Introduction

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

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.
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; Maileux 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; Wyssolmerski 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 Wnts 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 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-

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

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
Mammary induction requires WNT signaling (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% CO2.

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 NotI fragment was subcloned into the NotI site of pTRE2 vector (Clontech), providing a tetO promoter and β-globin polyA addition sequences. The tetO-Dkk1 transgene was released with AatII and SapI 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. 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).
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 35S-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) (Ser10) (Cell Signaling Technologies #9701) (1:100), followed by biotinylated goat anti-rabbit IgG antibody (Vector Labs) (1:200), and alkaline phosphatase-conjugated streptavidin (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-indoly1 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 lacZ 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; Fzs 1-9; Tcfss 1,3,4 and Left1; Dvl1; and Lrps 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. 2I). 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
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 mammmary 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
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 the 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-rtTAtetO-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
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), Left1 (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,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 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, 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).

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...
Mammary induction requires WNT signaling

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 Wnts 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 Wnts 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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