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The pattern of β-catenin responsiveness within the mammary gland is regulated by progesterone receptor

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Experiments involving β-catenin loss- and gain-of-function in the mammary gland have decisively demonstrated the role of this protein in normal alveologenesis. However, the relationship between hormonal and β-catenin signaling has not been investigated. In this study, we demonstrate that activated β-catenin rescues alveologenesis in progesterone receptor (*PR*; *Pgr*)-null mice during pregnancy. Two distinct subsets of mammary cells respond to expression of ΔN89β-catenin. Cells at ductal tips are inherently β-catenin-responsive and form alveoli in the absence of PR. However, PR activity confers β-catenin responsiveness to progenitor cells along the lateral ductal borders in the virgin gland. Once activated by β-catenin, responding cells switch on an alveolar differentiation program that is indistinguishable from that observed in pregnancy and is curtailed by PR signaling.

KEY WORDS: Beta-catenin, Breast, Progesterone receptor, Mammary gland, Wnt, Mouse, Stem cells

INTRODUCTION

The mammary gland is an epidermal appendage that undergoes both embryonic and postnatal development. During embryonic development, local signaling between the ectoderm and underlying mammary mesenchyme results in the formation of a rudimentary ductal tree embedded within a subdermal fat pad (Hens and Wysolmerski, 2005). Postnatal mammary development is temporally regulated by hormonal signaling (Lyons et al., 1958; Nandi, 1958). Puberty induces ductal elongation and bifurcation by stimulating cell proliferation within terminal end buds (TEBs) found at the ductal tips (Daniel and Silberstein, 1987; Bocchinfuso and Korach, 1997; Kleinberg et al., 2000). As puberty wanes, TEBs vanish and the ductal tree undergoes progressive estrus-induced maturation (Purnell and Saggers, 1974; Haslam, 1987). During early pregnancy, tertiary side-branches sprout from the ductal tree and alveolar buds form at their tips (Russo and Russo, 1978; Brisken, 2002). Alveolar differentiation proceeds in two stages termed lactogenesis I and II. Lactogenesis I occurs during mid-pregnancy and involves the expression of genes encoding the milk proteins β-casein and WDNM1 and accumulation of lipid droplets inside alveolar luminal cells. During late pregnancy, a lactogenic switch occurs that permits differentiation to proceed to lactogenesis II (Neville et al., 2002; Brisken and Rajaram, 2006). This stage involves expression of whey acidic protein (*Wap*) and α-lactalbumin genes, closure of tight junctions and secretion of milk and lipid droplets into the alveolar lumen (Robinson et al., 1995; Mather and Keenan, 1998; Nguyen et al., 2001). Lactation is followed by involution, which restores a pre-pregnant morphology to the parous gland (Lund et al., 1996; Wagner et al., 2002; Boulanger et al., 2005).

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Experiments involving hormonal supplementation of oophorectomized and hypophysectomized mice first established the contributions of individual hormones to specific stages of mammary development. Growth hormone and estrogen (E) promote ductal elongation. Progesterone (P) induces side-branching and alveologenesis and prolactin (PRL), in combination with P promotes alveolar development (Lyons et al., 1958; Nandi, 1958; Plaut et al., 1999; Shyamala, 1999; Atwood et al., 2000; Hovey et al., 2002; Aupperlee and Haslam, 2007). Recent studies on hormone receptor-null mice have confirmed and extended these findings. For example, mammary glands with targeted disruption of the estrogen receptor fail to undergo ductal extension (Bocchinfuso et al., 2000; Mallepell et al., 2006). Progesterone receptor (*PR*; *Pgr* – Mouse Genome Informatics)-null glands lack side-branches and alveoli (Lydon et al., 1995; Brisken et al., 1998). The PR-A isoform is dispensable to mammary development but *PR-B*$^{-/-}$ mice have fewer side-branches and alveoli resulting in impaired lactation, despite complete alveolar differentiation (Mulac-Jericevic et al., 2000; Shyamala et al., 2000; Mulac-Jericevic et al., 2003). Prolactin receptor (PRLR) is also essential for alveologenesis and is required for complete alveolar differentiation. *Prlr*$^{-/-}$ glands fail form alveoli when transplanted into pregnant hosts and *Prlr*$^{+/-}$ glands show arrested alveolar development at day 15.5 of pregnancy (Ormandy et al., 1997a; Brisken et al., 1999). The hormonal ablation and supplementation studies taken together with the genetic studies show that PR and PRLR co-operate in early pregnancy to promote alveologenesis. However, in late pregnancy, PR activity restrains alveolar differentiation as demonstrated by the observation that declining P levels, in the presence of high PRL, trigger the lactogenic switch (Deis and Delouis, 1983).

A series of studies have indicated that PR activities during pregnancy are mediated by several paracrine factors, including Wnts (Brisken et al., 2000; Brisken et al., 2002; Mulac-Jericevic et al., 2003). Seven Wnt proteins are expressed during mammary development (Gavin and McMahon, 1992; Weber-Hall et al., 1994). However, their contributions to specific mammary developmental processes remain unclear, and whether they signal through canonical β-catenin-mediated or non-canonical pathways has not been determined. β-catenin has been strongly implicated in pregnancy-induced mammary development by both gain- and loss-of-function experiments. MMTV-ΔN89β-catenin induces precocious

development in virgin females, whereas mice expressing suppressors of β-catenin signaling, such as axin or β-eng (in which the β-catenin C-terminal domain is replaced by the *Drosophila* Engrailed repressor domain), show impaired alveolar development in pregnancy (Hsu et al., 2001; Imbert et al., 2001; Hatsell et al., 2003; Rowlands et al., 2003; Tepera et al., 2003). These phenotypes suggest that hormonal and β-catenin pathways intersect to regulate postnatal mammary development.

To explore the relationship between PR and β-catenin signaling during mammary development, we tested the ability of MMTV-ΔN89β-catenin to rescue the $PR^{-/-}$ phenotype. Our results show that stabilized β-catenin rescues alveologenesis at ductal tips in $PR^{-/-}$ mammary glands. By contrast, PR is essential for the emergence or priming of a β-catenin-responsive subset of cells along the lateral borders of mammary ducts. In the presence of PR, ΔN89β-catenin-responding cells differentiate to lactogenesis I. In the absence of PR, ΔN89β-catenin-responding cell differentiation proceeds to lactogenesis II.

MATERIALS AND METHODS

Mouse breeding and genotyping

As $PR^{-/-}$ mice are infertile and MMTV-ΔN89β-catenin mice cannot feed their pups after the third pregnancy owing to progressive alveolar hyperplasia, the following breeding strategy was taken to generate mice for analyses. MMTV-ΔN89β-catenin females (Imbert et al., 2001) on an FVB/n strain background were mated to $PR^{-/-}$ males on a 129Sv X C57BL/6 strain background (Ismail et al., 2002), and the resulting F1 progeny were intercrossed. F2 $PR^{-/-}$ males were crossed to wild-type FVB/n to generate large numbers of F3 $PR^{+/-}$ female offspring, which were crossed to $PR^{-/-}$;ΔN89β-catenin males. All analyses were performed on the resulting F4 females of this cross. Mice were genotyped by PCR for the *PR-lacZ* allele using primers lacZF (5′-CATCCACGCGCGCGTACATC-3′) and lacZR (5′-CCGAACCATCCGCTGTGGTAC-3′) and for the wild-type allele using primers P1F (5′-TAGACAGTGTCTTAGACTCGTTGTTG-3′) and P2R (5′-GATGGGCACATGGATGAAATC-3′). Mice were screened for expression of stabilized β-catenin by Southern blot analysis (Imbert et al., 2001). Axin2dEGFP and conductin-*lacZ* mice were kind gifts of Dr Walter Birchmeier (Max Delbrueck Center, Berlin, Germany) and Dr Frank Costantini (Columbia University Medical Center, New York, NY). Conductin-*lacZ* mice were generated by homologous recombination using a bacterial *lacZ* gene, with a nuclear localization signal, to replace the second exon of the conductin (*Axin2*) gene as described (Lustig et al., 2002). These mice were genotyped by PCR for the conductin-*lacZ* allele using the lacZF and lacZR primers described above.

Carmine and X-Gal staining of mammary gland wholemounts

Inguinal mammary glands were fixed in Carnoy's solution and stained in carmine alum overnight. Alternatively, glands were fixed in 4% paraformaldehyde (PFA) in PBS for 1 hour, washed three times in rinse buffer (2 mM $MgCl_2$, 0.2% sodium deoxycholate, 0.2% NP40 in PBS) and stained overnight at room temperature in 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining solution (1 mg/ml X-Gal, 2 mM $MgCl_2$, 0.2% sodium deoxycholate, 0.2% NP40, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide). X-Gal-stained glands were post-fixed for 4 hours in 4% PFA and counterstained with carmine. Glands were then dehydrated in ethanol, cleared in Citrisolv (Fisher Scientific, Suwanne, GA) and mounted in Cytoseal (VWR, West Chester, PA).

Histology and immunohistochemistry

Mammary glands were dissected, fixed in 10% phosphate-buffered formalin overnight, processed and embedded in paraffin. Histological analyses were performed on Hematoxylin and Eosin-stained sections of paraffin-embedded tissues. Alveolar luminal area was quantified by thresholding the image and computing the luminal area and the number of alveoli in each field using ImageJ software (http://rsb.info.nih.gov/ij/; National Institutes of Health, Bethesda, Maryland, USA). The graph represents an average of five fields that were examined in three $PR^{+/-}$;ΔN89β-catenin and five $PR^{-/-}$;ΔN89β-catenin mice. Immunofluorescence was performed on formalin-fixed tissues and immunohistochemistry on X-Gal-stained, PFA-fixed, paraffin-embedded tissues after antigen retrieval. Antigen retrieval was performed by microwaving sections in 6.52 mM sodium citrate (pH 6.0) for 30 minutes. Primary rabbit antibodies directed against NKCC1 (gift of Dr Jim Turner, NIH, Bethesda, MD; 1:2000), PR (A0098, DAKO, Carpinteria, CA; which recognizes both PR-A and PR-B isoforms; 1:500), casein (gift of Dr Margaret Neville, University of Colorado, Denver, CO; 1:4000) and Ki67 (Novocastra, Newcastle, UK, NCL-Ki67p; gift of Dr Susan Logan; 1:1000), and mouse antibodies recognizing the Myc epitope (9E10, 1:100; a gift of Dr Harold Varmus, Memorial Sloan Kettering, New York, NY; 1:500) and PCNA (M0879, DAKO; 1:1000) were used for these analyses. For histochemical analyses, biotinylated secondary antibodies (anti-rabbit or anti-mouse IgG, Vector Laboratories) were used in conjunction with streptavidin peroxidase (Fisher Scientific) that was colorimetrically detected using diaminobenzidine (K3466, DAKO). Alternatively, FITC-conjugated goat anti-mouse and Cy3-conjugated donkey anti-rabbit secondary antibodies (Cappel, Solon, OH; 1:100 and 1:200, respectively) were used for immunofluorescence.

Oil Red O staining

Frozen sections (10 μm) were air-dried, fixed in 3.7% formaldehyde for 10 minutes and rinsed in deionized water and 60% triethylphosphate (TEP) (Fluka Chemie, Buchs, Switzerland). A working dilution of Oil Red O (Fluka Chemie, Buchs, Switzerland) was prepared fresh each time by diluting the stock solution (5 mg/ml in 60% TEP) to 36% with deionized water. Sections were stained for 25 minutes in the working solution of Oil Red O, counterstained with Hematoxylin 2 (Richard-Allan Scientific, Kalamazoo, MI), differentiated in running tap water, mounted in Gelmount (Fisher Scientific) and photographed immediately.

RNA isolation, northern blot analysis and real-time RT-PCR

Total RNA was extracted from frozen mammary gland samples, stored in liquid nitrogen, using the TotallyRNA Isolation Kit (Ambion, Austin, TX). Total RNA (20 μg) from all samples was separated by electrophoresis on a 1% phosphate-glyoxal agarose gel using the NorthernMax-Gly Kit (Ambion). 28S rRNA was used as a loading control for the total amount of RNA in the sample. Northern blot analyses were performed using end-labeled oligonucleotide probes for β-casein (5′-GTCTCTCTTGCA-AGAGCAAGGGCC-3′), *Wap* (5′-CAACGCATGGTACCGGTGTCA-3′) and *WDNM1* (5′-CAGAGCCCAGGCAGTAGTCATTGTC-3′) (http://mammary.nih.gov/tools/markers/molecular/MMD.html) and random-prime-labeled cDNA probes (Roche, Indianapolis, IN) for α-lactalbumin (GenBank BC069916) and *K18* (*Krt18*) (Imbert et al., 2001) using the NorthernMax-Gly Kit (Ambion). All analyses were performed on a single membrane. RNA (2 μg) was reverse transcribed using Superscript III reverse transcriptase (18080-044, Invitrogen, Carlsbad, CA) and 1 μl of the cDNA thus obtained was used in the real-time PCR reaction. Real-time reverse transcriptase (RT) PCR was performed according to the instructions provided with the SYBR Green Quantitative RT-PCR Kit (Sigma Aldrich, St Louis, MO) using a Light Cycler (Roche). The following primers were used: β-casein, 5′-GCCTTGCCAGTCTTGCTAAT-3′ and 5′-GGA-ATGTTGTGGAGTGGCAG-3′; *WDNM1*, 5′-ACTGCCTGGGCTCT-GTCTAA-3′ and 5′- TCTCCTGTGCATCGTTCATC-3′; α-lactalbumin 5′-CATAGCGTGTGCCAAGAAGA-3′ and 5′-CACATGGGCTTGTAG-GCTTT-3′; *Wap*, 5′-GTAGGACCCGCAAAACTCCT-3′ and 5′-TAGAT-TCCAAGGGCAGAAGC-3′; and *28S rRNA*, 5′-AAACTCTGGTGGA-GGTCCGT-3′ and 5′-CTTACCAAAAGTGGCCCACTA-3′ (Teuliere et al., 2005; Oxelmark et al., 2006). Analyses were performed in duplicate and values were normalized to those obtained for 28S rRNA. Fold changes were calculated relative to mRNA expression levels in a 12-week-old virgin mouse.

Mammary gland transplants

Three-week-old $Rag1^{-/-}$ recipient mice were anesthetized by intraperitoneal injection of ketamine (1 mg/g body weight) and xylazine (0.5 mg/g body weight). A vertical, midline, abdominal incision was made to expose both inguinal mammary glands. The mammary fat pad was

cleared of epithelium by resecting the gland lateral to the lymph node. Each recipient received 1 mm^3 epithelial fragments either from 6- to 10-week-old donor mice ($PR^{-/-}$ and $PR^{-/-}$;ΔN89β-catenin or $PR^{+/-}$ and $PR^{+/-}$;ΔN89β-catenin) in contra-lateral cleared fat pads. The abdominal incision was closed with 4.0 Ethilon interrupted sutures (http://mammary.nih.gov/tools/mousework/index.html). Transplanted fragments were allowed to grow for 6 weeks to reconstitute the mammary ductal system. Recipient mice were then mated and sacrificed at days 8.5 and 14.5 of pregnancy. Transplanted glands were stained with X-Gal (Applichem, Cheshire, CT) to differentiate between ductal systems generated from transplanted fragments and those arising from incompletely removed endogenous epithelium. Endogenous glands from the $Rag1^{-/-}$ mice were used to determine the extent of pregnancy-induced normal alveolar development and as negative controls for X-Gal staining.

Quantitation of branching

The extent of branching in transplanted glands was analyzed by counting the number branches per 1000 pixels of ductal length in four to seven fields. Ductal length was measured using ImageJ software. Secondary ducts are smaller than primary ducts and were distinguished based on this criterion. All branches arising from secondary ducts were designated as side-branches.

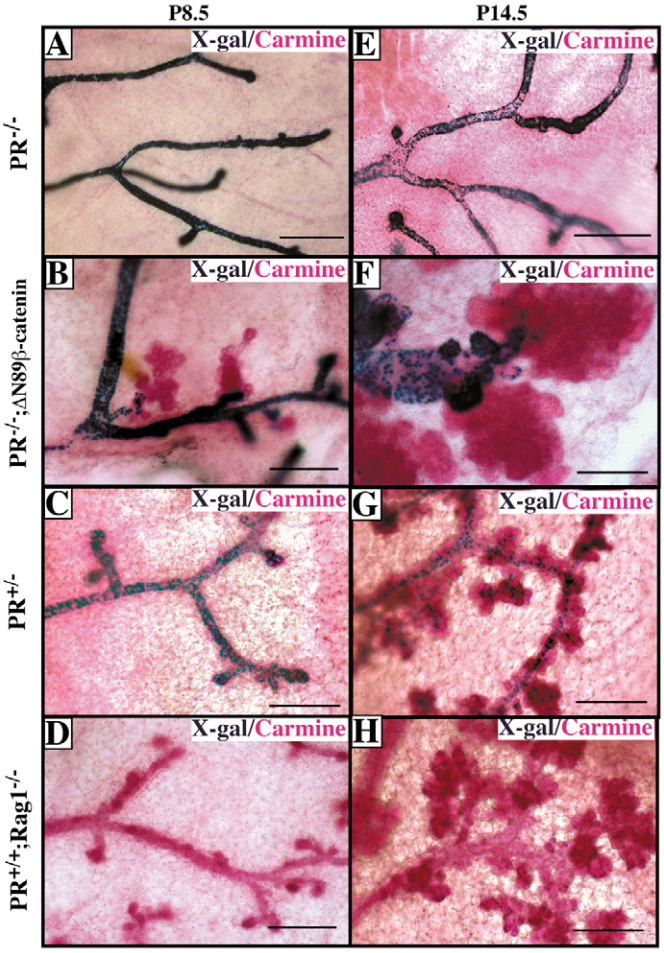
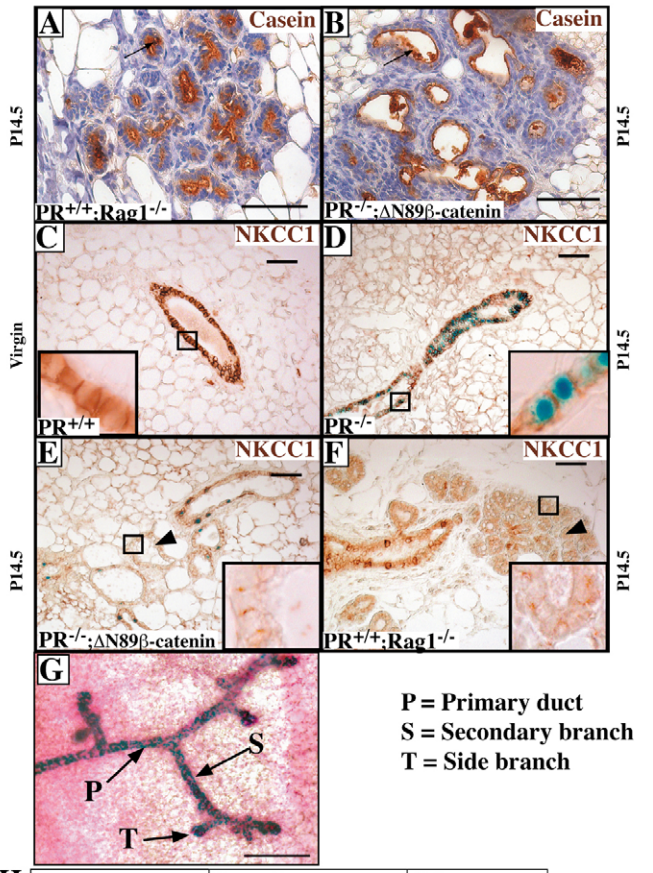


Fig. 1. ΔN89β-catenin expression rescues alveologenesis in $PR^{-/-}$ mice during pregnancy. Carmine and X-Gal-stained wholemounts of transplanted glands at 8.5 (**A-D**) and 14.5 (**E-H**) days of pregnancy. $PR^{-/-}$ glands do not develop alveoli (A,E). By contrast, alveoli are observed in $PR^{-/-}$;ΔN89β-catenin glands (B,F) that are consistently more distended than in control $PR^{+/-}$ (C,G) and endogenous $Rag1^{-/-}$ glands (D,H). Scale bars: 200 μm.

RESULTS

ΔN89β-catenin expression rescues alveologenesis but not side-branching in $PR^{-/-}$ glands

The requirement for PR in pregnancy-induced mammary development has been unequivocally demonstrated by studies in which transplanted $PR^{-/-}$ mammary glands fail to form side-branches



H

	Secondary Branches	Side Branches	
$PR^{+/-}$	2.86 ± 0.32	5.72 ± 0.90	p < 0.05
$PR^{+/-}$;ΔN89β-catenin	4.26 ± 1.40	7.97 ± 1.40	
$PR^{-/-}$	2.82 ± 0.36	1.44 ± 0.57	p > 0.05
$PR^{-/-}$;ΔN89β-catenin	4.03 ± 0.97	2.78 ± 0.97	

Fig. 2. ΔN89β-catenin expression induces alveolar development. Immunohistochemistry demonstrating expression of casein in alveoli from endogenous $Rag1^{-/-}$ (**A**) and transplanted $PR^{-/-}$;ΔN89β-catenin (**B**) glands at 14.5 days of pregnancy. Arrows point to luminal secretions containing casein. Immunohistochemical analysis demonstrating high levels of NKCC1 expression in young wild-type virgin (**C**) and transplanted $PR^{-/-}$ (**D**) glands at 14.5 days of pregnancy. Low levels of NKCC1 expression are observed in alveoli (arrowheads) generated in $PR^{-/-}$;ΔN89β-catenin (**E**) and endogenous $Rag1^{-/-}$ (**F**) glands at p14.5. Insets show higher magnifications of the boxed areas and demonstrate NKCC1 expression along the basolateral cell borders of ductal cells (insets in C,D) and absence of NKCC1 staining in alveoli (insets in E,F). (**G**) An example of the criteria used to identify primary ducts, secondary branches and side-branches. (**H**) Table of the number of secondary and side-branches observed per 1000 pixels of ductal length in $PR^{+/-}$ (n=7 fields), $PR^{+/-}$;ΔN89β-catenin (n=4 fields), $PR^{-/-}$ (n=7 fields) and $PR^{-/-}$;ΔN89β-catenin glands (n=6 fields). Differences in side-branches are statistically significant between $PR^{+/-}$ and $PR^{-/-}$ glands (P=0.008) and $PR^{+/-}$;ΔN89β-catenin and $PR^{-/-}$;ΔN89β-catenin glands (P=0.003), but not between $PR^{-/-}$ and $PR^{-/-}$;ΔN89β-catenin glands (P=0.057). Scale bars: 50 μm.

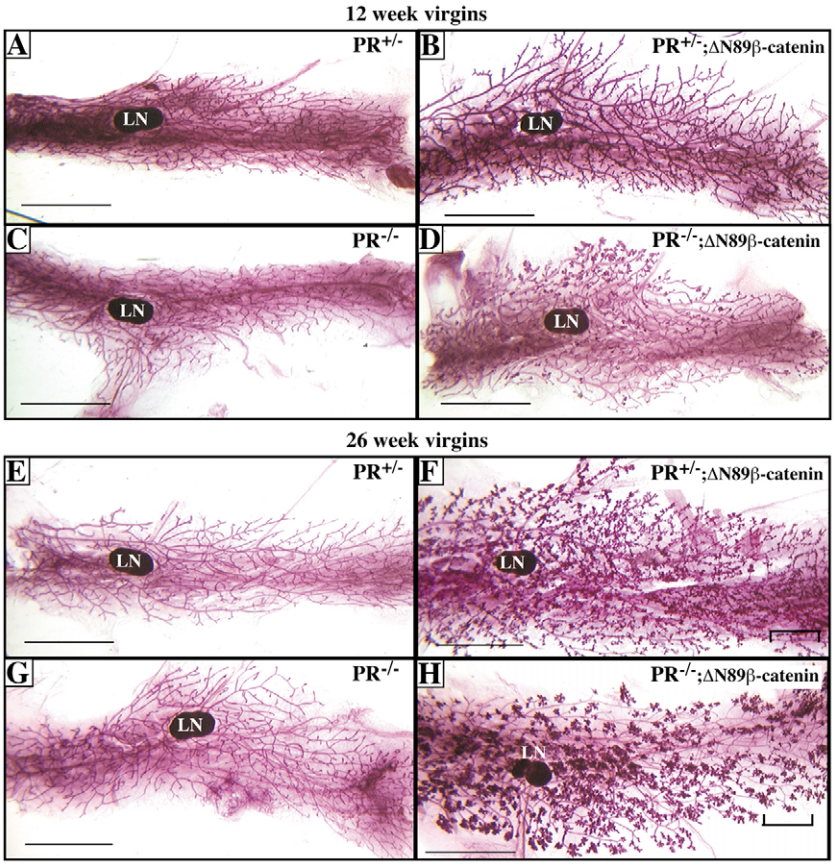


Fig. 3. Morphology of female virgin glands shows that ΔN89β-catenin induces precocious development in the presence and absence of PR. Carmine-stained wholemounts of inguinal mammary glands from 12-week-old (**A-D**) and 26-week-old (**E-H**) virgin mice. Precocious development is not observed in $PR^{+/-}$ (A,E) or $PR^{-/-}$ (C,G) glands but is seen in $PR^{+/-}$;ΔN89β-catenin (B,F) and $PR^{-/-}$;ΔN89β-catenin (D,H) glands, respectively. LN, lymph node. Brackets (F,H) indicate the regions that are magnified in Fig. 4. Scale bars: 5 mm.

or alveoli in response to E and P or to pregnancy (Lydon et al., 1995; Brisken et al., 1998). To investigate the intersections between β-catenin and PR signaling, we asked whether expression of ΔN89β-catenin could restore pregnancy-like morphogenesis to $PR^{-/-}$ glands. As $PR^{-/-}$ mice are infertile, these studies were performed in mammary glands transplanted into immunocompromised pregnant hosts.

As reported, $PR^{-/-}$ glands fail to develop in response to pregnancy (Fig. 1A,E). By contrast, rounded protrusions were observed in $PR^{-/-}$;ΔN89β-catenin glands (Fig. 1B,F) that resembled structures that formed in control transplanted $PR^{+/-}$ (Fig. 1C,G) and endogenous $Rag1^{-/-}$ glands (Fig. 1D,H) but which were consistently more distended. Wholemount analyses showed a significant increase in these structures between 8.5 and 14.5 days of pregnancy (compare Fig. 1B-D with F-H). Histological analysis identified the presence of alveolar features, such as casein expression, in these protrusions (Fig. 2A,B). To determine whether ΔN89β-catenin also rescued ductal side-branching, we analyzed the expression of the Na-K-Cl co-transporter, NKCC1, in transplanted mammary glands during pregnancy (Shillingford et al., 2003). In wild-type glands, NKCC1 is highly expressed along the basolateral membrane of ductal cells of virgin animals (Fig. 2C), but is reduced within alveoli during pregnancy (Shillingford et al., 2002). High levels of NKCC1 expression, indicative of ductal differentiation, were maintained in $PR^{-/-}$ glands (Fig. 2D). However, NKCC1 expression was reduced in the protrusions that develop in $PR^{-/-}$;ΔN89β-catenin glands (Fig. 2E) and in endogenous $Rag1^{-/-}$ alveoli (Fig. 2F). These data suggest that that ΔN89β-catenin rescues alveologenesis, but not side-branching, in $PR^{-/-}$ glands during pregnancy. Wholemounts of transplanted glands at 8.5 days of pregnancy showed no statistically significant difference in the number of secondary branches among the four genotypes of mice examined (Fig. 2G,H). Consistent with previous observations, $PR^{-/-}$ mice showed significantly fewer side-branches than $PR^{+/-}$ glands (*t*-test, P=0.008; Fig. 2H) (Lydon et al., 1995). Although on average ΔN89β-catenin glands contained more side-branches than their non-transgenic counterparts, these differences were not statistically significant (*t*-test, P=0.057).

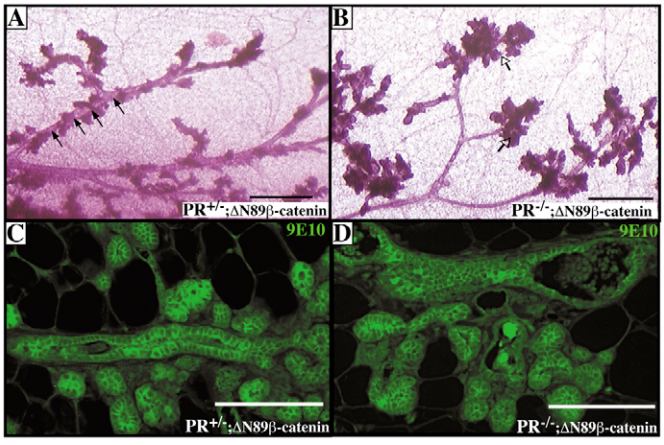


Fig. 4. Absence of PR restricts ΔN89β-catenin-induced precocious development to ductal tips. Carmine-stained wholemounts show precocious development along the lateral borders and tips of ducts in $PR^{+/-}$;ΔN89β-catenin glands (**A**) and terminally restricted precocious development in $PR^{-/-}$;ΔN89β-catenin glands (**B**). Indirect immunofluorescence for the N-terminal Myc tag epitope detected by the 9E10 antibody shows that transgene expression in all luminal cells is unaffected by *PR* genotype (**C**,**D**). Scale bars: 0.3 mm in A,B; 100 μm in C,D.

ΔN89β-catenin expression induces precocious mammary development in the absence of PR signaling

We then asked whether MMTV-ΔN89β-catenin expression could induce precocious alveolar development in virgin mice in the absence of PR. Wholemounts of virgin mammary glands from 12-week-old adolescent (Fig. 3A-D) and 26-week-old mature adult (Fig. 3E-H) female $PR^{+/-}$ and $PR^{-/-}$ mice were compared with those from mice transgenically expressing ΔN89β-catenin. Pubertal ductal extension was completed normally in virgin $PR^{+/-}$ and $PR^{-/-}$ glands and their transgenic counterparts. In adolescents, $PR^{+/-}$ (Fig. 3A) and $PR^{-/-}$ (Fig. 3C) ducts remained smooth but 5/6 $PR^{+/-}$;ΔN89β-catenin (Fig. 3B) and 2/6 $PR^{-/-}$;ΔN89β-catenin (Fig. 3D) glands showed precocious mammary development. By 26 weeks of age, $PR^{+/-}$ (Fig. 3E) and $PR^{-/-}$ (Fig. 3G) ducts remained smooth but all transgenic glands displayed precocious development regardless of PR status ($PR^{+/-}$;ΔN89β-catenin, *n*=7, Fig. 3F; and $PR^{-/-}$;ΔN89β-catenin, *n*=7, Fig. 3H). These data show that β-catenin signaling induces precocious mammary development in the absence of PR activity.

PR signaling alters the pattern of β-catenin response within the mammary gland

Further examination of wholemounts from 26-week-old virgin $PR^{+/-}$;ΔN89β-catenin and $PR^{-/-}$;ΔN89β-catenin mammary glands revealed striking differences in the pattern of development (Fig. 3F,H and Fig. 4A,B). In $PR^{+/-}$;ΔN89β-catenin glands, development occurred at regular intervals along the lateral borders of the secondary ductal branches as well as at ductal tips (Fig. 4A). By contrast, $PR^{-/-}$;ΔN89β-catenin mammary development occurred exclusively at the ends of ductal branches (Fig. 4B). Thus, a ΔN89β-catenin-responsive subset of cells exists at ductal tips regardless of PR activity, but the emergence of a second subset of ΔN89β-catenin-responsive cells along the lateral borders of ducts is strictly dependent upon PR activity within the virgin gland. As the MMTV-LTR is hormonally responsive, we examined whether PR produced these effects by altering ΔN89β-catenin expression (Shyamala and Dickson, 1976; Truss et al., 1992; Witty et al., 1995). However, indirect immunofluorescence detected Myc-tagged ΔN89β-catenin in all luminal cells regardless of PR expression (Fig. 4C,D). These data show that P/PR signaling has no effect on transgene expression but critically determines the pattern of response to ΔN89β-catenin in the adult mammary tree.

In other tissues it is known that only a subset of cells are capable of mounting a response to a uniformly expressed β-catenin transgene (DasGupta and Fuchs, 1999). To examine whether a similar phenomenon operates in the mammary gland, we investigated the expression of conductin-*lacZ*, a reporter of β-catenin signaling. Conductin (*Axin2*) is a target gene and negative-feedback regulator of the canonical Wnt signaling pathway. Expression of the conductin-*lacZ* knock-in allele provides a context-independent, reliable reporter of β-catenin signaling (Jho et al., 2002; Lustig et al., 2002; Yu et al., 2005). Analyses of $PR^{+/+}$;conductin$^{+/lacZ}$;ΔN89β-catenin mammary glands showed that, despite uniform ΔN89β-catenin transgene expression in all luminal cells (Fig. 5A,B), only a subset of cells expressed conductin-*lacZ* (Fig. 5B). Conductin-*lac*Z-positive cells were distributed at intervals along the ducts of $PR^{+/+}$;conductin$^{+/lacZ}$;ΔN89β-catenin glands (Fig. 5B,C) and were spatially distinct from cells expressing PR (Fig. 5C). Conductin-*lacZ* expression was prominent in all regions undergoing development, i.e. at ductal tips and along the ductal borders of $PR^{+/+}$;conductin$^{+/lacZ}$;ΔN89β-catenin glands (Fig. 5D). These results show that in $PR^{+/+}$ glands, the subset of cells capable of responding to β-catenin signaling is evenly patterned with respect to PR.

To determine which cell-types are proliferating in $PR^{+/+}$;conductin$^{+/lacZ}$;ΔN89β-catenin glands, we first analyzed the expression of PCNA and PR by double immunofluorescence. PR and PCNA were found in mutually exclusive expression patterns (Fig. 5E), reminiscent of previous reports in human breast and mouse mammary glands (Clarke et al., 1997; Seagroves et al., 2000). Thus, PR cells are quiescent in MMTV-ΔN89β-catenin virgin glands. By contrast, we observed that 34.9±11.3% of conductin-*lacZ*-positive cells express Ki67 (Fig. 5F,G). We conclude that the segregation between PR and proliferating cells is maintained in ΔN89β-catenin-expressing mice and that conductin-*lacZ*-positive cells are proliferating.

ΔN89β-catenin-induced alveolar differentiation is accentuated in the absence of PR signaling

β-catenin-induced structures faithfully recapitulate many features of normal alveolar development. For example, they are composed of bilayered structures (Fig. 6A,B) that contain eosinophilic secretions, and they express casein (Fig. 6C,D) and lipid droplets (Fig. 6E,F). In addition to having alveolar morphology, β-catenin-induced structures showed downregulation of *PR-lacZ* in a manner similar to that observed in pregnancy-induced alveoli (data not shown). We observed that alveoli from $PR^{-/-}$;ΔN89β-catenin glands were consistently larger than their counterparts in $PR^{+/-}$;ΔN89β-catenin glands (Fig. 6A-G), suggesting that they have progressed to a more advanced stage of lactogenic differentiation. To determine the extent to which ΔN89β-catenin induces alveolar differentiation, we analyzed milk protein gene expression by northern blotting (Fig. 7) and real-time RT-PCR (Table 1) performed on RNA isolated from virgin glands. Early milk protein genes including *WDNM1* and β-

Table 1. Relative levels of milk protein gene transcripts analyzed by RT-PCR

		WDNM1	β-casein	α-lactalbumin	*Wap*
1	12-week virgin	1±0.06	1±0.01	1±0.01	1±0.06
2	$PR^{+/-}$	1.12±0.17	17.63±6.38	0.16±0.03	0.35±0.05
3	$PR^{-/-}$	0.19±0.04	0.06±0.02	0.01±0.00	0.23±0.03
4	$PR^{+/-}$;ΔN89β-catenin	80.17±3.81	5712.87±53.98	0.56±0.02	24.68±1.76
5		16.45±2.42	3292.57±399.2	0.41±0.05	18.44±2.17
6		2.81±0.44	143.01±22.28	0.00±0.00	0.42±0.06
7	$PR^{-/-}$;ΔN89β-catenin	17.27±1.69	5976.15±470.3	3.36±0.31	167.15±14.66.
8		66.49±2.79	33689.23±601	18.44±0.77	968.76±40.64
9		1.44±0.11	319.57±17.67	0.05±0.00	1.53±0.14
10	Lactating	8.37±0.28	15339.88±543.3	23.02±0.94	2711.73±60.18

Expression of β-casein, *WDNM1*, α-lactalbumin and *Wap* transcripts in 12-week-old wild-type virgin (sample 1), 26-week-old $PR^{+/-}$ (sample 2), $PR^{-/-}$ (sample 3), $PR^{+/-}$;ΔN89β-catenin (samples 4-6), $PR^{-/-}$;ΔN89β-catenin (samples 7-9) and wild-type lactating mice. Samples 4-6 and 7-9 represent three mice of each genotype. All values represent samples analyzed in duplicate and normalized to *28S* ribosomal RNA.

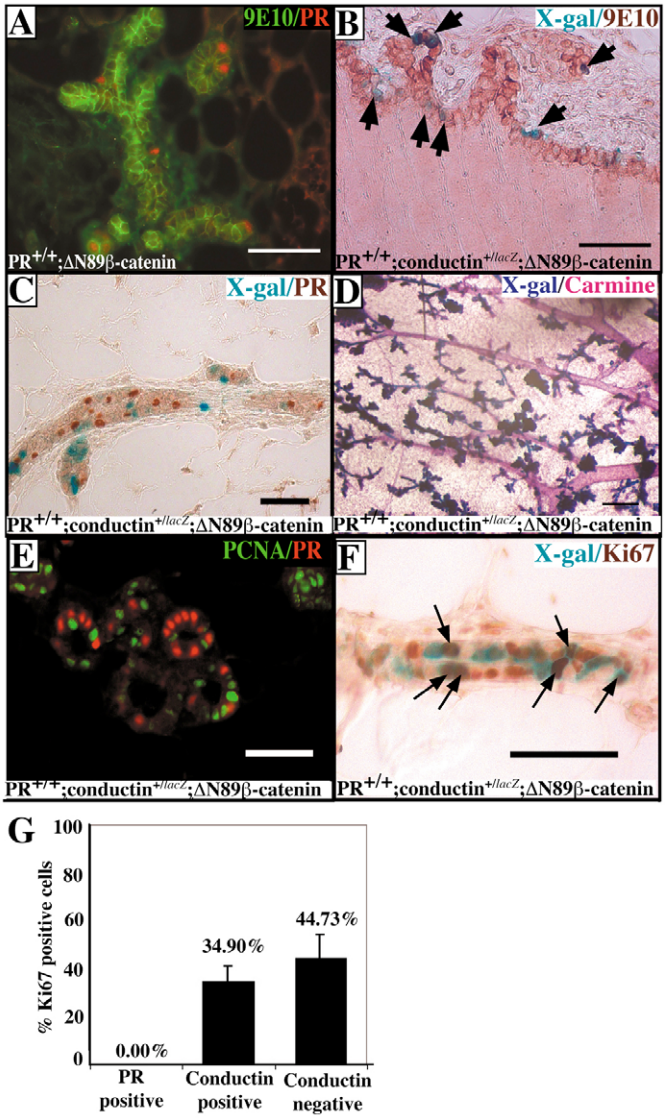


Fig. 5. β-catenin responsiveness is restricted to a subset of PR-negative mouse mammary cells, despite transgene expression in all cells. (**A**) Double immunofluorescence analysis of transgene detected by 9E10 (green) and PR expression detected by anti-PR (red) antibodies in a virgin MMTV-ΔN89β-catenin gland. (**B**) Expression of conductin-*lacZ* detected by X-Gal staining (blue, arrowheads) indicates that only a subset of ductal cells show a transcriptional response to uniform transgene expression immunohistochemically detected by 9E10 (brown) in a section of a $PR^{+/+}$;conductin$^{+/lacZ}$;ΔN89β-catenin gland. (**C**) PR immunohistochemistry on X-Gal-stained sections of $PR^{+/+}$;conductin$^{+/lacZ}$;ΔN89β-catenin glands demonstrates distinct populations of β-catenin-responsive and PR-expressing cells. (**D**) Carmine and X-Gal-stained wholemount of a $PR^{+/+}$;conductin$^{+/lacZ}$;ΔN89β-catenin gland showing conductin-*lacZ* expression at sites of alveolar development. (**E**) Indirect immunofluorescence for detection of PCNA (green) and PR (red) reveals that proliferating cells are segregated from PR-expressing cells. (**F**) Immunohistochemistry for Ki67 on $PR^{+/+}$;conductin$^{+/lacZ}$;ΔN89β-catenin gland showing proliferation of one third of conductin-*lacZ*-expressing cells (arrows). Adjacent cells that do not express conductin-*lacZ* also proliferate. (**G**) Graphical representation of the percentage of Ki67-positive cells within PR-positive, conductin-*lacZ*-positive and conductin-*lacZ*-negative populations. Scale bars: 50 μm in A-C,E,F; 0.3 mm in D.

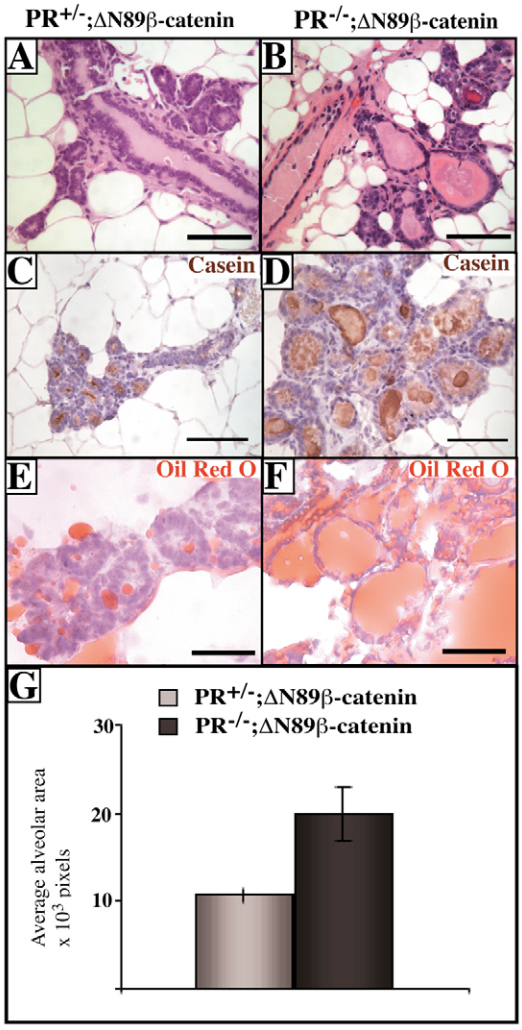


Fig. 6. A comparison of $PR^{+/-}$;ΔN89β-catenin and $PR^{-/-}$;ΔN89β-catenin glands. (**A**,**C**,**E**) $PR^{+/-}$;ΔN89β-catenin; (**B**,**D**,**F**) $PR^{-/-}$;ΔN89β-catenin. Hematoxylin and Eosin staining (A,B) shows that ΔN89β-catenin induces bilayered, secretion-filled structures. Note that alveolar lumen diameters are significantly larger in $PR^{-/-}$;ΔN89β-catenin glands. Functional alveolar differentiation is demonstrated by immunohistochemistry for casein expression (C,D) and Oil Red O staining (E,F) to detect lipid droplets in alveoli. (**G**) Average alveolar luminal area in $PR^{+/-}$;ΔN89β-catenin (gray) and $PR^{-/-}$;ΔN89β-catenin (black) glands. Scale bars: 50 μm.

casein were expressed in all $PR^{+/-}$;ΔN89β-catenin (n=3) and $PR^{-/-}$;ΔN89β-catenin (n=3) mice at levels above those detected in their non-transgenic virgin littermates. However, transcripts for late milk protein genes, including α-lactalbumin and *Wap*, which were present in barely detectable amounts in $PR^{+/-}$;ΔN89β-catenin glands (n=3), were expressed at higher levels in 2/3 $PR^{-/-}$;ΔN89β-catenin glands (Fig. 7 and Table 1). We conclude that ΔN89β-catenin-induced alveoli undergo secretory differentiation but are restrained by PR activity from undergoing the lactogenic switch.

DISCUSSION

Mammary gland development is critically dependent on hormonal signaling. Previous studies have shown that β-catenin signaling is required for alveologenesis and alveolar survival (Hsu et al., 2001; Imbert et al., 2001; Tepera et al., 2003). However, connections

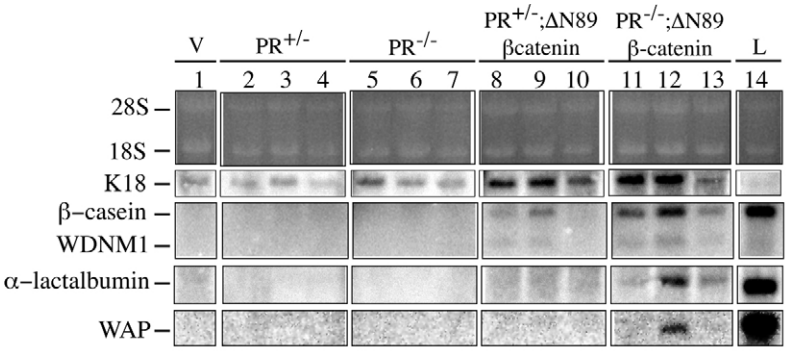


Fig. 7. Northern blot analysis of milk protein RNA levels in 26-week-old virgin female mice. 28S and 18S rRNA subunits visualized by ethidium bromide staining provided loading controls. In addition, *K18* (*Krt18*), a luminal cell marker, was used as a positive control for the integrity of the RNA and epithelial content. Three mice of each genotype were examined as indicated: $PR^{+/-}$ (lanes 2-4), $PR^{+/-}$;ΔN89β-catenin (lanes 8-10), $PR^{-/-}$;ΔN89β-catenin (lanes 11-13) and $PR^{-/-}$ (lanes 5-7). RNA from lactating (L, lane 14) and virgin (V, lane 1) mice were used as positive and negative controls, respectively.

between hormonal and β-catenin signaling have not been investigated. Here, we show that two distinct subsets of mammary cells are responsive to β-catenin. One subset, located at ductal tips, is intrinsically β-catenin-responsive. A second subset, distributed at intervals along the lateral ductal borders, requires PR signaling to become β-catenin-responsive. ΔN89β-catenin-induced alveoli commence secretory differentiation but are restrained by PR from undergoing the lactogenic switch (Fig. 8).

ΔN89β-catenin specifically restores alveologenesis to $PR^{-/-}$ mammary glands

Our results show that although luminal expression of ΔN89β-catenin specifically restores alveologenesis to ductal tips, it does not rescue side-branching in $PR^{-/-}$ mice. Side-branching and alveologenesis are abolished in $PR^{-/-}$ glands but can be restored by placing $PR^{-/-}$ cells in the proximity of $PR^{+/+}$ cells, thus indicating that PR exerts its effects by inducing paracrine factors (Brisken et al., 1998). Wnt and RANKL have been implicated as downstream paracrine effectors in these processes (Gavin and McMahon, 1992; Weber-Hall et al., 1994; Bradbury et al., 1995; Humphreys et al., 1997; Brisken et al., 2000; Fata et al., 2000; Grimm et al., 2002; Mulac-Jericevic et al., 2003). β-catenin is the central transducer of the canonical Wnt pathway and can also synergize with RANKL signaling (Fig. 8) (Fantl et al., 1999; Shtutman et al., 1999; Tetsu and McCormick, 1999; Cao et al., 2001; Lamberti et al., 2001; Rowlands et al., 2003). The observation that ΔN89β-catenin rescues alveologenesis but not side-branching in $PR^{-/-}$ mice is entirely consistent with previous findings that β-catenin signaling suppressors specifically impair alveologenesis (Hsu et al., 2001; Tepera et al., 2003). From these results, we conclude that PR induction of side-branching must be transduced through β-catenin-independent pathways in luminal cells, or, through effects on other cell types (Fig. 8). Alternatively, cell-fate choice could be determined in the mammary gland by specific β-catenin levels as is the case for other epidermal appendages, where lowering the β-catenin activity promotes sebaceous and epidermal fate and raising β-catenin signaling induces hair-cell fate (Gat et al., 1998; Niemann et al., 2002; Niemann and Watt, 2002).

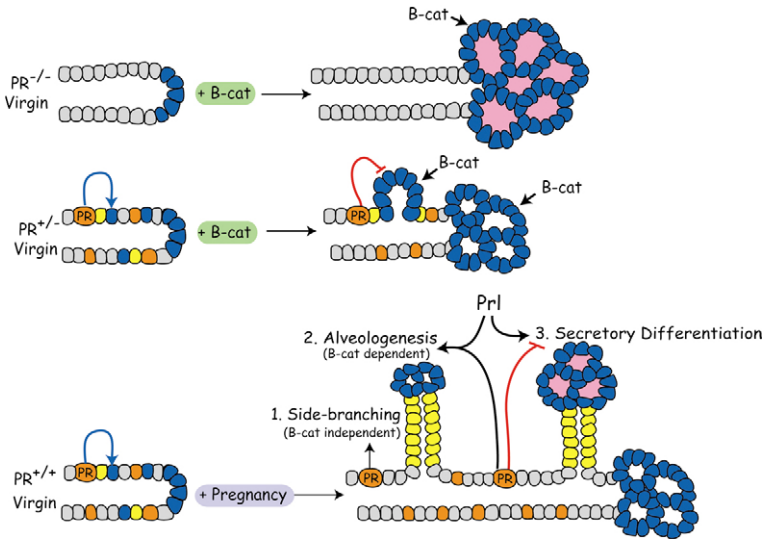


Fig. 8. Schematic of the proposed interactions between PR and β-catenin signaling. In $PR^{-/-}$ glands, cells at ductal tips are intrinsically responsive to β-catenin signaling (blue). In $PR^{+/-}$ glands, PR induces competence to respond to β-catenin signaling within a subset of alveolar progenitors along lateral borders (blue) and possibly designates other bipotent (gray) and ductal (yellow) progenitor populations. β-catenin induces alveologenesis at ductal tips of $PR^{-/-}$ mice and at tips and along lateral borders of $PR^{+/-}$ mice. It also triggers the secretory differentiation program, resulting in distended secretion-filled lumen (pink) in $PR^{-/-}$ mice. Secretory differentiation is restrained by PR. In the normal virgin mammary gland, PR induces a non-uniform *PR* expression pattern and competence to respond to β-catenin in alveolar progenitors (blue) during ductal maturation. During early pregnancy, PR-WNT4 signaling induces expansion of ductal progenitors (yellow) to form side-branches through β-catenin-independent routes. PR and PRL cooperate to induce alveologenesis, a process that is β-catenin-dependent. Later in pregnancy, PR restrains and PRL promotes alveolar differentiation. β-catenin is required for alveologenesis and may participate at multiple steps in the secretory differentiation pathway.

PR signaling is essential for β-catenin responsiveness along lateral ductal borders

The most surprising finding of the current study is that PR signaling induces dramatic changes in the pattern of β-catenin responsiveness within the virgin gland. Using conductin-*lacZ* as a reporter of β-catenin signaling, we identified a subset of luminal cells that are capable of responding to β-catenin. These proliferative responder cells are regularly distributed with respect to PR-positive cells and appear to be precursors of conductin-*lacZ*-positive precocious alveoli. Alveolar development is found along the lateral ductal borders in $PR^{+/+}$;ΔN89β-catenin and $PR^{+/-}$;ΔN89β-catenin glands but is restricted to the ductal tips in $PR^{-/-}$;ΔN89β-catenin glands. One interpretation of our findings is that PR is essential for the spatial patterning of a β-catenin-responsive cell type along the lateral ductal borders (Fig. 8). Precedence for PR mediating patterning comes from analyses of PR expression in $PR^{-/-}$ mice. $PR^{-/-}$ ducts fail to undergo the transition from a juvenile, uniform pattern of PR expression to an adult, non-uniform PR expression pattern that is seen in wild-type and $PR^{+/-}$ animals (Ismail et al., 2002; Shyamala et al., 2002). Our results suggest that PR activity plays a more global role in patterning the virgin gland during the process of ductal maturation and might regulate the positioning of multiple progenitors (bipotent, ductal and alveolar) along lateral borders. As alveoli emerge directly from lateral borders in $PR^{+/-}$;ΔN89β-catenin virgin glands (Fig. 4A), their formation is dependent upon PR activity but not upon the prior formation of a ductal side-branch.

An alternative interpretation of our data is that PR acts downstream of β-catenin. For example, temporal increases in PR activity during estrus and pregnancy could control β-catenin responsiveness by regulating the availability of a limiting factor required for β-catenin signaling (Fig. 8). At a molecular level, a number of factors are known to regulate β-catenin signaling. Regulated nuclear entry of β-catenin has been observed in *Xenopus* embryos and the β-catenin partners, Legless (LGS; BCL9)/BCL9-2 and Pygopus (PYGO), are required for this step in flies (Schneider et al., 1996; Kramps et al., 2002; Thompson et al., 2002; Hoffmans and Basler, 2004; Townsley et al., 2004; Stadeli et al., 2006). Once inside the nucleus, assembly of β-catenin transcription complexes is dependent upon the expression of DNA-binding partners, such as Tcf proteins/LEF1, which play important roles in mammary development. $Lef1^{-/-}$ mice fail to develop mammary buds and *Tcf1* and *Tcf4* are expressed in adult mammary glands (van Genderen et al., 1994; Korinek et al., 1997; Barker et al., 1999; Roose et al., 1999). Intriguingly, recent studies have shown that P primes uterine cells for β-catenin signaling by increasing Tcf/Lef and reducing GSK3β levels but E is required for nuclear entry (Rider et al., 2006).

β-catenin responsiveness of cells at ductal tips is PR-independent, as they undergo alveologenesis in $PR^{-/-}$ as well as $PR^{+/-}$ mice. We do not know why these cells are intrinsically responsive to β-catenin. It is possible that these cells are pre-existing alveolar progenitors. However, in rats it has been proposed that ductal tips contain a remnant of TEB stem cells (Russo and Russo, 1978). Previous studies have suggested that only stem cells are capable of responding to β-catenin signaling. For example, although keratin-14-ΔN87β-catenin is expressed throughout the basal epidermal layer, TOPGAL transcriptional response is restricted to epidermal and hair follicle stem cells (DasGupta and Fuchs, 1999). Similarly, despite elevation of β-catenin in all intestinal cells of Apc^{min} mice, conductin-*lacZ* expression is restricted to crypt stem cells and premalignant adenomas (Lustig et al., 2002; Maretto et al., 2003). These studies show that only stem cells and possibly early progenitors are β-catenin-responsive. Consistent with these observations, ΔN89β-catenin expression has been shown to expand the mammary stem/progenitor pool (Liu et al., 2003; Liu et al., 2004). Thus, the inherent ability of cells at ductal tips to respond to β-catenin, without prior priming by PR, is in keeping with their suggested designation as residual TEB stem cells, as proposed by Russo (Russo and Russo, 1978).

ΔN89β-catenin-induced secretory alveolar differentiation is restrained by PR

Our previous studies have shown that β-catenin stimulation results in the emergence of structures that are morphologically indistinguishable from normal alveoli (Imbert et al., 2001; Rowlands et al., 2003). Here we report that in addition to initiating alveologenesis and causing alveolar progenitor expansion and survival, β-catenin induces a significant degree of alveolar differentiation that progresses to lactogenesis II in the absence of PR (Fig. 8). It is not clear whether β-catenin initiates alveologenesis and subsequent differentiation proceeds by default, or, whether β-catenin acts at multiple steps to facilitate the differentiation process. During pregnancy, P and PRL intersect in complex ways to regulate alveologenesis, differentiation and expansion. PR and PRLR reciprocally regulate one another's expression and both pathways phosphorylate and activate STAT5A, an important mediator of alveolar development (Edery et al., 1985; Ormandy and Sutherland, 1993; Gouilleux et al., 1995; Groner and Gouilleux, 1995; Ormandy et al., 1997b; Richer et al., 1998). Moreover, both PR and PRLR increase the levels of RANKL and cyclin D1, which are essential for alveologenesis and alveolar maturation, respectively (Brisken et al., 2002; Brockman et al., 2002). However, numerous studies indicate that PRLR promotes and PR activity restrains the lactogenic switch (Neifert et al., 1981; Graham and Clarke, 1997; Ormandy et al., 1997a; Nguyen and Neville, 1998; Buser et al., 2007). For example, in the presence of a differentiated and secretion-competent mammary epithelium, P withdrawal triggers the lactogenic switch (Neville et al., 2002). Ovariectomy or treatment with the P antagonist RU486 in late pregnancy induces lactation (Nguyen et al., 2001). Decreasing P levels coincide with lactogenesis in many species and P secretion from retained placental fragments delays lactogenesis in women (Martin et al., 1978; Neifert et al., 1981). Some degree of PRLR activity, either through placental lactogen (PL) or PRL, is required for the lactogenic switch as abolishing PL, by hysterectomy, and PRL, by bromocryptine treatment, completely prevents tight-junction closure (Nguyen et al., 2001). The fact that β-catenin promotes the lactogenic switch in the absence of PR and is restrained from doing so by the presence of PR raises the possibility that β-catenin additionally functions downstream of PRL (Fig. 8). Future experiments will focus upon elucidating the relationship between β-catenin signaling and the PRL-JAK-STAT axis in secretory differentiation.

In summary, our data show that although β-catenin signaling is sufficient to induce alveologenesis at ductal tips, it is insufficient to stimulate alveolar development along lateral ductal borders. We propose that a β-catenin-independent PR activity is required to generate and/or maintain stem/progenitor cell competence along lateral ductal borders. Once rendered competent by this mechanism, a progenitor subset is poised to undergo alveologenesis in response to β-catenin signals.

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