. Beads were implanted in the flank region along the mammary line of E10.75 mouse embryos.

### Histological observation

Cultured tissues were fixed in 4% formamide in PBS (pH 7.4) overnight at 4 °C. After washing in PBS, tissues were treated with 20% sucrose in PBS overnight at 4 °C and subsequently embedded in gelatin. Ten-micrometre-thick frozen sections were cut and de-gelatinized in warm PBS for 30 min. After tap-water, the sections were stained with haematoxylin and eosin. The tissue was mounted using DAKO glycergel mountant (DAKO Corp.).

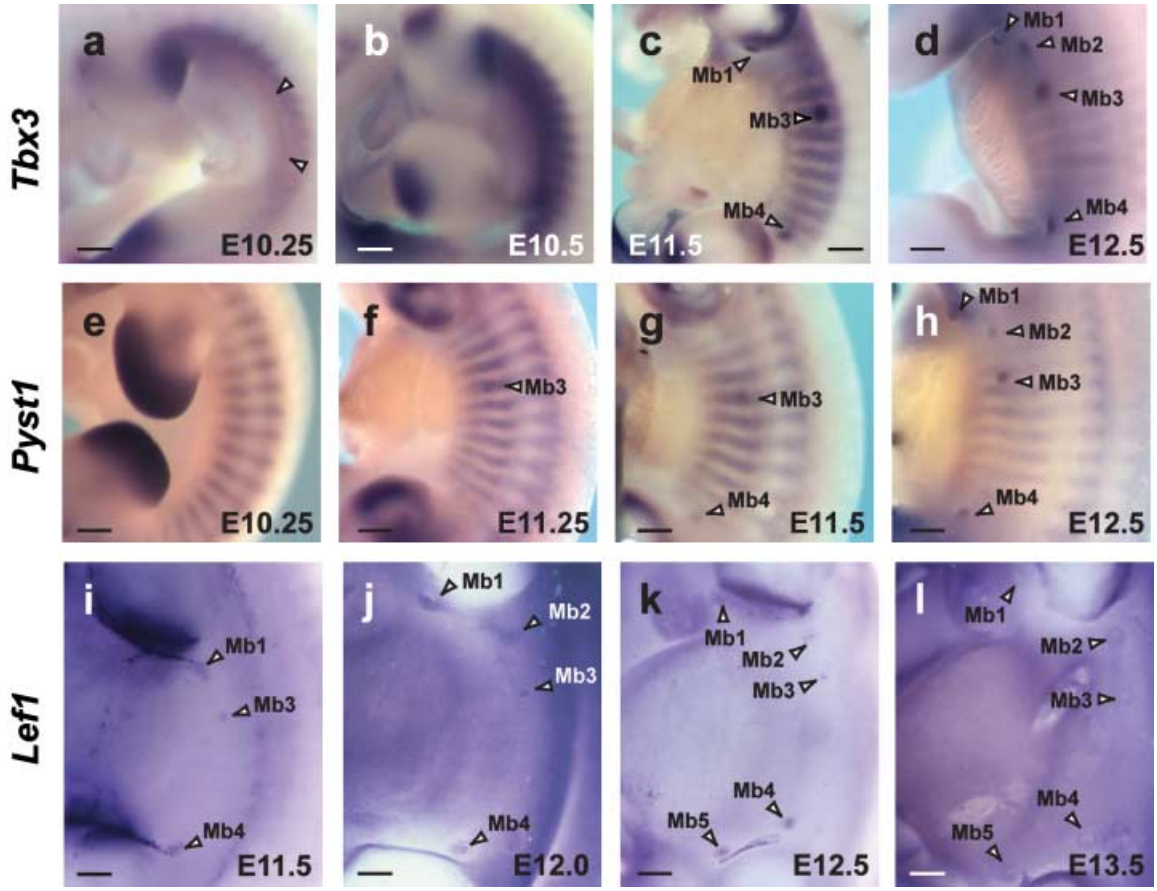


**Fig. 1** Expression of *Tbx3*, *Pyst1* and *Lef1* from E10.25 to E13.5. (a) *Tbx3* expressed in a thin line marking what appears to be the mammary line (between two arrowheads). *Tbx3* is also expressed in a graded fashion in a thicker band more dorsally with transcripts being more abundant anteriorly. (b) *Tbx3* expression at E10.5 is stronger than E10.25 but the line seen at E10.25 cannot be distinguished. (c) E11.5; three pairs of mammary buds expressing *Tbx3* become apparent in a dot-like pattern (Mb1, Mb3 and Mb4). (d) E12.5; five pairs of mammary buds expressing *Tbx3*. (e) E10.25; *Pyst1* expressed as stripes marking the somites, and not yet in mammary rudiments. (f) *Pyst1* expression first detected in a mammary rudiment (Mb3); at E11.25 it is also in stripes of somitic expression. (g) *Pyst1* expression in Mb3 and Mb4 at E11.5. (h) *Pyst1* expressed in full complement of mammary buds at E12.5 (Mb1–Mb5). (i) E11.5; *Lef1*, known as early marker of mammary placode formation, expressed in Mb3 in a dot-like pattern; in Mb1 and Mb4 in a comet-like pattern with a tail. (j) *Lef1* expressed separately in Mb2 and Mb1 at E12. (k–l) E12.5 and E13.5; *Lef1* expression detected in five pairs of mammary buds. Note that Mb5 is last to form. Mb1–5, mammary buds. Scale bars, 200 μm.

## Results

### *Tbx3* and *Pyst1* expression in early mammary bud development

The first signs of mammary gland development can be recognized around E10.5–E11. At E10.25, *Tbx3* transcripts are detected in a very thin line, more ventrally, marking what appears to be the mammary line (Fig. 1a). *Tbx3* is also expressed more dorsally in a band along the flank between the limb buds. This expression is graded from anterior to posterior, with highest levels of transcripts adjacent to the posterior margin of the forelimb bud (at the level of somite 12), gradually diminishing down the flank to the anterior limit of the hindlimb bud (somite 23). No expression is observed in the flank anterior to the level of the forelimb (somite level 8), although *Tbx3* is expressed caudal to the hindlimb. At a similar stage in development, *Pyst1* appears as stripes of somitic expression marking epaxial and hypaxial myotome (Dickinson et al. 2002; Fig. 1e).

By E10.5, no localized *Tbx3* expression is seen in mammary buds but the flank expression of *Tbx3* intensifies into a thick, more ventral band (Fig. 1b) and is uniform along the anterior–posterior length of the flank. At this stage, *Pyst1* expression remains as stripes marking the somites (data not shown). A few hours of development later (approximately E11), the first mammary bud expressing *Tbx3* can be seen in flank

ectoderm at the level of somite 16/17, corresponding to the third mammary bud (Mb3) (data not shown). *Pyst1* transcripts are also first detected in Mb3 ground (E11.25) (Fig. 1f); at this stage, the stripes of myotomal expression of *Pyst1* extend more ventrally along the flank of the embryo marking intercostal muscles.

By E11.5, three mammary buds expressing *Tbx3* become apparent – Mb1, Mb3 and Mb4 (Fig. 1c). At this stage, the robust band of *Tbx3* expression is reduced to stripes marking what appears to be emerging ribs. *Pyst1* expression is first detected in Mb3 at E11.25 (Fig. 1f) then in Mb4 at E11.5 (Fig. 1g). *Lef1*, reported as one of the earliest genes expressed in early mammary bud rudiments (van Genderen et al. 1994), is also first expressed in Mb3 at E11/11.5, then Mb4 and Mb1 at E11.5/12.

By E12.5, the last mammary buds (Mb2 and Mb5) form. Mb2 emerges in the axillary region at the level of somite 13/14, whereas Mb5 develops as an inguinal rudiment inside the anterior margin of the hindlimb (at the level of somite 23). Both *Tbx3* and *Pyst1* expression becomes more robust in the full complement of mammary buds (Mb1–Mb5), whereas the stripy expression in the trunk is reduced (Fig. 1d,h). At this stage, mammary bud expression of *Tbx3* is more intense. *Tbx3* expression persists longer than *Pyst1* expression, which is eventually lost shortly after E13. *Tbx3* transcripts are still present by E14 (data not shown). *Lef1* is still expressed in all five mammary buds at E13.5 (Fig. 1l). Based on these expression patterns, it appears that the mammary buds are initiated along the main body axis in the sequence Mb3, Mb4, Mb1, Mb2 and Mb5.

### Expression of *Fgf4*, *Fgf8*, *Fgf9*, *Fgf17* and *Fgfr1* in embryonic mammary gland

Previous studies on FGF signalling in embryonic mammary gland development focused on the FGF10/FGFR2b signalling pathway (Spencer-Dene et al. 2001; Mailleux et al. 2002). However, the transient expression of *Pyst1* in E11.5–E13 mouse mammary buds suggests that a non-FGFR2b signalling pathway may also be involved in early stages of embryonic mammary gland development. We therefore examined expression of *Fgfr1* and FGF ligands specific for FGFR1, including *Fgf4*, *Fgf8*, *Fgf9* and *Fgf17* (Ornitz et al. 1996) in sections of mammary glands at early stages of development.

At E12, *Fgfr1* is expressed in ectoderm and mammary epithelium (Fig. 2a). Expression of *Fgf8* and *Fgf9* could also be detected in mammary epithelium and lower-level transcripts in adjacent ectoderm (Fig. 2c,d). Expression of *Fgf4* and *Fgf17* was detected in mammary epithelium, but not in ectoderm (Fig. 2b,e). We also observed that *Fgf7* and *Fgf10* are both expressed in mammary epithelium at E12, as previously reported (reviewed in Veltmaat et al. 2003). *Fgf7*, but not *Fgf10*, has also been reported to be expressed in surface ectoderm (Mailleux et al. 2002). *Fgf7* is also expressed in mesenchyme before it condenses into mammary mesenchyme, whereas mesenchymal *Fgf10* expression only appears much later in the fat pad layer at E15.5.

### FGF8 maintains and/or induces ectopic expression of *Tbx3*, *Pyst1* and *Lef1*

Beads soaked in various FGF proteins, including FGF-1, FGF-2, FGF-4, FGF-8 and FGF-10, can induce ectopic limb buds when grafted into lateral plate mesoderm of early (prelimb bud) chick embryos (Cohn et al. 1995; Ohuchi et al. 1995; Vogel et al. 1996; Crossley et al. 2001) or into mouse flank (Tanaka et al. 2000). In mice, five pairs of mammary epithelial buds emerge at E11.5/12 after limb buds have formed at E9.5 (forelimbs) and E10 (hindlimbs). To test whether exogenous application of FGF on beads could induce ectopic mammary glands, FGF-8 (1 mg $mL^{-1}$)-soaked beads were grafted onto E10.75 mouse flank just prior to the first signs of mammary gland development. After 48 h of *in vitro* culture, *Tbx3* expression is maintained in flank tissue around FGF-8 bead (7/7 cases; Fig. 3b). A section through the bead reveals *Tbx3* in mesenchyme ($n = 3$; compare Fig. 3b′ with Fig. 3b″). In controls, *Tbx3* expression is not maintained around PBS beads (3/3 cases; Fig. 3a) but is confined to mammary buds. Ectopic expression of *Pyst1* is also induced in mesenchyme around FGF-8 bead (6/6; Fig. 3d,d′,d″) as is mesenchymal expression of *Lef1* (4/6; Fig. 3f,f′,f″). FGF8 induction of *Tbx3*, *Pyst1* and *Lef1* occurs predominantly on one side of the FGF bead. However, we could not observe any ectopic mammary buds 48 h after FGF8 bead implantation nor any changes in epithelial morphology.

### SU5402 inhibits the effects of adding FGF-8 and affects line-to-bud transition of mammary buds

To test the requirement of FGF for maintenance and induction of *Tbx3*, *Pyst1* and *Lef1* in mammary gland initiation, we grafted beads soaked in FGFR1 inhibitor,

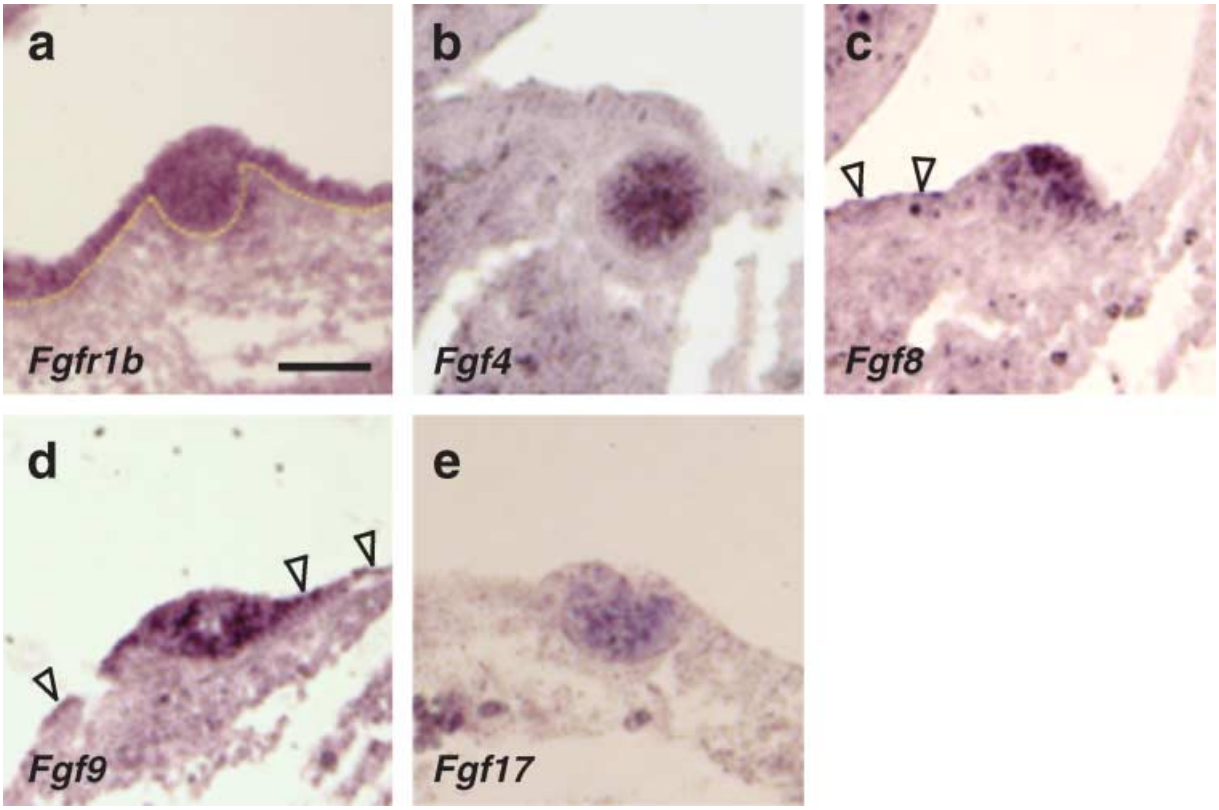


**Fig. 2** Expression of *Fgfr1* and FGF ligands specific for FGFR1 in mammary glands at E12. (a) *Fgfr1* expression at high levels in ectoderm and mammary epithelium. (b) *Fgf4* expression in centre of mammary bud, but not in epidermis. (c) *Fgf8* more highly expressed in mammary epithelium than ectoderm (arrowheads). (d) *Fgf9* strongly expressed in ectoderm (arrowheads) and in mammary epithelium. (e) *Fgf17* expression only detected in mammary epithelium. Scale bars, 100 μm.

SU5402 (10 mM; Mohammadi et al. 1997) onto the presumptive mammary-forming region of cultured flanks from E10.75 mouse embryos. When two beads were applied for 48 h, *Tbx3* expression is completely abolished in flank and mammary buds (3/4 cases; Fig. 4b), compared with DMSO control beads, which have no effect ($n = 4$; Fig. 4a). Figure 4(a) shows a culture in which three DMSO beads had been grafted and mammary buds 1, 3 and 4 still developed normally. Expression of *Pyst1* ($n = 5$; Fig. 4d) and *Lef1* ($n = 5$; Fig. 4f) also disappears 48 h after SU5402 treatment, whereas *Pyst1* ($n = 4$; Fig. 4c) and *Lef1* ($n = 4$; Fig. 4e) expression is detected in DMSO-treated tissue and corresponds to normal mammary buds. Interestingly, when one SU5402 bead was grafted into flank, *Lef1* was expressed in a comet shape (48–72 h, $n = 2$, Fig. 4g,h). These results indicate that Fgf signalling is required for maintenance of *Tbx3*, *Pyst1* and *Lef1* expression in developing mammary buds and may also be involved in the line to bud transition.

### Inhibiting Wnt signalling abolishes *Tbx3* expression and mammary bud formation

To determine whether Wnt signalling is involved in maintenance and/or induction of *Tbx3* and *Pyst1* expression in early mammary bud formation, we treated flanks with beads soaked in Wnt inhibitor, CK1-7 (20 mM), for 48 h. Expression of *Lef1*, a gene coincident with many known sites of endogenous Wnt signalling and a component of the canonical Wnt pathway, is reduced (compare DMSO control, $n = 6$, Fig. 5a with CK1-7 bead, 4/6 cases, Fig. 5b). When CK1-7 beads were implanted, *Tbx3* expression is completely lost in flank and mammary bud (4/6 cases; Fig. 5f); with DMSO control beads, *Tbx3* is expressed in mammary buds as normal ($n = 6$; Fig. 5e). However, in contrast to the inhibition of *Tbx3* expression, *Pyst1* expression, an immediate early response to FGFR1 signalling (Eblaghie et al. 2003), is unaffected by implantation of CK1-7 beads ($n = 4$, Fig. 5d; DMSO control, $n = 4$, Fig. 5c).

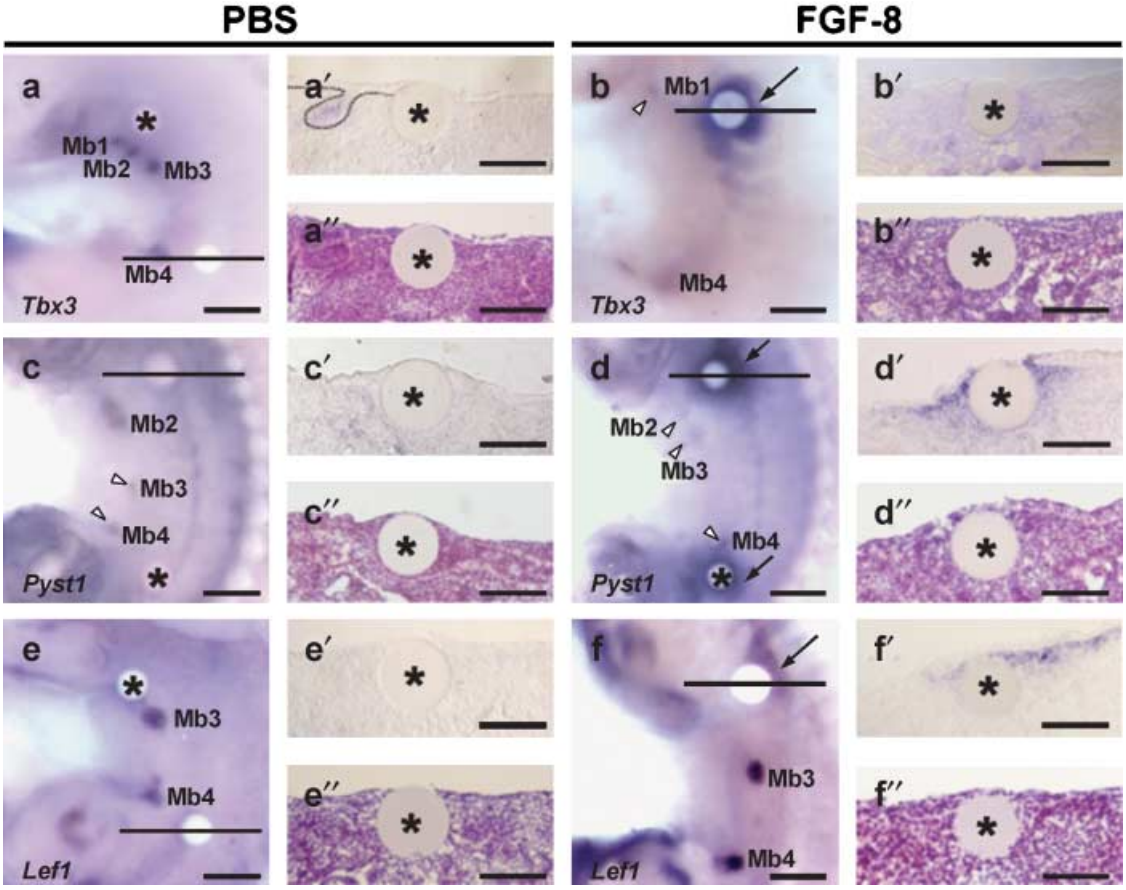


**Fig. 3** Effects of FGF8 on gene expression in devloping mammary glands. *In vitro* organotypic cultures 48 h after implanting PBS (a,c,e) or FGF-8 (b,d,f) soaked beads. After whole-mount *in situ* hybridization for *Lef1*, *Pyst1* and *Tbx3* (a,b,c,d,e,f), cultured tissue was sectioned through the bead (a′,b′,c′,d′,e′,f′). Haematoxylin and eosin-stained sections of organ cultures (a″,b″,c″,d″,e″,f″). (a) PBS bead; *Tbx3* expression confined to mammary bud. No *Tbx3* expression around the bead see also (a′). Ectoderm morphology unaffected by bead (a″). (b) FGF8 bead; *Tbx3* expression maintained around FGF-8 bead predominantly dorsally. (b′,b″) Section through the FGF-8 bead reveals *Tbx3* in mesenchyme but no change in ectodermal morphology. (c) PBS bead; *Pyst1* expression detected in mammary buds but not induced near PBS bead. (c′,c″) No *Pyst1* transcripts in either epithelium and mesenchyme near bead and no change in ectoderm. (d) FGF-8 beads; strong *Pyst1* expression around the beads (arrows). (d′,d″) *Pyst1* expression in mesenchyme and no change in ectodermal morphology. (e) PBS bead; *Lef1* expression not induced by PBS bead, but confined to mammary buds. (e′,e″) Section showing no expression of *Lef1* in either epithelium and mesenchyme; ectoderm morphology normal. (f) FGF8; *Lef1* induced in mesenchyme predominantly dorsal to bead (arrow). (f′,f″) Section through FGF-8 bead; *Lef1* expressed in mesenchyme; no change in ectoderm morphology. Mb1–5, mammary buds; arrow, ectopic expression around FGF-8 beads; asterisk, PBS or FGF-8 soaked beads; arrowheads, mammary buds. Scale bars (a–f), 300 μm; (a′–f′, a″–f″), 150 μm.

## Discussion

### Timing of gene expression in mammary gland initiation

We detected *Tbx3* expression in developing mammary glands earlier than expression of *Lef1* and *Pyst1*. *Lef1* has previously been reported as the earliest gene to be expressed in mammary gland development (van Genderen et al. 1994). In our study, a line of *Tbx3* expression running down the flank and concentrated expression of *Tbx3* in the third mammary gland (Mb3) seems to be found at stages prior to expression of *Lef1* in Mb1, Mb3 and Mb4 (and *Pyst1* in Mb3). The temporal pattern of expression of *Tbx3*, *Lef1* and *Pyst1* in the different mammary glands suggests that the sequence of mammary gland initiation is 3, 4, 1, 2, 5. Using *Bmp4*, *Msx1* and *Msx2* to visualize mammary buds, we found that the sequence of bud emergence appears to be 3, 1, 2, 4, 5 (data not shown). Based on their observations using *Lef1* to visualize mammary glands, Mailleux et al. (2002) reported that mouse mammary buds appear in the order of 3, 4, 5, 1, 2. Of all the genes mentioned, *Tbx3* seems to be expressed earliest in developing mammary buds, and the differences in the sequence of

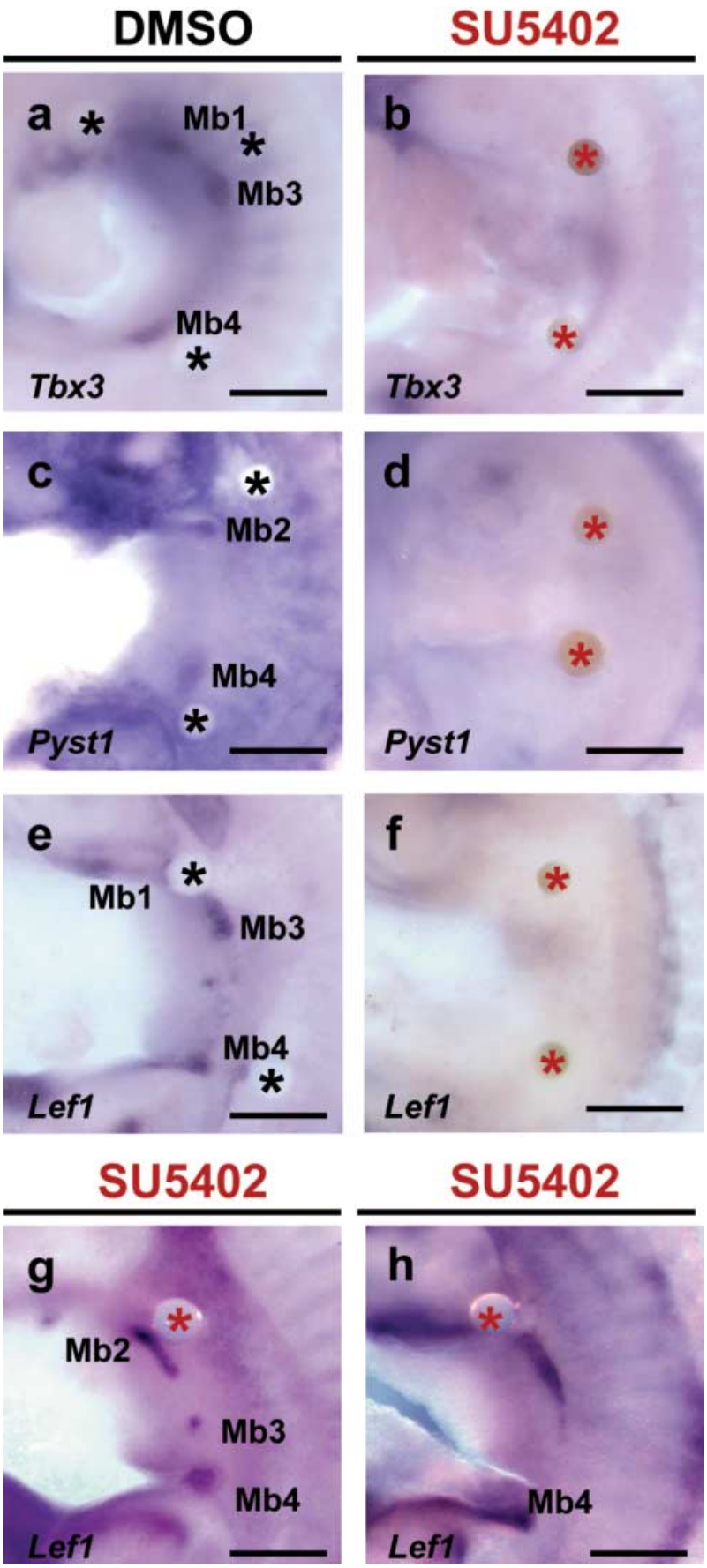


**Fig. 4** Effects of FGFR1 inhibitor (SU4502) on gene expression in mammary buds. Flank cultures 48 h after implanting either DMSO beads (a,c,e) or two SU5402 soaked beads (b,d,f). One SU4502 soaked bead applied (g,h). (a) *Tbx3* expression; DMSO beads had no effect and *Tbx3* expressed normally in mammary buds. (b) *Tbx3* expression completely abolished in flank and mammary buds by SU5402 treatment. (c) *Pyst1* expressed in mammary buds, but not around DMSO bead. (d) Expression of *Pyst1* lost after SU5402 treatment. (e) *Lef1* not expressed around DMSO bead. (f) Complete absence of *Lef1* expression after SU5402 treatment. (g) One SU5402 bead applied for 48 h to flank; *Lef1* in Mb2 remains as comet shape. (h) 72 h culture treated with one SU5402 bead. *Lef1* expression in the Mb2 still remains as comet shape. Mb1–5, mammary buds; black asterisk, DMSO soaked beads; red asterisk, SU5402 soaked beads. Scale bars, 300 μm.

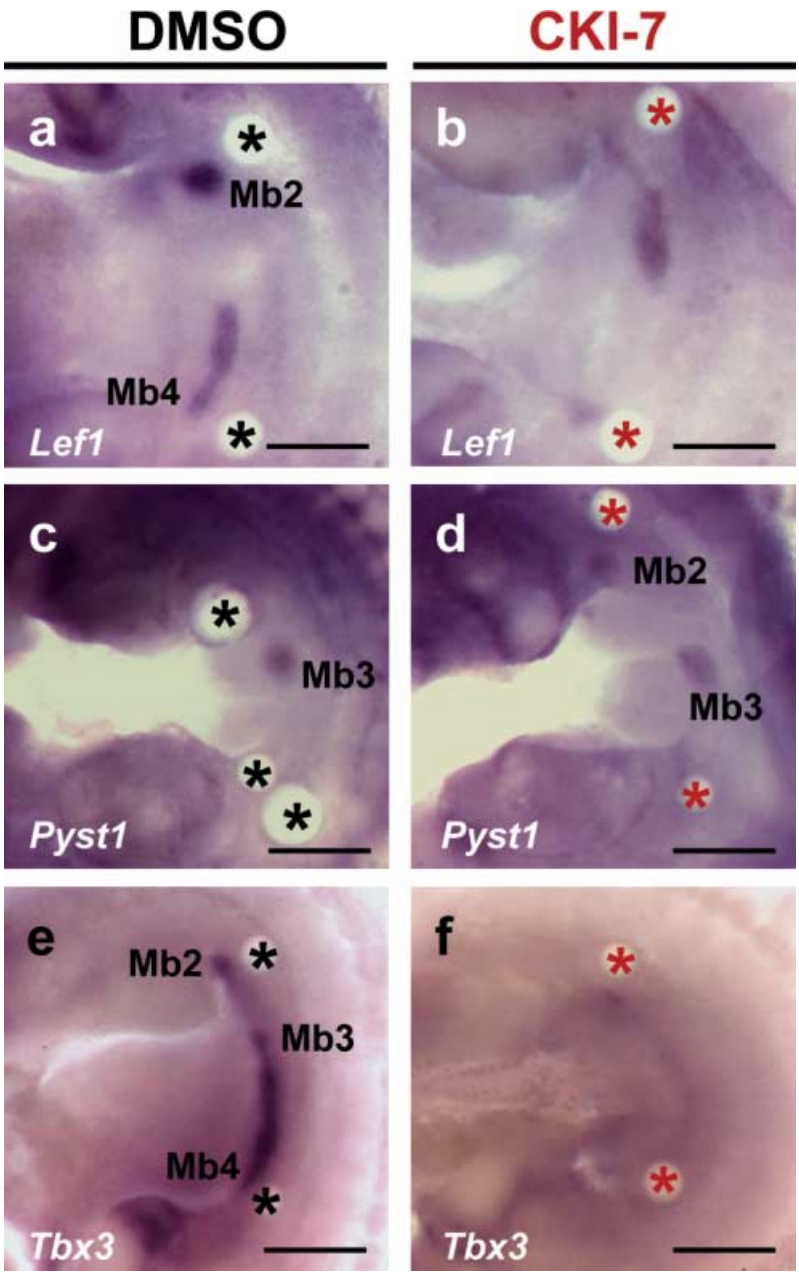


**Fig. 5** Effects of Wnt inhibitor (CK1-7) on gene expression in mammary glands. Flank cultures 48 h after implanting either two DMSO beads (a,c,e) or two CK1-7 beads (b,d,f). (a) *Lef1* expressed normally in DMSO treated tissue. (b) *Lef1* expression reduced by CK1-7. Note for example weaker expression in Mb4 region. (c) *Pyst1* expression; no change with PBS beads. (d) *Pyst1* is also not affected by CK1-7 beads. (e) *Tbx3* expression; DMSO had no effect. (f) *Tbx3* expression completely lost in flank and mammary buds. Mb1–5, mammary buds; black asterisk, DMSO soaked beads; red asterisk, CK1-7 soaked beads. Scale bars, 300 μm.

mammary bud appearance are probably due to the expression markers used to monitor the development of the glands. Nonetheless, Mb3 is found consistently to be the first to form.

Our analysis of the timing of expression of *Tbx3*, *Lef1* and *Pyst1* is consistent with a scheme in which Tbx3 lies upstream of both Wnt and Fgf signalling in mammary gland initiation (see later and Fig. 6). Indeed, in *Tbx3* knockout mice, which lack mammary glands, *Lef1* and *Wnt10b* are not expressed (Davenport et al. 2003; see later). Furthermore, in other organs in which Tbx genes are involved in initiation, such as development of limbs (Takeuchi et al. 2003) and lung (Kawashima et al. 1988; Cebra-Thomas et al. 2003; Sakiyama et al. 2003), Tbx genes again seem to lie upstream of Wnt and Fgf signalling. Thus, for example, when dominant negative forms of *Tbx5* and *Tbx4* are misexpressed in the prospective limb regions of chick embryos, a limbless phenotype results and both *Wnt* and *Fgf* expression is repressed (Takeuchi et al. 2003).

*Tbx3* is initially expressed broadly in ventral mouse flank and then is expressed in a thin line, as has also been reported for *Lef1*. In contrast, *Pyst1* expression is fairly transient and is associated with discrete mammary

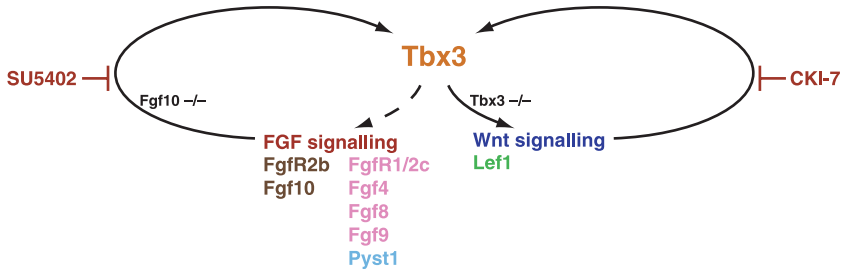


**Fig. 6** Genetic pathway integrating FGF, Wnt and Tbx3 in epithelial–mesenchymal interactions regulating mammary gland initiation. Fgf signalling through FgfR1, blocked by SU5402, maintains *Tbx3* expression and leads to expression of *Pyst1* and *Lef1*. Fgf10 signalling is also required for mammary gland development as shown in knock-out mice. Wnt signalling, blocked by CK1-7, also maintains *Tbx3* expression. *Tbx3* null mutants do not form mammary buds and expression of *Wnt10b* and *Lef1* is absent (Davenport et al. 2003), suggesting that *Tbx3* controls their expression. *Fgf*8, *Fgf9, Fgf4, Fgf17* are expressed in ectoderm or mammary knob epithelium. *Pyst1* expression depends on signalling through FgfR1.

glands rather than being in a line. The thin line of *Tbx3* and *Lef1* expression seems likely to represent the so-called mammary line along which discrete mammary glands subsequently develop (reviewed in Veltmaat et al. 2003). The milk line was first seen using scanning electron microscopy in rabbits (Propper, 1978), although this was rather controversial. It is interesting that these patterns of gene expression, together with the line of blue cells seen in TOPGAL mice, provide a visual marker for this structure.

An initial line of gene expression has also been detected in the development of the dorsal feather tract in chick embryos (Jung et al. 1998). A continuous stripe of *Shh* expression is observed in the dorsal midline of the embryo, where the primary row of feather buds will form. This linear pattern subsequently breaks up into a series of spots of expression. It is not clear whether this change in pattern from a line to a series of spots involves switching off gene expression in intervening cells. Another possibility is that cells expressing the gene aggregate to form spots. In other systems, FGF signalling has been shown to direct cell movement. Interestingly, in some of our experiments in which we inhibited Fgf signalling, the mammary line to spot transition did not occur.

### FGFR1 signalling pathway is involved in early mammary gland development

Previous studies have implicated FGF10/FGFR2b signalling in mammary gland development, and expression of genes encoding both ligand and receptor has been reported in mammary epithelium at E11.5 (Mailleux et al. 2002). Our data suggest that FGFR1 signalling is also involved. This possibility was first suggested to us because of the mammary gland expression of *Pyst1*, which is associated with signalling via FGFR1/FGFR2c in other regions of mouse embryos (Dickinson et al. 2002). Using section *in situ* hybridization, we were able to detect expression of genes encoding FGF ligands *Fgf4*, *Fgf8*, *Fgf9* and *Fgf17* in the tight mammary knob and/or overlying ectoderm and also expression of the gene encoding the *Fgfr1* receptor. Furthermore, the involvement of this *Fgf* signalling system in mammary gland initiation is supported by the effects of the SU5402 inhibitor on mammary gland development in cultured flanks. This inhibitor, which specifically interferes with FGFR1 signalling, abolished expression of *Tbx3*, *Lef1* and *Pyst1* in developing mammary glands. These results suggest that in the mammary gland, as in the developing limb, both *FGFR2b* and *FGFR1/2c* signalling are involved in initiation of organ development.

Consistent with a role for *FGFR1/2*c signalling in mammary gland initiation, we observed ectopic expression of *Tbx3*, *Lef1* and *Pyst1* in cultured flanks of mouse embryos in tissue adjacent to beads soaked in FGF8. In the case of *Tbx3*, this may represent local maintenance of the widespread expression seen prior to mammary gland development. Interestingly, sections showed that the ectopic expression of *Tbx3* is in the mesenchyme. Ectopic expression of *Lef1* and *Pyst1* is also mesenchymal and no ectopic epithelial thickenings were observed. Thus, the issue of whether FGF8 (or other FGFs) can induce ectopic mammary gland formation in the same way as FGF8 (and other FGFs) can induce ectopic limb buds still remains to be resolved (see also Spencer-Dene et al. 2001). The idea that both organs are initiated in the same way is an attractive possibility,

but using the same signal to initiate two different structures in a similar region of the embryo during development could present problems. However, work on chick embryos shows that limb induction in response to FGF application can only occur at stages prior to limb development long before mammary glands form.

### Mammary gland initiation involves Wnt-dependent regulation of *Tbx3*

Our results show that inhibition of Wnt signalling abolishes *Tbx3* expression in mouse flank region cultured just prior to mammary gland formation. This suggests that Wnt signalling in addition to Fgf signalling is required to induce and/or maintain *Tbx3* expression in early mammary glands. Moreover, we found that *Pyst1* expression is not affected by the Wnt inhibitor, suggesting that an Fgfr1/2c pathway may be acting independently of a Wnt signal during these early stages of mammary gland development. Although *Lef1* expression is reduced when CK1-7 beads are implanted, it does not seem as sensitive to Wnt inhibition as *Tbx3* expression. During early tooth development, a canonical Wnt/Lef1 pathway was reported (Kratochwil et al. 2002), with the suggestion that the role of Lef1 is to activate *Fgf4* in the dental epithelium, thus connecting the WNT and FGF signalling pathways and allowing sequential and reciprocal communication between epithelium and mesenchyme. It should also be pointed out, however, that the FGF and WNT signalling pathways we are discussing here are found in the epithelial component of the early mammary gland and not in the mesenchyme.

Figure 6 presents a synthesis of our results in the context of previous work. Although there are still many uncertainties, the data point to interactions between *Tbx3*, *Wnt* and *Fgf* signalling being involved in early mammary gland initiation. These interactions are strikingly similar to those that have been dissected out in early limb initiation but that involve *Tbx5* and *Tbx4* rather than *Tbx3*. In our scheme, we suggest that *Fgf* signalling lies both upstream and downstream of *Tbx3* expression. It is difficult to distinguish between initiation of *Tbx3* expression and maintenance of expression first in the mammary line and then in the bud, but there is good evidence for autoregulation of expression of the *Tbx* family of genes by *Fgf* signalling (Isaacs et al. 1994; Schulte-Merker & Smith, 1995; Casey et al. 1998). It is also possible that *Wnt* signalling also lies both upstream and downstream of *Tbx3* expression because in the *Tbx3*$^{-/-}$ mouse embryos, *Wnt10b* is not expressed (Davenport et al. 2003). Another common feature between the signalling interactions suggested by results on mammary gland initiation and those in the limb is that FGF signalling through at least two different classes of receptors seems to be involved. FGF signalling not only via FGFR1/2c as we have shown here, but also via FGFR2b (Spencer-Dene et al. 2001; Mailleux et al. 2002), seems to play a role. It is striking that even the same sets of FGF ligands are expressed in both mammary gland and limb, including *Fgf4*, *Fgf8*, *Fgf9*, *Fgf10* and *Fgf17.* In the limb, there is a signalling loop that maintains, reciprocally, *Fgf8* and *Fgf10* expression. Finally, it is worth bearing in mind that other signalling systems have also been implicated in early mammary gland development, for example Bmp signalling (Phippard et al. 1996; reviewed in Veltmaat et al. 2003). The challenge will be to work out how all these signalling systems act together to initiate mammary gland development.

## Acknowledgements

This research was supported by a Royal Society Korea–UK joint project grant to C.T. and H.-S.J. C.T. is also supported by The Royal Society. M.C.E. was supported by a PhD studentship from the Anatomical Society of Great Britain and Ireland, and the British Council. H.-S.J. and S.-J.S. were supported by grant number R13-2003-13 from the Basic Research Programme of the Korea Science & Engineering Foundation.

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