Canonical WNT signaling promotes mammary placode development and is essential for initiation of mammary gland morphogenesis

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Summary

Mammary glands, like other skin appendages such as hair follicles and teeth, develop from the surface epithelium and underlying mesenchyme; however, the molecular controls of embryonic mammary development are largely unknown. We find that activation of the canonical WNT/β-catenin signaling pathway in the embryonic mouse mammary region coincides with initiation of mammary morphogenesis, and that WNT pathway activity subsequently localizes to mammary placodes and buds. Several *Wnt* genes are broadly expressed in the surface epithelium at the time of mammary initiation, and expression of additional *Wnt* and WNT pathway genes localizes to the mammary lines and placodes as they develop. Embryos cultured in medium containing WNT3A or the WNT pathway activator lithium chloride (LiCl) display accelerated formation of expanded placodes, and LiCl induces the formation of ectopic placode-like structures that show elevated expression of the placode marker *Wnt10b*. Conversely, expression of the secreted WNT inhibitor Dickkopf 1 in transgenic embryo surface epithelium in vivo completely blocks mammary placode formation and prevents localized expression of all mammary placode markers tested. These data indicate that WNT signaling promotes placode development and is required for initiation of mammary gland morphogenesis. WNT signals play similar roles in hair follicle formation and thus may be broadly required for induction of skin appendage morphogenesis.

Key words: Mammary gland, Placode, Mammary bud, WNT, TOPGAL, *Dkk1*, Mouse

Introduction

Mammary glands, like hair and teeth, are epithelial appendages that originate from the surface ectoderm and develop through epithelial–mesenchymal interactions (Hardy, 1992; Thesleff et al., 1995; Veltmaat et al., 2003). The first histological sign of mammary morphogenesis is a thickening of the surface epithelium in bilateral curved ridges between the limb buds. In mouse embryos, these mammary lines form at approximately embryonic day (E) 10.5 (Fig. 1A) (Hardy, 1992; Thesleff et al., 1995; Veltmaat et al., 2003; Veltmaat et al., 2004) and give rise to five pairs of lens-shaped mammary placodes between E11 and E11.5 (Fig. 1A) in response to signals from the underlying mesenchyme (Veltmaat et al., 2003). Observations in rabbit embryos suggest that mammary placodes may form by the migration and accumulation of motile cells at defined locations along the mammary lines (Propper, 1978). Between E11.5 and E12.5, placodal epithelial cells invade the mesenchyme to form bud structures similar to those seen in hair follicle and tooth development. However, at this point the development of these various appendages diverges: mammary bud epithelium continues downward growth in female embryos, forming a mammary sprout between E15.5 and E16.5. By E16.5 a lumen develops within the sprout, and the nipple forms by differentiation of the overlying epidermis in response to signals from specialized mammary mesenchyme. On E16, the sprout starts to branch, and by birth the mammary gland is composed of a ductal tree consisting of the primary duct and 15-20 secondary branches invested in the mammary fat pad. In male embryos the bud separates from the epithelium by testosterone-dependent mechanisms between E14.5 and E15.5, and subsequently degenerates. By contrast, hair follicle and tooth buds in embryos of both sexes undergo folding morphogenesis and very specific programs of epithelial and mesenchymal differentiation (Hardy, 1992; Jernvall and Thesleff, 2000).

Postnatal mammary development is regulated by ovarian and pituitary hormones (Hennighausen and Robinson, 2001). At puberty, the ducts elongate and bifurcate until they fully

penetrate the fatty stroma. In early to mid-pregnancy, extensive ductal side-branching occurs, alveolar structures develop and mammary epithelial cells differentiate and gain the ability to synthesize milk in preparation for lactation. After weaning of pups the alveoli involute and the gland is remodeled to a series of ducts similar to that seen in mature virgins.

While several hormones and paracrine factors have been implicated in the regulation of postnatal mammary gland development (Hennighausen and Robinson, 2001), less is known about the signals that control embryonic mammary morphogenesis. The genes encoding fibroblast growth factor 10 (FGF10), FGF receptor 2B (FGFR2B) and the T-box transcription factor TBX3 are required for the formation of most mammary placodes (Davenport et al., 2003; Mailleux et al., 2002). The lymphoid enhancer factor 1 (LEF1) transcription factor is essential for the maintenance of mammary placodes and buds (van Genderen et al., 1994) (K. Kratochwil, personal communication), and the MSX1 and MSX2 transcription factors are necessary for development beyond the placode stage (Satokata et al., 2000). Differentiation of the mammary mesenchyme, downgrowth of the mammary sprout and formation of the nipple require parathyroid hormone-related protein (PTHrP) and the type 1 parathyroid hormone/parathyroid hormone-related protein receptor (PTH1R) (Foley et al., 2001; Wysolmerski et al., 1998). Little is known about the molecular pathways regulating the formation of the initial branched ductal network.

WNT paracrine signaling molecules play key roles in the development of most organ systems, regulating cell fate decisions, proliferation, adhesion, cell shape and cell movements (Huelsken and Birchmeier, 2001; Niehrs, 2001). WNTs form a large family of related proteins and signal through several different pathways (Niehrs, 2001). The most extensively studied, 'canonical' pathway involves binding of WNT to Frizzled (FZ) receptors and to obligate co-receptors of the LDL receptor related protein (LRP) family, leading to inactivation of a complex of proteins that degrades cytoplasmic β-catenin. As a result, β-catenin accumulates in the cytoplasm, translocates to the nucleus and forms active transcriptional complexes with members of the LEF/TCF transcription factor family (Huelsken and Birchmeier, 2001). Non-canonical WNT pathways require FZ, but not LRP, β-catenin or LEF/TCF factors (Niehrs, 2001).

It is well known that activation of canonical WNT signaling can cause mammary tumors (Imbert et al., 2001; Michaelson and Leder, 2001; Robinson et al., 2000). However, the functions of *Wnt*s in normal mammary development are less well defined. The phenotype of *Lef1*-null mice suggests that WNT signals might regulate embryonic mammary development (van Genderen et al., 1994). *Wnt4* mediates progesterone-induced ductal side-branching in early pregnancy (Brisken et al., 2000), and transgenic expression of *Wnt1* or *Wnt10b* causes excessive branching and precocious alveolar development in virgin mice (Robinson et al., 2000). Conversely, expression of *Axin*, which acts to destabilize β-catenin, causes defective alveolar formation during pregnancy (Hsu et al., 2001). Thus, WNT signaling may be important at multiple steps in normal mammary gland development.

We show here that the canonical WNT signaling pathway is prominently activated during embryonic mammary morphogenesis, and is also active during alveolar development

Table 1. Expression of WNT pathway genes in dissected mammary buds at E12.5 and E15.5, assayed by RT-PCR using primers that amplify the nucleotides indicated

Gene	E12.5	E15.5	Accession Number	Nucleotides amplified
Wnt1	+	+	XM_128048	1469-1859
Wnt2	+	+	BC026373	376-891
Wnt2a	–	–	NM_009520.2	144-558
Wnt3	+	+	NM_009521.1	1103-1514
Wnt3a	–	+	NM_009522.1	1161-1581
Wnt4	–	+	NM_009523.1	1-430
Wnt5a	+	+	NM_009524	522-870
Wnt5b	–	+	NM_009525.2	135-425
Wnt6	+	–	NM_009526.1	1-298
Wnt7a	–	–	NM_009527.2	762-1311
Wnt7b	+	+	NM_009528.1	1009-1339
Wnt8a	–	–	NM_009290.1	1031-1440
Wnt8b	–	–	NM_011720	39-285
Wnt10a	+	–	NM_009518.1	1589-1944
Wnt10b	+	+	NM_011718.1	176-343
Wnt11	+	+	NM_009519	849-1341
Fz1	+	+	NM_021457	879-1152
Fz2	+	+	AF139183	76-420
Fz3	+	+	U43205	1708-2309
Fz4	+	+	U43317	1710-2189
Fz5	+	+	NM_022721.1	767-972
Fz6	+	+	U43319	1535-1923
Fz7	+	+	NM_008057	1654-1996
Fz8	+	+	NM_008058.1	1523-1757
Fz9	+	+	XM_284144.1	1278-1508
Tcf1	+	+	XM_122244	1224-1622
Tcf3	+	+	XM_132668	640-839
Tcf4	+	+	NM_013685	2442-2639
Lef1	+	+	NM_010703	1859-2210
Lrp5	+	+	NM008513	2894-3254
Lrp6	+	+	NM008514	2941-3290
Dvl1	+	+	U10115	2674-3225

A positive result is indicated as '+'; absence of an amplified band is indicated as '–'.

at mid-pregnancy. WNT signaling activity is detected in the mammary region from the time of mammary initiation, providing the earliest known molecular marker for the mammary line, and subsequently localizes to mammary placodes and buds. We demonstrate that forced activation of the WNT pathway in cultured embryos accelerates the development of mammary placodes in their normal locations, and induces the formation of ectopic placode-like structures. Analysis of early stages of mammary development in transgenic embryos ectopically expressing the secreted WNT inhibitor Dickkopf 1 (DKK1) in the surface epithelium reveals the absence of localized expression of all molecular placode markers tested, and a complete failure to form any mammary structures. These results indicate that activation of WNT signaling promotes the development of mammary placodes in competent epithelium, and is required for the initiation of mammary gland morphogenesis.

Materials and methods

Analysis of TOPGAL expression

TOPGAL mice (DasGupta and Fuchs, 1999) were maintained on a CD-1 background. Noon of the day of vaginal plug appearance was designated E0.5. Embryonic stages were confirmed by analysis of limb morphology. Whole embryos, embryonic ventral skin or adult number 4 inguinal mammary glands were fixed and stained with X-

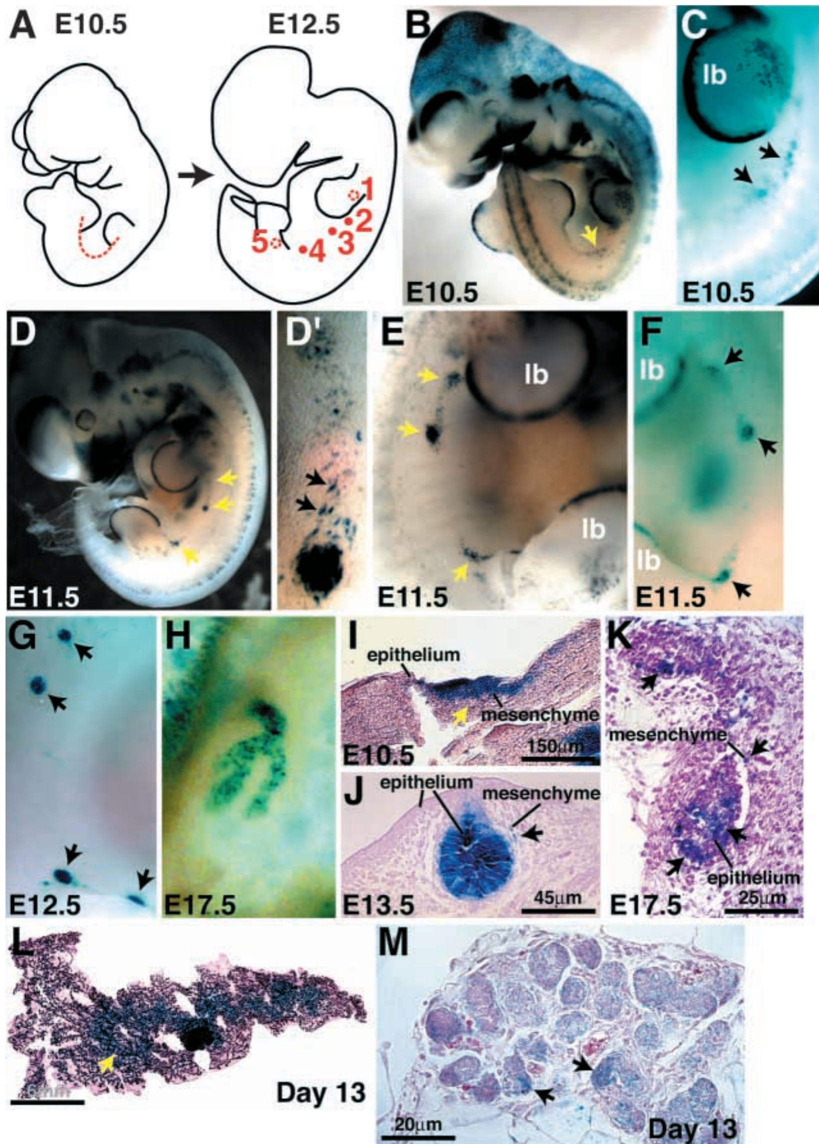

Fig. 1. TOPGAL expression during mammary gland development. (A) Development of mammary buds (red circles) from the presumptive mammary line (broken red line) during mouse embryogenesis (E10.5-E12.5). Mammary buds 1 and 5 develop underneath the limb buds and are depicted as open circles with a broken circumference. Placodes arise asynchronously, with placode pair number 3 detected first, followed by number 4, then numbers 1 and 5, and finally pair number 2 (Veltmaat et al., 2003). (B-G) Whole-mount X-gal stained TOPGAL embryos at E10.5 (B,C); E11.5 (D-F); and E12.5 (G). Cells expressing the TOPGAL transgene appear blue. At E10.5, TOPGAL activity appears as a streak in the mammary region between the fore- and hind-limb buds (lb), indicated by arrows (B,C). By E11.5, TOPGAL-positive cells accumulate in placodes at defined locations that are reproducible between different embryos (D-F). D' shows a higher magnification view of placode number 3 from the embryo shown in D. Blue-stained cells are seen on the surface of the embryo adjacent to the developing placode (arrows). At E12.5, almost all the blue-staining cells in the mammary region are concentrated within well-defined mammary buds (G, arrows). (H) X-gal-stained whole-mount of E17.5 ventral skin showing punctate staining throughout the mammary epithelial ducts. (I) 20 µm section through the area of the mammary line in an E10.5 TOPGAL embryo. Note TOPGAL activity in both the surface epithelium and the underlying mesenchyme (arrow). (J) 5 µm section through a mammary bud from an E13.5 embryo. The epithelial cells and a few surrounding mammary mesenchymal cells (arrow) express TOPGAL. (K) 5 µm section through the mammary ducts from an E17.5 embryo. A subset of epithelial cells and a few mesenchymal cells express TOPGAL (arrows). (L) Whole-mount preparation of X-gal and carmine aluminum (red)-stained mammary gland from a TOPGAL transgenic mouse on day 13 of pregnancy. TOPGAL is active in developing alveoli (arrow). (M) 5 µm section through the mammary gland shown in L. TOPGAL is active in epithelial cells of developing alveolar structures (arrows).

gal (Furth et al., 1994), photographed and/or paraffin-embedded, sectioned and counterstained with eosin. Mammary gland whole mounts were counterstained with 0.2% carmine, 0.5% aluminum potassium sulfate.

RT-PCR analysis of gene expression in isolated mammary buds

RNA was extracted from mammary buds microdissected from E12.5 and E15.5 CD-1 embryos using Trizol (Invitrogen), and treated with DNAse I (GenHunter). First strand cDNA synthesis was performed using Superscript™ One-Step RT-PCR with Platinum® *Taq* kit (Invitrogen) using 100 ng of RNA per reaction. Eighteen base pair primers were used to amplify the sequences indicated in Table 1.

Production of conditioned media and embryo cultures

L cells stably transfected with *Wnt3a* cDNA (ATCC) were maintained in DMEM/4 mM L-glutamine/10% fetal bovine serum/400 µg/ml G-418. Conditioned media collected after 4 and 7 days in the absence of G418 were pooled and filtered (0.22 µm). To test WNT activity, 3T3 cells (ATCC) were transfected using GenePorter2 transfection reagent (Gene Therapy Systems), with 0.2 µg pcDNA3.1/β-galactosidase plasmid and 1.8 µg TOPFLASH or FOPFLASH plasmids that contain LEF/TCF binding sites or mutated, nonfunctional LEF/TCF binding sites, and a luciferase reporter gene (Korinek et al., 1997). Transfected cells were treated with 50% WNT3A-conditioned medium, 50% control-conditioned medium (from untransfected L cells), or 25 mM lithium chloride (LiCl) (positive control). Cell lysates were analyzed for luciferase activity and values normalized to β-galactosidase activity and protein concentration. TOPGAL embryos were cultured in glutamine-supplemented DMEM/F12, 10% FBS on cell culture inserts (Falcon) in the presence or absence of 50% WNT3A-conditioned medium, 50% control-conditioned medium, or 50 mM LiCl at 37°C, 5% CO_2.

Generation of mice inducibly expressing *Dkk1* in surface epithelium and mouse genotyping

A *β-globin* intron and mouse *Dkk1* cDNA, PCR-amplified from a *K14-Dkk1* transgene (Andl et al., 2002), were cloned into pGEMTeasy (Promega), and a 1.5 kb *Not*I fragment was subcloned into the *Not*I site of pTRE2 vector (Clontech), providing a *tetO* promoter and *β-globin* polyA addition sequences. The *tetO-Dkk1* transgene was released with *Aat*I and *Sap*I and injected into fertilized eggs from a B6SJLF1/J × B6SJLF1/J cross (Jackson Laboratories). Transgenic founders were identified by Southern blotting of tail biopsy DNA. To

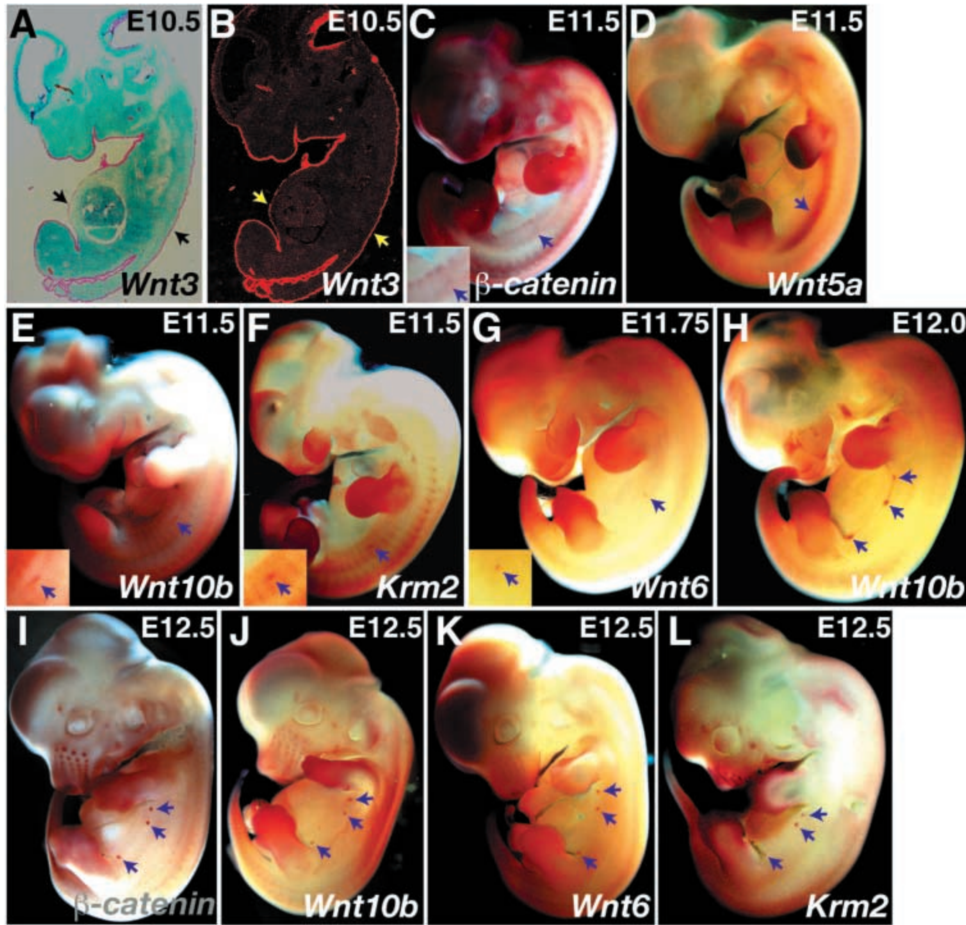

Fig. 2. Expression of WNT pathway genes at early stages of mammary development. (A,B) In-situ hybridization of a ^{35}S-labeled probe for *Wnt3* to a parasagittal paraffin section of an E10.5 embryo photographed in bright field (A) and dark field (B) with red stage illumination. The signal appears as red grains and is indicated by arrows. (C-L) Whole-mount in-situ hybridization using the probes indicated. The signal appears red-brown and mammary region expression is indicated by arrows in each panel. Insets in panels C,E,F,G represent higher magnification photographs of the regions indicated by arrows in each panel.

generate *Dkk1*-expressing double transgenic and control littermates, heterozygous *tetO-Dkk1* transgenic mice were crossed with heterozygous *K5-rtTA* mice (Diamond et al., 2000), and pregnant females were placed on chow formulated with 1 g/kg doxycycline (BioServ, Laurel, MD) immediately after observation of a copulation plug. Progeny were genotyped by PCR analysis of tail biopsy DNA. *Wnt5a* mutant mice were genotyped as described previously (Yamaguchi et al., 1999).

In-situ hybridization and immunohistochemistry

Section in-situ hybridization with ^{35}S-labeled probes and whole-mount in-situ hybridization using digoxigenin-labeled probes were carried out according to published protocols (Decimo et al., 1995; Millar et al., 1999). Probes for *Wnt* genes, *Fgf10*, *Dkk1*, β-catenin, *Lef1* and *Tbx3* have been described previously (Andl et al., 2002; Chapman et al., 1996; Gavin et al., 1990; Kispert et al., 1996; Millar et al., 1999; Mucenski et al., 2003; Parr et al., 1993; Wang and Shackleford, 1996). Probe templates for *Krm1*, *Krm2* were synthesized by PCR of E14.5 embryo cDNA with 18 bp primers designed to amplify nucleotides 838-1516 of *Krm1* and 843-1620 of *Krm2* cDNAs (Genbank accession numbers NM 032396, NM 028416). T7 RNA polymerase-binding sites were added to 3′ primers to create templates for synthesis of antisense probes and to 5′ primers to create sense probe templates.

Whole-mount immunohistochemistry of cultured embryos was carried out according to Hogan et al. (1994). Fixed embryos were incubated in anti-phospho-histone H3 (Ser10) (Cell Signaling Technologies #9701) (1:100), followed by biotinylated goat anti-rabbit IgG antibody (Vector Labs) (1:200), and alkaline phosphatase-conjugated streptavadin (Vector Labs) (1:200), and were developed in NBT/BCIP (Roche).

Scanning electron microscopy

E10.5-E12.5 embryos were washed in filtered PBS five times, and fixed for 1 hour at room temperature, followed by overnight at 4°C, in 0.1 M sodium cacodylate pH 7.6, 2% glutaraldehyde. After fixation, embryos were washed in distilled water, dehydrated in graded ethanols, transferred to freon overnight, and then air dried. Embryos were mounted using carbon putty on aluminum stubs, sputter coated with gold to approximately 100 angstroms thickness, and viewed using a JEOL 330 scanning electron microscope.

Results

Canonical WNT signaling is active at multiple stages of mammary development

To determine when WNT signaling is active during mammary development, we utilized transgenic mice carrying a TOPGAL *lacZ* reporter gene that is expressed at sites of canonical WNT pathway activity (DasGupta and Fuchs, 1999). Reporter gene expression, detected by 4-chloro-5-bromo-3-indoyl β-D-galactopyranoside (X-gal) staining for β-galactosidase, was first apparent in the mammary region at approximately E10.5 in a punctate fashion along bilateral, curved lines connecting the fore- and hind-limb buds (Fig. 1B,C). These appeared to be analogous to the milk or mammary lines (Propper, 1978). Histologic examination of these regions at E10.5 demonstrated TOPGAL expression in the surface epithelium and underlying mesenchyme (Fig. 1I). By E11.5, TOPGAL-expressing cells had coalesced into distinct mammary placodes (Fig. 1D-F). At this stage, most placodes had a comma-shaped tail of

TOPGAL-expressing cells, and there was often a thin line of staining connecting buds 2 and 3 and buds 4 and 5. Examination at higher magnification revealed that some TOPGAL-expressing cells near the forming placodes lay on the surface of the epithelium, consistent with a prior report of apparently motile cells that lie atop the mammary line in rabbit embryos (Propper, 1978) (Fig. 1D').

By E12.5, TOPGAL expression in the mammary region was almost completely restricted to mammary bud epithelium, which showed intense *lac*Z staining (Fig. 1G,J). A few mesenchymal cells surrounding the buds were also *lacZ*-positive (arrow in Fig. 1J). TOPGAL expression persisted within the buds until E15.5 and disappeared on E16.5. Staining reappeared at E17.5 in the epithelial ducts (Fig. 1H,K). Rare mesenchymal cells also stained at this stage. This staining pattern persisted through the first few days of postnatal life.

No TOPGAL activity was detected during adolescent development, early pregnancy, late pregnancy or in lactating and involuting mammary glands (data not shown). Reporter gene expression appeared transiently during mid-pregnancy between days 12 and 13 of gestation within epithelial cells of the developing alveoli (Fig. 1L,M), consistent with prior findings that overexpression of the canonical WNT antagonist Axin in the mammary glands of transgenic mice inhibits the development of alveolar structures (Hsu et al., 2001), while expression of stabilized β-catenin results in precocious alveolar development (Imbert et al., 2001).

Expression of WNT pathway genes at early stages of mammary development

The above data indicated that WNT activity is pronounced during early mammary development. To identify *Wnt* and WNT pathway genes that might participate in WNT signaling at these stages, we first used RT-PCR to survey *Wnt*, *Fz*, *Tcf* and *Lrp* gene expression in mammary buds microdissected from embryos at E12.5 and E15.5. As indicated in Table 1, multiple *Wnt* genes; *Fz*s *1-9*; *Tcf*s *1,3,4* and *Lef1*; *Dvl1*; and *Lrp*s *5* and *6* showed expression in mammary buds by this assay (Table 1).

To determine the localization and timing of *Wnt* gene expression in the mammary region, we performed section and whole-mount in-situ hybridization of embryos between E10.5 and E12.5 using probes for *Wnt* genes that were positive for expression by RT-PCR at E12.5, and *Wnt2b* and *Wnt16*, which had not been tested by RT-PCR. *Wnt3*, *Wnt6* and *Wnt10b* showed generalized expression in the surface epithelium (Fig. 2A,B and data not shown). *Wnt10b* expression showed the earliest specific localization to the mammary epithelium at E11.5, when darker streaks of signal were noted along the mammary line (Fig. 2E, see inset). By E12.0, *Wnt10b* expression was elevated in nascent mammary buds as well as in remnants of the mammary line (Fig. 2H); at E12.5, elevated expression was confined to the buds (Fig. 2J). Mammary expression of *Wnt10a* was similar to, but weaker than, that observed for *Wnt10b* (data not shown). Expression of *Wnt6* was slightly elevated in developing placodes at E11.75 (Fig. 2G, see inset) and clearly localized to mammary buds by E12.5 (Fig. 2K). Similar expression of *Wnt10b* and *Wnt6* in the mammary region was recently reported by Veltmaat et al. (Veltmaat et al., 2004). *Wnt7b* was expressed at low levels in mammary buds (data not shown), and *Wnt11* was very weakly expressed in ring shapes around the buds (see Fig. 6G, blue arrows) and also showed weak expression in a broad stripe of mesenchyme between the limb buds (see Fig. 6G, yellow arrow). *Wnt5a* was strongly expressed in a broad band of mesenchyme underlying the mammary region at E10.5-E12.5 (Fig. 2D and data not shown) but was not specifically upregulated in mammary placodes and buds. We did not detect expression of *Wnt1*, *Wnt2*, *Wnt2b* or *Wnt16* in the mammary region by in-situ hybridization. The discrepancy in the results obtained for *Wnt1* and *Wnt2* expression in RT-PCR and in-situ hybridization experiments was probably due to the less sensitive nature of the whole-mount in-situ hybridization assay.

Several additional WNT pathway genes were expressed in developing mammary placodes. Although β-catenin mRNA was broadly expressed at E11.5, it was slightly upregulated in the mammary region (Fig. 2C, see inset). By E12.5, β-catenin mRNA was clearly elevated in mammary buds (Fig. 2I). A similar pattern of localized upregulation of β-catenin mRNA has previously been described in developing hair follicle placodes (Andl et al., 2002; Huelsken et al., 2001). Kremen 2 (*Krm2*), encoding a cell surface receptor required for activity of DKK1 (Mao et al., 2002), was expressed in a similar pattern to β-catenin and showed elevated expression in the mammary line by E11.5 (Fig. 2F, see inset). *Krm2* expression localized strongly to mammary buds by E12.5 (Fig. 2L). The related gene *Krm1* showed a similar expression pattern to *Krm2* (data not shown). By contrast, expression of *Dkk1* was not specifically elevated in the mammary region (data not shown).

Forced activation of the canonical WNT pathway promotes placode development

The presence of WNT reporter activity in the mammary line and the dynamic expression pattern of WNT pathway components during mammary placode formation suggested that WNT signaling might regulate the development of mammary placodes. To begin to test this hypothesis, we asked whether forced activation of the WNT pathway affects placode formation. For these experiments, we developed a culture system permitting formation of mammary placodes in vitro. E10.5-E11.0 TOPGAL embryos were dissected and cultured for 0-48 hours and stained with X-gal to visualize the mammary line and the developing placodes. When embryos were initially harvested, the expected punctate X-gal staining within the mammary line was observed (Fig. 3A). Over the next 24-48 hours in culture, TOPGAL activity consistently and progressively coalesced into individual oval structures at points on the ventral-lateral surface corresponding to the location of mammary placodes in vivo (n=72 from 24 different litters) (Fig. 3A-C). Histological examination of these structures revealed that they consisted of thickened epithelium that formed shallow invaginations rather than full bud development. This was reflected by the intensity of X-gal staining, which was less in cultured embryos than in similarly aged embryos in vivo. Culture times longer than 48 hours resulted in deterioration of the embryos and shedding of surface epithelium.

To examine the effects of activating the WNT pathway on placode formation, we cultured embryos for 24-48 hours in the presence of either WNT3A-conditioned medium (Fig. 3E) or control-conditioned medium (Fig. 3D). The biological activity of the WNT3A medium was demonstrated by its ability to

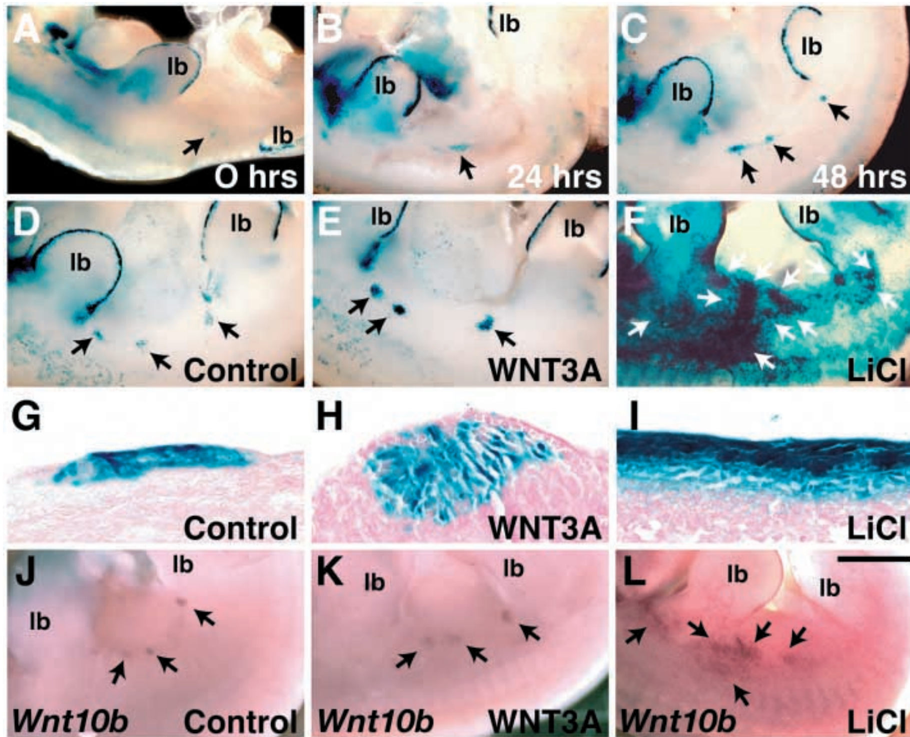

Fig. 3. Mammary placode formation in cultured embryos. (A-C) X-gal-stained E10.5 TOPGAL embryos after 0 hours (A), 24 hours (B) or 48 hours (C) culture in control medium. Arrows indicate developing placodes. (D-F) X-gal-stained E11 TOPGAL embryos after 24 hours culture in control-conditioned medium (D), WNT3A-conditioned medium (E) or LiCl-supplemented medium (F). WNT3A treatment causes the formation of placodes that are larger and more distinct than in the littermate control (arrows in D,E). LiCl treatment causes activation of TOPGAL transgene expression along the lateral aspect of the embryo, and formation of multiple intensely blue-staining clusters of cells (F, arrows). (G-I) Histological sections (counterstained with eosin) through the mammary placodes of E11 X-gal-stained embryos cultured for 24 hours in control medium (G), WNT3A-conditioned medium (H) or LiCl (I). (J-K) Whole mount in-situ hybridizations for *Wnt10b* on E11 embryos cultured for 48 hours (J,K) or 24 hours (L) in control medium (J), WNT3A-conditioned medium (K) or 50 mM LiCl (L). LiCl treatment was limited to 24 hours, as we found 48 hours' exposure to LiCl to be toxic. Arrows indicate *Wnt10b* expression in placodes (J-L) and ectopic placode-like structures (L). Scale bar: 0.65 mm in A; 0.75 mm in B; 0.9 mm in C; 0.5 mm in D-F, 33 μm in G-I; 0.6 mm in J-L.

activate a canonical WNT reporter gene (TOPFLASH) (Korinek et al., 1997) in NIH 3T3 cells. WNT3A-conditioned medium activated TOPFLASH 4-5-fold over baseline levels, while control-conditioned medium did not activate the reporter (data not shown). WNT3A accentuated mammary placode development such that the individual placodes formed sooner and were larger than those seen in controls (compare Fig. 3E,D). Histological analysis demonstrated that WNT3A treatment promoted the invagination of the placodes into the underlying mesenchyme, forming structures that resembled mammary buds (Fig. 3H), rather than the shallow invaginations formed in embryos cultured in control, or control-conditioned, media (Fig. 3G and data not shown). Accelerated placode development and/or the formation of larger, thicker placodes relative to littermate controls was observed in 70-80% of embryos cultured with WNT3A-conditioned medium (n=29, from 11 separate litters).

The actions of WNT3A on mammary placode development may be modulated by the local expression of specific receptors and WNT pathway inhibitors. Therefore we also examined the effects of post-receptor activation of WNT signaling on placode formation in cultured embryos using LiCl, which activates WNT signaling by inhibiting GSK3-β and stabilizing cytoplasmic β-catenin (Hedgepeth et al., 1997). LiCl treatment induced TOPGAL expression in a wide swath along the ventral-lateral surface of the embryo (Fig. 3F). Mammary placodes developing at their normal locations appeared larger and more irregular than in controls. In addition, accumulations of TOPGAL-positive cells were formed at ectopic locations, both along and lateral to the mammary line (100% of treated embryos, n=9 from four different litters). Histologically, the areas of X-gal staining consisted of multilayered regions of surface epithelium that resembled the placodes formed in control embryos, but were thicker and covered a much larger surface area (compare Fig. 3G and I). Portions of these placodes contained undulating regions of invagination into the mesenchyme, but not to the degree seen in WNT3A-treated embryos. These results suggested that post-receptor activation of WNT signaling promotes placode formation in extended regions of the ventral-lateral surface of the embryo.

To investigate whether activation of WNT signaling by WNT3A and LiCl induced the expression of molecular placodal markers, we examined the expression of *Wnt10b* in the embryo cultures by in-situ hybridization. Expression of *Wnt10b* was specifically elevated within the placode-like structures in control-cultured embryos, and in the larger placodes formed in WNT3A- and LiCl-treated embryos (Fig. 3J-L). In addition, LiCl treatment induced *Wnt10b* expression in patchy pattern over a wide area of the ventral-lateral surface of the cultured embryos (Fig. 3L), similar to the pattern of LiCl-induced TOPGAL staining (Fig. 3F). Similar results were obtained in experiments examining expression of the early placode marker *Tbx3* (data not shown). These data demonstrate that stimulation of WNT signaling promoted both the morphogenesis of mammary placodes and the expression of molecular placode markers.

LiCl-mediated activation of the WNT signaling pathway might promote mammary placode formation by stimulating the proliferation of specific placode precursor cell populations, by altering cell movements in the mammary region, or by causing larger than usual numbers of surface epithelial cells to adopt a placodal fate. To begin to distinguish among these

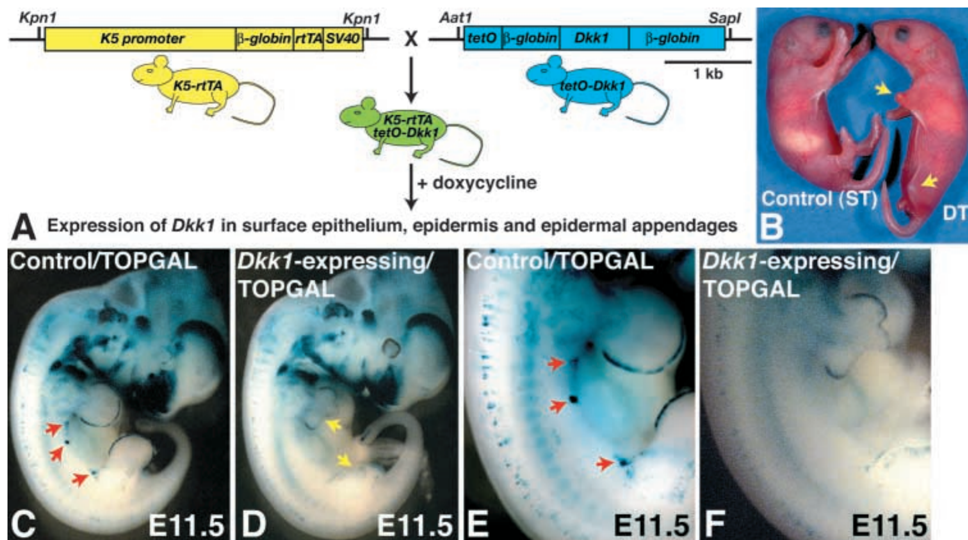

Fig. 4. Inducible expression of *Dkk1* in the surface epithelium and inhibition of TOPGAL activity. (A) Scheme for doxycycline-inducible expression of *Dkk1*. Mice carrying the *K5-rtTA* transgene (yellow) are mated to mice carrying the *tetO-Dkk1* transgene (blue). In double transgenic offspring (green), expression of *Dkk1* can be induced by doxycycline in cells where the *K5* promoter is active. (B) Phenotype of newborn double transgenic (DT) pup (right) compared with control single transgenic (ST) littermate (left) after doxycycline treatment from E0.5. The DT pup has severe limb defects (arrows), open eyes and lacks vibrissa follicles. (C-F) A *K5-rtTA*, *tetO-Dkk1*, TOPGAL (*Dkk1*-expressing/TOPGAL) embryo (D,F) and *tetO-Dkk1*, TOPGAL (Control/TOPGAL) control littermate (C,E) were doxycycline-treated throughout gestation, harvested at E11.5 and stained with X-gal to reveal sites of TOPGAL WNT reporter expression. (E,F) Higher-magnification photographs of the mammary regions of the embryos shown in C,D, respectively. Strong staining for β-galactosidase is visible in the mammary region of the control at sites of mammary placode development (C,E, red arrows) and other sites, including the apical ectodermal ridge of each limb bud. TOPGAL activity was severely reduced in the mammary region of the *K5-rtTA*, *tetO-Dkk1*, TOPGAL embryo (D,F). Deformities of the limb buds correlate with loss of β-galactosidase expression from the limb edge (yellow arrows in D), consistent with the known requirement for canonical WNT signaling in maintenance of the apical ectodermal ridge (Barrow et al., 2003).

mechanisms, we examined proliferation in embryos that had been sacrificed at E11 and cultured for 24 hours in control medium or medium supplemented with 50 mM LiCl. Whole-mount immunohistochemistry for the mitotic marker phospho-histone H3 revealed that the number of proliferating cells in the surface epithelium was slightly decreased in LiCl-treated embryos, possibly due to toxicity of LiCl. In both control- and LiCl-treated embryos, proliferating cells were largely excluded from sites of placode formation, consistent with previous reports that mammary placodes are relatively non-proliferative (Balinsky, 1950; Veltmaat et al., 2003) (data not shown). These observations indicate that LiCl does not stimulate the proliferation of specific placode precursor cell populations, but instead may act by altering the fates or movements of cells in the mammary region.

A system for reproducible, efficient WNT inhibition in the surface epithelium in vivo

To determine whether canonical WNT signaling is required for the initiation of mammary placode development in vivo, we ectopically expressed the secreted WNT inhibitor DKK1 in embryonic surface epithelium. DKK1 acts by binding to LRP and KRM1 or KRM2, resulting in internalization of a DKK1/LRP/KRM complex and removal of LRP from the plasma membrane (Mao et al., 2002; Zorn, 2001). DKK1-mediated inhibition of canonical WNT signaling is rapid, potent and specific (Mao et al., 2002; Zorn, 2001). We have previously shown that constitutive *K14* promoter-driven ectopic expression of *Dkk1* in transgenic surface epithelium blocks mammary bud development (Andl et al., 2002). However, determination of the precise mammary defects in these mice was complicated by variability in *Dkk1* expression levels between different founder embryos and by the fact that high levels of expression caused perinatal lethality and precluded the establishment of transgenic lines (Andl et al., 2002). To avoid this problem we developed a doxycycline-inducible bi-transgenic system that allowed us to consistently produce embryos expressing high levels of ectopic *Dkk1* (Fig. 4A).

We generated mice carrying a transgene comprised of the *Dkk1* coding region downstream of a tetracycline/doxycycline-responsive promoter (*tetO*) (Gossen and Bujard, 1992). Seven transgenic founder animals were obtained and had grossly normal phenotypes. These founders were mated to mice carrying a transgene in which coding sequences for the reverse tetracycline-dependent transactivator (*rtTA*) (Kistner et al., 1996) were placed downstream of a keratin 5 (*K5*) promoter that, like the *K14* promoter, is active in the surface epithelium from E9.5 (Byrne et al., 1994; Diamond et al., 2000). Pregnant females were placed on doxycycline immediately after detection of a copulation plug to induce *Dkk1* expression in double transgenic *K5-rtTA*/*tetO-Dkk1* embryos. Three of the seven founders produced double transgenic offspring with hair and tooth phenotypes similar to those shown by mice constitutively expressing the *K14-Dkk1* transgene. A *tetO-Dkk1* founder line that produced offspring with strong phenotypes similar to those seen in mice expressing high levels of the *K14-Dkk1* transgene was used for the experiments described below. Induced double transgenic offspring of this line reproducibly lacked hair follicles, teeth and mammary glands at birth, and displayed limb defects; by contrast, single-transgenic control littermates and uninduced double transgenic mice were phenotypically normal (Fig. 4B and data not shown).

Whole-mount and section in-situ hybridization revealed ectopic expression of *Dkk1* in the surface epithelium of

doxycycline-treated double transgenic E10.5-E12.5 *K5-rtTA/tetO-Dkk1* embryos (data not shown). To determine whether ectopic *Dkk1* efficiently inhibited canonical WNT signaling in the developing mammary region we crossed *K5-rtTA*, *tetO-Dkk1* double transgenic mice to homozygous TOPGAL mice and placed the pregnant females on doxycycline until they were sacrificed at E10.5-E12.5. Control doxycycline-treated *K5-rtTA*, TOPGAL and *tetO-Dkk1*, TOPGAL embryos showed TOPGAL reporter activity in the mammary line at E10.5 and in developing mammary placodes and buds at E11.5 and E12.5 as expected (Fig. 4C,E and data not shown). By contrast, in induced *K5-rtTA*, *tetO-Dkk1*, TOPGAL triple transgenic embryos, TOPGAL activity was markedly reduced in the mammary region at E10.5-E11.0 (*n*=2) and was either completely, or almost completely, absent at E11.5 (*n*=5) and E12.5 (*n*=5), indicating that WNT/β-catenin signaling was efficiently and consistently inhibited (Fig. 4D,F and data not shown).

Canonical WNT signaling is required for mammary placode morphogenesis and localized expression of placode markers

We used scanning electron microscopy to examine mammary placode development in E11.5 double transgenic *K5-rtTA/tetO-Dkk1* and single transgenic or non-transgenic littermate control embryos. The initial indication of mammary development in controls was the appearance of a mound of cells at approximately E11.5 corresponding to placode number 3, the first to develop (Fig. 5A,C). No such structure was visible at the same position in *Dkk1*-expressing littermate embryos (Fig. 5B,D), indicating that placode formation was inhibited. Examination of *Dkk1*-expressing embryos 1 day later demonstrated that all five pairs of placodes were absent in the *Dkk1*-expressing embryos (data not shown).

To determine if *Dkk1* perturbed the expression of molecular markers of placode development, we used whole-mount in-situ hybridization to examine the expression of β-catenin, *Wnt10b* (Christiansen et al., 1995), *Lef1* (van Genderen et al., 1994) and *Krm2* at E12.5, and *Tbx3* (Chapman et al., 1996) at E11.75 in *Dkk1*-expressing and control embryos. None of these markers was expressed in a localized fashion in the mammary region of *Dkk1*-expressing embryos (Fig. 6A-D,I-L and data not shown). Weakly localized expression of *Wnt11* in ring shapes around placodes in control embryos (Fig. 6G, blue arrows) was also absent from *Dkk1*-expressing embryos (Fig. 6H), although faint *Wnt11* expression was maintained in a broad stripe between the limb buds (Fig. 6G,H, yellow arrows). Localized upregulation of *Wnt10b* was absent from *Dkk1*-expressing embryos even at E11.5, a stage at which *Wnt10b* expression is usually visible as a faint line in controls (Fig. 6M,N). This result indicates that *Dkk1* blocks WNT signaling upstream of mammary-specific expression of *Wnt10b*, and is consistent with our observation that TOPGAL expression appears in the mammary line approximately 1 day before *Wnt10b* expression (compare Fig. 1B and Fig. 2E). By contrast, ectopic *Dkk1* did not affect expression of *Wnt5a* in the mammary region at E11.5 or E12.5 (Fig. 6E,F and data not shown). These results suggested *Wnt5a* as a possible candidate for a locally expressed regulator of mammary development whose actions might be blocked by *Dkk1*. However, histological examination of the mammary region in *Wnt5a*-null (Yamaguchi et al., 1999) female embryos revealed that mammary primordia were present at E15.5 (Fig. 6Q,R). Furthermore the pattern of mammary placode induction, revealed by in-situ hybridization with a *Wnt10b* probe, was similar to that seen in control embryos (Fig. 6S,T).

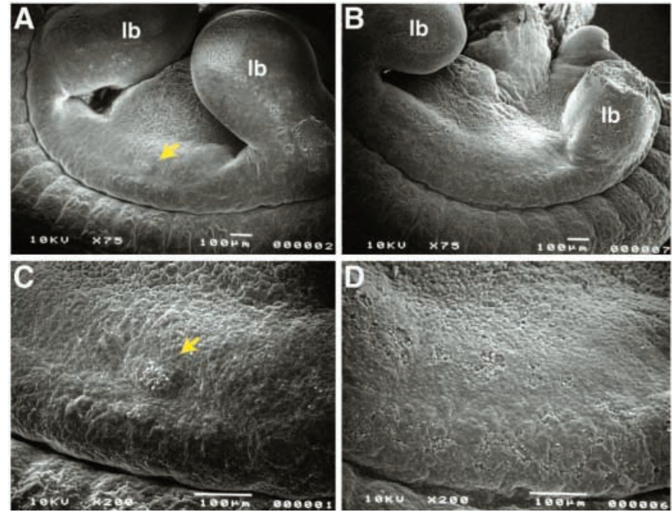

Fig. 5. Mammary placodes are absent in *Dkk1*-expressing embryos. Scanning electron microscopy of the ventral-lateral surface of induced *K5-rtTA*, *tetO-Dkk1* (B,D) and control single transgenic littermate (A,C) embryos at E11.5. Placode number 3 is visible in the control as a mound of cells protruding above the surface of the embryo (arrows in A,C) and is absent in the induced double-transgenic embryos (B,D). A,B were photographed at 75× magnification and C,D at 200× magnification. Scale bars: 100 μm. lb, limb bud.

Ectopic *Dkk1* causes a more complete block to mammary placode development than that observed in embryos lacking FGF10 or the FGF receptor, FGFR2b, in which one pair of mammary buds is still formed (Mailleux et al., 2002). While *Fgfr2b* expression localizes to mammary placodes at E11-E12, *Fgf10* is expressed transiently at E10.5 in the most ventral epithelial regions of the dermamyotome, and may signal to cells that give rise to mammary region dermis (Mailleux et al., 2002). To begin to investigate the relationships between FGF10 and WNT signaling, we asked whether expression of *Fgf10* in the dermamyotome was affected by ectopic expression of *Dkk1* in the surface epithelium. Expression of *Fgf10* in the somites of control embryos appeared as a thin, broken line of staining between the anterior and posterior limb buds at E10.5 (Fig. 6O). Similar staining was seen in *Dkk1*-expressing bi-transgenic littermates (Fig. 6P), suggesting that WNT/β-catenin signaling activity in the mammary region either lies downstream of *Fgf10* or acts in an independent fashion.

Discussion

Our experiments demonstrate that activation of the TOPGAL WNT reporter gene (DasGupta and Fuchs, 1999) occurs in the presumptive mammary lines of mouse embryos and that canonical WNT activity subsequently localizes to developing mammary placodes and buds. Forced activation of WNT signaling promotes the development of mammary placodes; conversely, inhibition of canonical WNT signaling by *Dkk1*

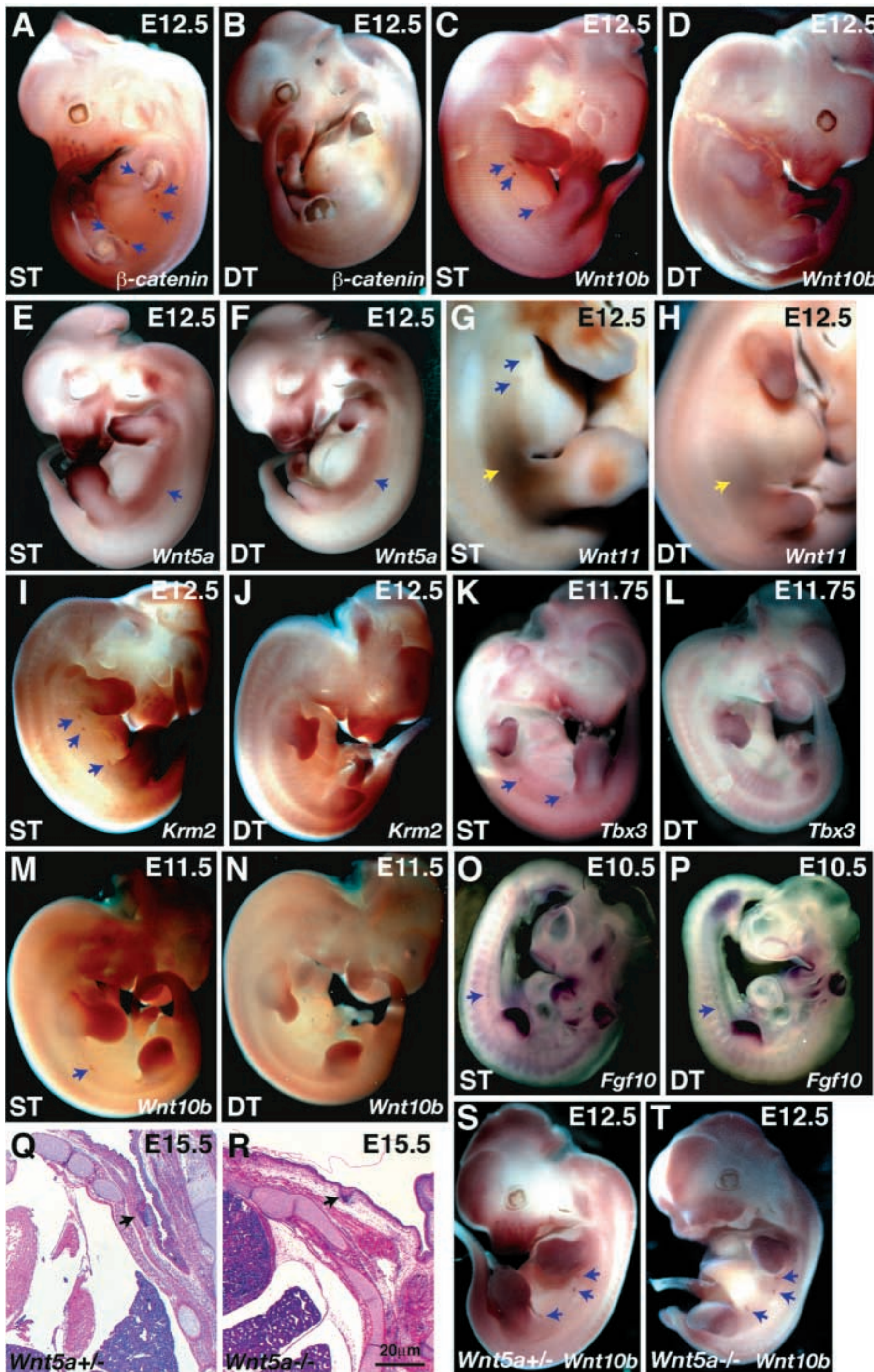

Fig. 6. Absence of localized expression of mammary placode markers in induced *K5-rtTA*, *tetO-Dkk1* embryos and presence of mammary rudiments in *Wnt5a*-null embryos. (A-P) *K5-rtTA*, *tetO-Dkk1* double transgenic (DT) (B,D,F,H,J,L,N,P) and single transgenic (ST) control littermate (A,C,E,G,I,K,M,O) embryos that were doxycyline-treated throughout gestation, harvested at E12.5 (A-J), E11.75 (K,L), E11.5 (M,N) and E10.5 (O,P), and subjected to whole-mount in-situ hybridization with the probes indicated. Control E12.5 and E11.75 embryos show discrete upregulation of β-catenin (A), *Wnt10b* (C), *Krm2* (I) and *Tbx3* (K) in mammary buds (A,C,I) and placodes (K). Limbs were removed from the embryo in A to show all five buds on the left side. Localized expression of all of these genes is absent from the mammary region of induced *K5-rtTA*, *tetO-Dkk1* double transgenic littermates (B,D,J,L). *Wnt11* is weakly expressed in ring shapes around the buds of control (G) (blue arrows) but not *Dkk1*-expressing (H) embryos. Weak expression of *Wnt11* in a broad stripe of mesenchyme is present in both control and *Dkk1*-expressing embryos (G,H, yellow arrows). Expression of *Wnt5a* in mammary region mesenchyme is unaffected by ectopic *Dkk1* (E,F) (arrows). Upregulated *Wnt10b* expression is first apparent in the mammary line of controls at E11.5 (M, arrow), and is absent in the induced *K5-rtTA*, *tetO-Dkk1* double transgenic littermate (N). *Fgf10* is expressed in somites of doxycyline-treated control and *K5-rtTA*, *tetO-Dkk1* double transgenic littermates at E10.5 (O,P, arrows). (Q,R) Transverse hematoxylin and eosin-stained paraffin sections of E15.5 *Wnt5a*$^{+/-}$ control (Q) and *Wnt5a*$^{-/-}$ (R) female embryos showing thoracic mammary rudiments (arrows). (S,T) *Wnt10b* expression in mammary buds of *Wnt5a*$^{+/-}$ (S) and *Wnt5a*$^{-/-}$ (T) E12.5 embryos (arrows).

blocks the initiation of placode development and the localized expression of all molecular placode markers examined.

Activation of TOPGAL expression in the mammary line occurs earlier than localized expression of other known markers for embryonic mammary development, including those identified in this study. This localized activation of the canonical WNT pathway might be achieved by restricted expression of a specific *Wnt* gene. Interestingly, however, we were not able to identify a *Wnt* that was an obvious candidate for this role. Of the *Wnt* genes analyzed, *Wnt10b* showed the earliest and most marked localization to the mammary line, but its expression was apparent 1 day later than TOPGAL expression. Furthermore, *Dkk1*-mediated WNT inhibition blocked localized *Wnt10b* expression. These results suggest that the initial localized expression of TOPGAL in the mammary line is not driven by mammary-specific *Wnt10b* expression. Although expression of *Wnt5a* in cells underlying the mammary region is apparent by E10.5 (Yamaguchi et al., 1999) and is unaffected by ectopic *Dkk1*, we found that *Wnt5a* is not essential for initiation of mammary development. It remains possible that *Wnt5a* acts redundantly with another *Wnt* gene expressed in mammary region dermis; for example, *Wnt11* shows weak, broad expression in this region. However, recent evidence suggests that in most developmental contexts *Wnt5a* and *Wnt11* signal via alternate pathways (Veeman et al., 2003) and antagonize canonical WNT signaling (Maye et al., 2004; Weidinger and Moon, 2003).

Instead, localized canonical WNT signaling may be achieved through regulation of responsiveness to a generally expressed WNT. We find that *Wnt6*, *Wnt3* and *Wnt10b* are broadly expressed in the surface epithelium at the time of mammary initiation. One or more of these might act in concert with localized non-WNT signals to induce TOPGAL activation within the mammary line. It has been postulated that FGF10 signals to the developing mammary dermis to induce the local production of an unknown secreted factor that is necessary for placode formation (Veltmaat et al., 2003). Signaling by FGF10 or an FGF10-induced factor might be required for epithelial and/or mesenchymal cells in the mammary region to respond to a broadly expressed WNT.

Interestingly, LiCl treatment of cultured embryos does not induce uniform TOPGAL activation, instead producing a swath of high activity between the limb buds and another more dorsal domain. Endogenous β-catenin mRNA is expressed at elevated levels in these regions (compare Fig. 2C and Fig. 3F), suggesting that β-catenin mRNA levels might modulate the LiCl response. Determining whether β-catenin mRNA levels are regulated by *Fgf10*, and/or can influence mammary development, will require further investigation.

We find that TOPGAL expression persists in developing mammary placodes and buds until the sprout stage. Together with the observation that placodes and buds are formed but not maintained in the absence of *Lef1* (van Genderen et al., 1994) (K. Kratochwil, personal communication), these data indicate that WNT signaling is required for the continued development of mammary placodes as well as for their induction. *Wnt10b* is a candidate for the signal that maintains TOPGAL expression in established mammary placodes and buds, but is unlikely to act alone as expression of *Wnt*s *6*, *10a* and *7b* also localizes to placodes. Elevated expression of *Wnt10b* and *Lef1* in developing placodes is known to require *Tbx3* (Davenport et al., 2003). Since we find that ectopic *Dkk1* blocks localized epithelial expression of *Tbx3*, it is likely that WNT signaling lies both upstream and downstream of *Tbx3* in mammary placode development.

Embryos expressing the canonical WNT inhibitor *Dkk1* display a complete block in the formation of all five pairs of mammary placodes, based on the results of scanning electron microscopy and whole-mount in-situ hybridization with several different placodal markers. This phenotype contrasts with those of embryos lacking functional *Lef1*, *Fgfr2*, *Fgf10* or *Tbx3*, in which initial development of some placode pairs occurs (Davenport et al., 2003; Mailleux et al., 2002; van Genderen et al., 1994). In *Lef1*-null embryos most pairs of placodes are formed and develop to the bud stage, but the buds are not maintained (van Genderen et al., 1994) (K. Kratochwil, personal communication). Our data indicate that canonical WNT signaling is required for the induction of all of the mammary placodes, and suggest that LEF1 acts redundantly with other LEF/TCF family members at early stages of placode formation. We detect expression of *Tcf1*, *Tcf3* and *Tcf4* in isolated mammary buds, indicating that additional TCF family members are expressed in the mammary region (Table 1).

The requirement for canonical WNT signaling in mammary placode induction is strikingly similar to the role of this pathway in the initiation of hair follicle morphogenesis (Andl et al., 2002). In both cases, morphological changes in the epithelium and the localized expression of early molecular placode markers are blocked by ectopic *Dkk1*. Furthermore, in both mammary and hair follicle placode development, localized activation of the WNT pathway appears to precede localized expression of *Wnt* genes, suggesting that localized pathway activation is initially regulated by broadly expressed *Wnt*s in conjunction, or in competition, with other factors. Forced activation of WNT signaling causes the de novo formation of hair follicles and tooth-bud-like structures in vivo (Gat et al., 1998; Zhou et al., 1995). Our results suggest that WNT pathway stimulation can also promote placode development in mammary region epithelium in vitro. Taken together, these data suggest that WNT signaling is not merely permissive for appendage induction, but instead, once activated, directs multipotent stem cells in the surface epithelium to adopt an appendage fate rather than becoming stratified epidermis. Little is known about regulation of the timing and location of development of various appendages. The apparent cooperation of WNT signaling with a regionally restricted FGF signal in mammary development, and our observation that responsiveness to extracellular and intracellular activation of WNT signaling is regionally restricted, may provide clues to the mechanisms that direct appendage formation at particular developmental stages and in specific regions.

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