

Targeted activation of β -catenin signaling in basal mammary epithelial cells affects mammary development and leads to hyperplasia

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Summary

Wnt/ β -catenin signaling pathway is involved in the maintenance of the progenitor cell population in the skin, intestine and other tissues, and its aberrant activation caused by stabilization of β -catenin contributes to tumorigenesis. In the mammary gland, constitutive activation of Wnt/ β -catenin signaling in luminal secretory cells results in precocious lobuloalveolar differentiation and induces adenocarcinomas, whereas the impact of this signaling pathway on the function of the second major mammary epithelial cell lineage, the basal myoepithelial cells, has not been analyzed. We have used the keratin (K) 5 promoter to target the expression of stabilized N-terminally truncated β -catenin to the basal cell layer of mouse mammary epithelium. The transgenic mice presented an abnormal mammary phenotype: precocious lateral bud formation, increased proliferation and premature differentiation of luminal epithelium in pregnancy, persistent proliferation in lactation and

accelerated involution. Precocious development in pregnancy was accompanied by increased Myc and cyclin D1 transcript levels, and a shift in p63 variant expression towards the Δ Np63 form. The expression of ECM-degrading proteinases and their inhibitors was altered in pregnancy and involution. Nulliparous transgenic females developed mammary hyperplasia that comprised undifferentiated basal (K5/14-positive, K8- and α -smooth muscle-actin-negative) cells. Multiparous mice, in addition, developed invasive basal-type carcinomas. Thus, activation of β -catenin signaling in basal mammary epithelial cells affects the entire process of mammary gland development and induces amplification of basal-type cells that lack lineage markers, presumably, a subpopulation of mammary progenitors able to give rise to tumors.

Key words: β -catenin, Progenitor cell, Mammary gland, Basal epithelial cell, Hyperplasia, Mouse

Introduction

The pseudostratified mammary epithelium comprises a luminal layer of secretory cells and a basal layer of myoepithelial cells that are responsible for milk expulsion during lactation. Owing to their specific location, the myoepithelial cells can integrate multiple signals from the luminal cells, the underlying basement membrane and the connective tissue, thereby playing a central role in the control of epithelial-stromal interactions in the mammary gland. However, apart from their contractile function, the myoepithelial cells remain poorly studied, and currently very little is known about their role in mammary development and the mechanisms that control their phenotype. Like other basal epithelial cells, mammary myoepithelial cells express basal keratins (in particular, K5 and K14) and P-cadherin, and, in addition, contractile smooth muscle (SM) proteins, such as α -SM-actin. By contrast, luminal cells express K8 and K18, which are characteristic of simple epithelia.

The origin of the two major mammary epithelial cell lineages remains poorly understood. Serial transplantation

experiments have suggested that the adult mouse mammary epithelium harbors long-lived, bipotent and lineage-restricted progenitors with a high proliferative potential (Smalley and Ashworth, 2003; Smith and Boulanger, 2003). Moreover, a technique that allows the maintenance of early mammary progenitors in vitro was recently developed (Dontu et al., 2003). However, the location of the progenitor cell population in the mammary epithelium and its phenotypic characteristics remain to be established. Ultrastructural studies revealed a candidate cell type – undifferentiated ‘pale’ or ‘light’ cells resting on the basement membrane or the suprabasal surface of myoepithelial cells (Smith and Boulanger, 2003; Smith and Medina, 1988). These cells are rare, but they are present at all stages of mammary development and are distributed throughout the mammary tree. Welm et al. have shown that the cell population expressing Sca1, a presumable progenitor cell marker, is located in the luminal layer of mammary gland (Welm et al., 2002). Similarly, the experiments performed in vitro with separated luminal and myoepithelial cells have suggested that bipotent mammary precursor cells belong to the

luminal compartment (Pechoux et al., 1999; Smalley et al., 1999). However, other studies provided evidence that luminal cells in the human and mouse mammary glands originated from progenitors with basal characteristics (Bocker et al., 2002; Jonkers et al., 2001). We recently described a mouse mammary epithelial cell line with progenitor properties (Deugnier et al., 2002a). In vitro, these cells exclusively express basal cell markers, such as P-cadherin and K5/14, whereas in vivo, when injected into the cleared mammary fat pad, they are able to self-renew and to produce differentiated progeny restricted to the luminal secretory lineage. On the contrary, a cell line displaying progenitor properties isolated by Gudjonsson et al. from human breast presents essentially luminal characteristics. In clonal cultures or when grafted subcutaneously in nude mice, these cells are able to differentiate into myoepithelium (Gudjonsson et al., 2002). These apparently contradictory data suggest that phenotypically diverse progenitor populations may exist in the mammary epithelium, either permanently or at specific developmental stages.

Numerous studies have shown that the Wnt/ β -catenin signaling pathway is involved in the maintenance of the progenitor cell population in the skin, intestine and other tissues (Alonso and Fuchs, 2003; Sancho et al., 2003). In epithelial cells, β -catenin is engaged in the formation of cadherin-containing cell-cell junctions, whereas non-junctional β -catenin is rapidly degraded by the ubiquitin-proteasome system. Activation of Wnt signaling results in stabilization of β -catenin, its translocation to the nucleus, binding to Lef/Tcf transcription factors and the transactivation of target genes, including those that encode important regulators of growth, survival and differentiation (see Wnt gene homepage at <http://www.stanford.edu/~rnusse/pathways/targets.html>). Wnt/ β -catenin signaling is involved in the regulation of cell fate during development, and its aberrant activation due to β -catenin stabilization contributes to tumorigenesis (Nelson and Nusse, 2004; Polakis, 2000).

Several members of the Wnt family are differentially expressed during mammary development (Buhler et al., 1993; Gavin and McMahon, 1992; Lane and Leder, 1997; Weber-Hall et al., 1994). However, with the exception of Wnt4, their functions have not been studied yet. Overexpression of Wnt4 in mouse mammary epithelial cells grafted into cleared mammary fat pads of virgin mice results in the formation of ramified outgrowths, mimicking the branching pattern typically seen during early pregnancy (Bradbury et al., 1995). Furthermore, Brisken et al. have reported that Wnt4 acts downstream of the progesterone receptor to induce ductal side branching during pregnancy (Brisken et al., 2000).

Expression of Wnt1 and Wnt10b in luminal epithelial cells, driven by the mouse mammary tumor virus (MMTV)-promoter, affects mammary development, and induces hyperplasia and mammary tumors (Lane and Leder, 1997; Tsukamoto et al., 1988). Similarly, activation of β -catenin signaling by stabilization of β -catenin in luminal epithelial cells results in the development of mammary tumors, including adenocarcinoma and squamous metaplasia (Imbert et al., 2001; Michaelson and Leder, 2001; Miyoshi et al., 2002a; Miyoshi et al., 2002b; Rowlands et al., 2003). Conversely, overexpression of axin, which favors β -catenin degradation in luminal cells, impairs lobuloalveolar development (Hsu et al.,

2001). The expression of a dominant-negative β -catenin/engrailed chimera in luminal cells induces apoptosis, showing that the transactivation capacity of β -catenin is essential for mammary epithelial cell survival (Tepera et al., 2003).

We used the K5 promoter to target the expression of stabilized N-terminally truncated β -catenin to the basal epithelial cell layer of the mammary gland. The resulting activation of β -catenin signaling was accompanied by precocious lobuloalveolar development in pregnancy, persistent luminal cell proliferation in lactation and accelerated involution, and led to basal-type mammary hyperplasia and invasive carcinomas.

Materials and methods

Generation of transgenic mice

cDNA coding for N-terminally truncated (lacking amino acids 1 to 57) mouse β -catenin (Δ N57- β -catenin) was HA-tagged and inserted into the blunt ended *Bam*HI site of the keratin 5 expression cassette kindly provided by Dr J. Jorcano (Ramirez et al., 1994). The resulting construct was designated K5- Δ N57- β cat (Fig. 1A).

Transgenic mice were generated by pronuclear injection of the purified K5- Δ N57- β cat construct into fertilized oocytes from C57BL/6 mice. Animals were screened for transgene integration by PCR, performed on genomic DNA isolated from tail snips. The transgene copy number was determined by Southern blotting followed by quantification of the radioactive signal using a Storm 860 phosphorimager (Molecular Dynamics) and an Image Quant program.

Whole-mount mammary gland staining, histology, BrdU-incorporation and TUNEL assays

In all cases, only mice with litters of seven or eight pups were used for analysis. For whole-mount staining, the fourth mammary glands were spread on microscope slides, fixed overnight in methacarn (60% methanol, 30% chloroform, 10% acetic acid), rehydrated and stained with carmine alum overnight. After dehydration, samples were cleared in xylene and digital images were acquired with a JVC KYF50 color video camera in a Leica MZ8 binocular using the Scion Image software.

Prior to embedding in paraffin, mammary gland specimens were fixed in methacarn or 4% paraformaldehyde in PBS and dehydrated. For histological analysis, 6 μ m sections were cut and stained with Hematoxylin and Eosin.

To assess cell proliferation, mice were injected intraperitoneally with 0.25 mg 5-Bromo-2'-deoxyUridine (BrdU)/g body weight 2 hours prior to sacrifice. BrdU incorporation was detected on sections by immunohistochemistry. After light counterstaining with Hematoxylin, 1000-1500 nuclei per sample were counted.

To detect apoptotic nuclei, paraformaldehyde-fixed paraffin sections were analyzed by TdT digoxigenin nick-end labeling with Apoptag kit (Serologicals) following manufacturer's instructions.

Digital images were acquired with a JVC KYF50 color video camera in a Nikon Optiphot-2 microscope using the Scion Image software.

Immunohistochemistry

For immunohistochemistry, the sections were incubated in 1% H₂O₂ to block endogenous peroxidase activity. To retrieve nuclear antigens on paraffin-embedded skin sections, slides with sections were incubated for 20 minutes in 10 mM sodium citrate buffer, pH 6.0 at 90°C. Furthermore, the sections were incubated for 60 minutes in 5% FCS, overnight with primary antibodies and for 2 hours at room temperature with appropriate secondary antibodies. Nuclei were stained with 1 μ g/ml DAPI (Sigma) for immunofluorescence studies,

or counterstained with Hematoxylin for immunohistochemistry. An epifluorescence Leica DMRBE microscope and a CCD Hamamatsu C5985 camera were used for image acquisition.

Antibodies

The following primary antibodies were used for immunostaining: rabbit polyclonal anti-β-catenin, anti-laminin (Sigma-Aldrich, 1/100), anti-keratin 5 (Covance, 1/2000) and anti β-casein (a gift from Dr D. Medina); rat monoclonal anti-HA tag (Roche, 1/100); and mouse monoclonal anti-β-catenin (Transduction Laboratories, 1/100), anti-BrdU (Pharmingen, 1/100), anti keratin 8 (Progen, 1/100), anti-α-SM-actin (Sigma-Aldrich, 1/100), anti-cyclin D1, anti-p63 (Pharmingen, 1/100), anti-keratin 10 (Dako, 1/100) and anti-hair keratin AE13 (a gift from Dr T. T. Sun, New York, USA; 1/20).

Alexafluor-conjugated secondary antibodies (1/1000, Molecular Probes) were used for immunofluorescence labeling. The Dako Envision + System HRP kit (Dako) was used for immunohistochemistry.

RT-PCR

Total RNA was isolated from fourth (abdominal) and third (thoracic) frozen mammary glands with RNA-plus reagent (Bioprobe Systems, Montreuil-Sous-Bois, France). Two µg of total RNA was treated with RNase-free DNase (10 U; Roche) for 10 minutes at 37°C to remove any contaminating DNA, and reverse transcribed with Mo-MuLV reverse transcriptase (200 U, Promega) primed with random hexamers (1 µg, Roche). Control reactions in which reverse transcriptase was omitted were included for each sample.

PCRs included 0.5 µl of cDNA from the reverse transcriptase reaction, 5 U Hotgoldstar (Eurogentec), 1.5 mM MgCl₂, 0.4 µM primers, 200 µM deoxy-NTP, in a total volume of 25 µl. PCR conditions were as follows: 95°C for 10 minutes (one cycle); 94°C for 30 seconds; Tm, 30 seconds; 72°C for 1 minute. PCR products were detected by electrophoresis in agarose gels and photographed using a Vilber Lourmat TFX20 system.

Quantitative PCR was performed by monitoring in real time the increase in fluorescence of the SYBR Green dye on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). The thermal cycling conditions included an initial denaturation step at 95°C for 10 minutes and 40 cycles of 95°C for 15 seconds followed by 1 minute at either 65°C for cyclin D1, or 60°C for the rest of the transcripts. The target gene transcript levels relative to the glyceraldehyde phosphate dehydrogenase (Gapd) transcript levels were calculated as 2^(Ct Gapd-Ct target gene). Two independent experiments were performed in duplicate in each case.

Gene-specific primers (Eurogentec) were designed using the Oligo 4.0 software and are listed in Table 1.

Results

Generation of transgenic mice and transgene expression in the mammary gland

The K5 promoter was used to target the expression of ΔN57-β-catenin to the basal layer of the mammary epithelium. Four founders with genomic integration of the transgene were

Table 1. Primers for PCR

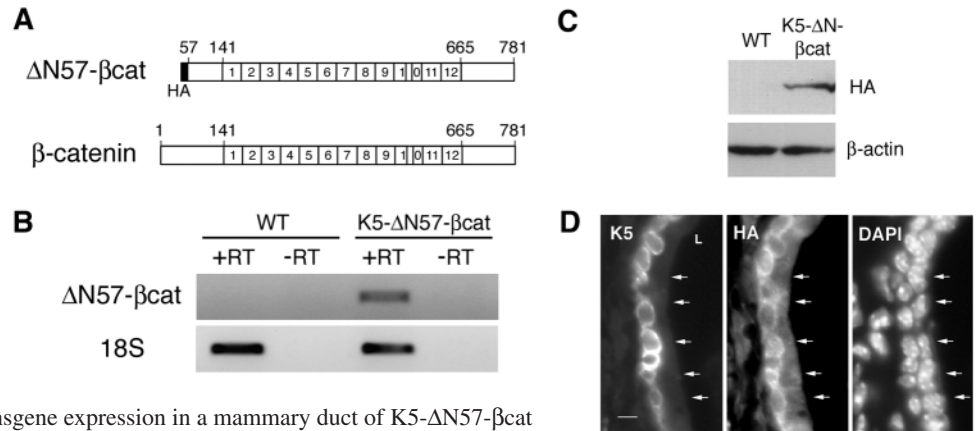
Transcript	Primers sequence	Tm used (°C)	Amplicon size (bp)
β-Casein	5'-GCCTTGCCAGTCTTGCTAAT-3' 5'-GGAATGTTGTGGAGTGGCAG-3'	60	251
WAP	5'-AAAAGCCAGCCCCATTGAGG-3' 5'-AGGGTTATCACTGGCACTGG-3'	60	294
α-Lactalbumin	5'-TCGTTCCCTTTGTTCTGGTG-3' 5'-TCGTTCCCTTTGTTCTGGTG-3'	60	377
ΔN57-β-catenin	5'-ATCCTTATGACGTGCCTGAC-3' 5'-GCCCGTCAATATCAGTACT-3'	58	126
Mmp2	5'-TGGGTGGAAATTCAGAAGGTGC-3' 5'-ATCTACTTGTCTGGACATCAGGGGG-3'	60	694
Mmp3	5'-TGTACCCAGTCTACAAGTCTCCAC-3' 5'-CTGCGAAGATCCACTGAAGAAGTAG-3'	60	658
MT1-Mmp	5'-GTGATGGATGGATAACCAATGC-3' 5'-GAACGCTGGCAGTAAAGCAGTC-3'	60	685
Timp1	5'-CTTTGCATCTCTGGCATCTGG-3' 5'-AAGTAGACAGTGTTCAGGC-3'	60	655
Timp3	5'-TGACAGGGCGCTGTATGAAGG-3' 5'-GTGGTAGCGGTAATTGAGGCC-3'	60	110
18 S rRNA	5'-TCGGAAGTGAAGCCATGATT-3' 5'-CCTCCGACTTTCGTTCTTGATT-3'	60	146
ΔNp63	5'-TGCCCAGACTCAATTTAGTGA-3' 5'-GAGGAGCCGTTCTGAATCTG-3'	60	83
TAp63	5'-CTCGTGGAAAGAAAGTTATTAC-3' 5'-GGGCTGTACTGAGCATATAG-3'	60	127
Gapd	5'-CCAATGTGTCCGTCGTGGATC-3' 5'-GTTGAAGTCGAGGAGACAAC-3'	60	149
Myc	5'-CACCAGCAGCGACTCTGAA-3' 5'-CCCGACTCCGACCTCTTG-3'	60	99
Cyclin D1	5'-CATCAAGTGTGACCCGGACTG-3' 5'-CCTCCTCCTCAGTGGCCCTTG-3'	65	116

Fig. 1. Transgene and its expression in the mouse mammary gland. (A) The β -catenin construct used for expression under the control of the K5 promoter in the basal mammary epithelial cells.

(B) RT-PCR analysis of transgene expression in 7.5-day-pregnant mouse mammary glands. 18S RNA was used as a normalization control for RT-PCR.

(C) Western blotting analysis of 7.5-day-pregnant wild-type and transgenic mouse mammary gland protein extracts performed with anti-HA-tag antibody. β -Actin served as loading control.

(D) Double immunofluorescence labeling showing transgene expression in a mammary duct of K5- Δ N57- β cat mouse. The transgene product detected with anti-HA-tag antibody is found in the basal cell layer of the mammary epithelium in correlation with the K5 expression, whereas the luminal epithelial cell layer is not labeled (arrows). DAPI was used to stain the nuclei. L, lumen. Scale bar: 10 μ m.



obtained. Two of them (#7 and #33) displayed Mendelian transmission to the F1 generation and expressed the transgene, as determined by RT-PCR (Fig. 1). Southern blotting analysis demonstrated that the transgenic mice of the established lines carried 10-20 copies of the transgene (not shown). Western blotting performed with anti-HA-tag antibody detected the transgene product in the K5- Δ N57- β cat mammary gland protein extract (Fig. 1C). Double immunofluorescence labeling revealed the transgene product in the basal mammary epithelial cell layer in correlation with the K5 expression. Luminal epithelial cells did not express transgene (Fig. 1D).

K5- Δ N57- β cat mice were viable and fertile. As expected, they presented skin lesions similar to those observed previously in mice expressing N-terminally truncated β -catenin in basal epithelial cells (Celso et al., 2004; Gat et al., 1998; Van Mater et al., 2003). Homozygous and heterozygous transgenic animals were used for further analysis.

Expression of Δ N57- β -catenin in basal mammary cells induces precocious side branching and secretory cell differentiation in pregnancy

K5- Δ N57- β cat females were able to feed normal-sized litters, suggesting that, overall, mammary differentiation was complete. However, K5- Δ N57- β cat mouse mammary glands presented several developmental abnormalities. In particular, at 7.5, 10.5 and 13.5 days of pregnancy, transgenic mouse glands contained significantly more lateral buds and short side branches than did those from wild-type animals (Fig. 2A). A BrdU incorporation assay revealed considerably more proliferating luminal epithelial cells in K5- Δ N57- β cat glands than in wild-type mammary glands on day 7.5 of pregnancy (20.5 \pm 0.2% versus 11.4 \pm 1.9%, respectively). An anti- β -casein antibody stained wild-type glands only weakly at 7.5 days of pregnancy, whereas numerous alveoli from K5- Δ N57- β cat mice were positive for β -casein at this developmental stage (Fig. 2B). Semi-quantitative analysis of milk gene expression by RT-PCR confirmed that at 7.5 days of pregnancy transgenic glands contained higher levels of β -casein transcripts than their wild-type littermates (Fig. 3). Furthermore, at 13.5 days of pregnancy, WAP and α -lactalbumin mRNA levels were higher in K5- Δ N57- β cat glands than in those from the wild-type mice (Fig. 3).

Thus, lateral bud emergence from the mammary ducts was accelerated in pregnant K5- Δ N57- β cat mice, leading to precocious side branching and premature differentiation of the secretory epithelium.

Expression of Δ N57- β -catenin in basal mammary cells leads to persistent proliferation in lactation and premature mammary involution

Whole-mount staining did not reveal any significant difference between lactating wild-type and transgenic glands. However, at peak lactation (six days postpartum), when cell division had stopped in wild-type glands, BrdU-incorporating luminal epithelial cells were still present in K5- Δ N57- β cat glands, revealing that cells were still proliferating (Fig. 2C, upper panel). Similar to wild-type animals, apoptotic nuclei were extremely rare in mammary glands from 6-day-lactating K5- Δ N57- β cat mice (Fig. 2C, lower panel). Semi-quantitative RT-PCR showed that levels of β -casein, WAP and α -lactalbumin mRNA were not altered in lactating transgenic mouse glands (Fig. 3).

To induce involution, pups were removed from their mothers after 6 days of lactation. Three days later, alveoli from wild-type mice were still full of residual milk. Transgenic mouse glands regressed faster, and in 3-day-old involuting glands, most alveoli were collapsed and contained multiple apoptotic cells, as revealed by TUNEL assay (Fig. 2D). Accordingly, at post-weaning day 3, the amount of milk gene transcripts was significantly lower in transgenic glands than in wild-type glands (Fig. 3).

Altered expression of Mmp and Timp in developing K5- Δ N57- β cat mouse mammary glands

ECM-degrading metalloproteinases (Mmp) and their inhibitors are essential for the control of mammary development (Wiseman and Werb, 2002). We therefore compared Mmp and Timp expression in mammary glands from wild-type and transgenic mouse by semi-quantitative RT-PCR. On day 7.5 of pregnancy, MT1-Mmp transcript levels were higher in transgenic glands than in wild-type glands (Fig. 4). Although Timp1 transcript levels were also elevated in transgenic mouse glands at 7.5 days of pregnancy, they were significantly lower than in wild-type animals at later time points (10.5 and 13.5

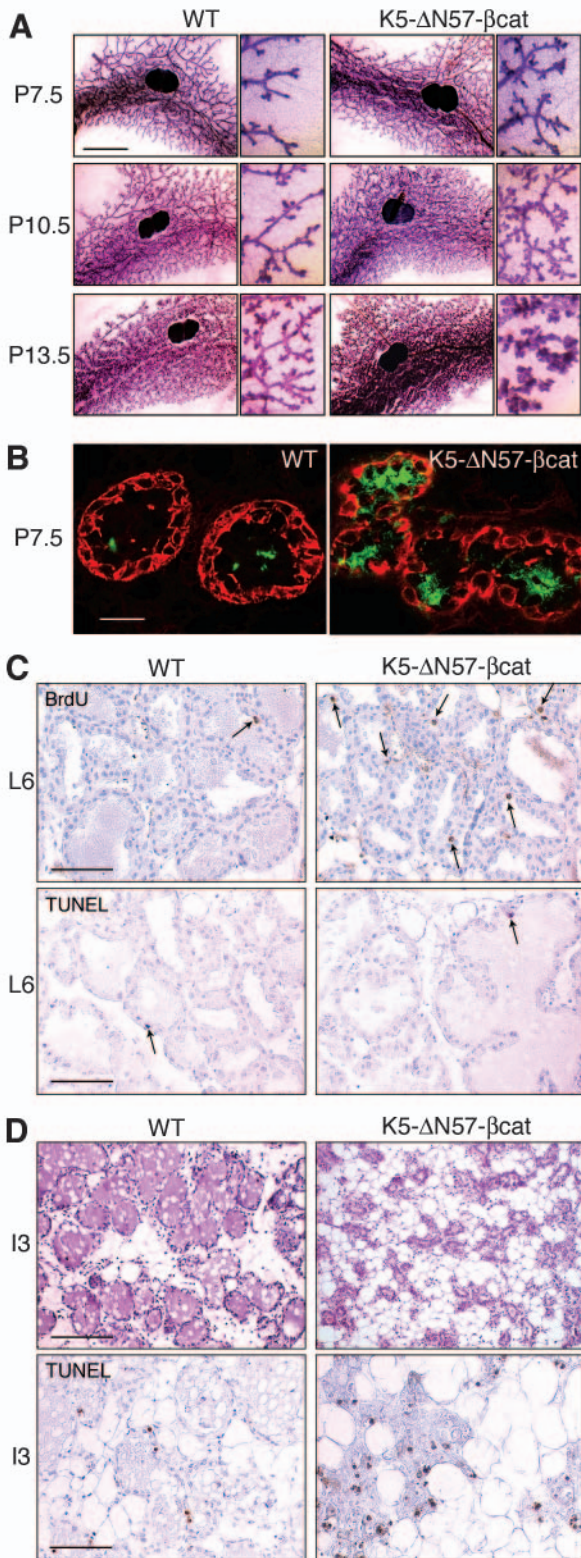


Fig. 2. Expression of $\Delta N57$ - β -catenin in basal mammary cells induces precocious development in pregnancy, persistent proliferation in lactation and accelerated involution. (A) Precocious lobuloalveolar development in pregnant K5- $\Delta N57$ - β cat mice. Whole-mount staining of mammary glands from pregnant wild-type (WT) and transgenic mice. (B) Premature secretory cell differentiation in pregnant K5- $\Delta N57$ - β cat mice. β -Casein expression in 7.5-day-pregnant mouse mammary glands. Alveoli from K5- $\Delta N57$ - β cat mice contain more β -casein than those from wild-type littermates. Immunostaining with anti- β -casein (green) and anti- α -SM-actin (red) antibodies. (C) Persistent proliferation in K5- $\Delta N57$ - β cat mouse mammary glands at peak lactation. Staining with anti-BrdU antibodies (upper panel) and TUNEL assay (lower panel). The number of BrdU-positive cells (arrows) is greater in lactating transgenic mouse mammary glands after 6 days of lactation than in wild-type animals, whereas apoptosis rates are not altered. (D) Accelerated involution in K5- $\Delta N57$ - β cat mammary glands as revealed by Hematoxylin and Eosin staining (HE, top panel) and TUNEL assay (bottom panel). Sections through 3-day-old involuting mammary glands. P7.5, P10.5 and P13.5 correspond to 7.5, 10.5 and 13.5 days of pregnancy; L6, to 6 days of lactation; I3, to 3 days of involution. Scale bars: 4 and 0.5 mm in A; 20 μ m in B; 100 μ m in C; 200 μ m in D (upper panel), 100 μ m in D (lower panel).

counterparts, whereas Timp1 levels were lower, and Mmp2, Mmp3 and Timp3 transcript levels were considerably higher (Fig. 4). These data show that activation of β -catenin signaling in basal mammary epithelial cells affects the expression of Mmps and their inhibitors during pregnancy, lactation and involution.

Increased expression levels of Myc and cyclin D1, and a shift in p63 variant expression in pregnant K5- $\Delta N57$ - β cat mouse mammary glands

Myc and cyclin D1, important regulators of epithelial cell proliferation, are target genes of the Tcf/ β -catenin pathway (He et al., 1998; Shtutman et al., 1999; Tetsu and McCormick, 1999). Using real-time RT-PCR, we found that the levels of Myc and cyclin D1 transcripts in pregnant K5- $\Delta N57$ - β cat mouse mammary glands were ~2.2- and 3-fold higher than in their wild-type littermates (Fig. 5).

p63 (Trp63 – Mouse Genome Informatics), a member of the p53 transcription factor family expressed in the basal cell layer,

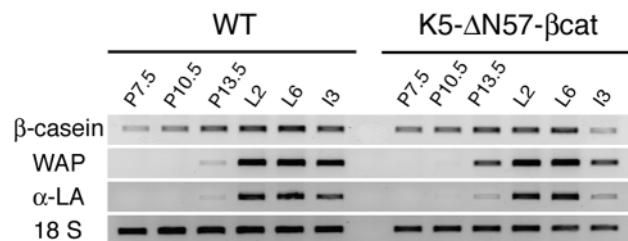


Fig. 3. Milk gene expression in wild-type (WT) and K5- $\Delta N57$ - β cat mammary glands estimated by semi-quantitative RT-PCR at different developmental stages. There are increased levels of β -casein in 7.5-day-pregnant, increased levels of WAP and α -lactalbumin (α -LA) in 13.5-day-pregnant, and an accelerated decrease of β -casein, WAP and α -LA in involuting K5- $\Delta N57$ - β cat mouse mammary glands. 18S RNA was used as a normalization control for RT-PCR. P7.5, P10.5 and P13.5 correspond to 7.5, 10.5 and 13.5 days of pregnancy; L2 and L6, to 2 and 6 days of lactation; I3, to 3 days of involution.

days) (Fig. 4). Mmp2, Mmp3, Timp2 and Timp3 transcript levels were similar in pregnant transgenic and wild-type mouse glands (not shown). No changes in Mmp/Timp expression were detected in lactating transgenic mouse glands (Fig. 4; data not shown). MT1-Mmp levels were slightly higher in 3-day-old involuting K5- $\Delta N57$ - β cat glands than in their wild-type

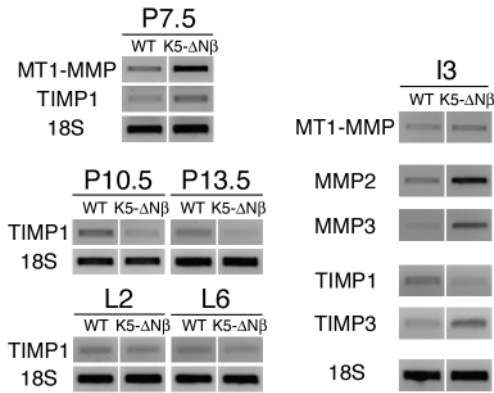


Fig. 4. Altered expression of matrix-degrading metalloproteinases and their inhibitors in K5- Δ N57- β cat mouse mammary glands. Semi-quantitative RT-PCR analysis of Mmp and Timp transcript levels in wild-type (WT) and K5- Δ N57- β cat (K5- Δ N β) mouse mammary glands. 18S RNA was used as a normalization control for RT-PCR. Samples from wild-type and K5- Δ N57- β cat glands were analyzed in the same gel. P7.5, P10.5 and P13.5 correspond to 7.5, 10.5 and 13.5 days of pregnancy; L2 and L6, to 2 and 6 days of lactation; I3, to 3 days of involution.

is thought to play an essential role in differentiation and growth control in stratified epithelia (Courtois et al., 2004; Koster et al., 2004). Two major p63 variants, TAp63, which contains a transactivation domain, and Δ Np63, which lacks this domain, are functionally distinct and, in many cases, exert opposite effects on target gene transcription (Wu et al., 2003). At 7.5 days of pregnancy, in wild-type mouse mammary glands, the amount of Δ Np63 variant mRNA was 14.7-fold higher than that of TAp63 mRNA. In K5- Δ N57- β cat glands, the difference between TAp63 and Δ Np63 transcript levels was even greater, as the amount of TAp63 transcript was 2.4-fold lower, and that of Δ Np63 transcript 2.4-fold higher, than in wild-type glands (Fig. 5).

Thus, in pregnant mice, activation of β -catenin signaling in basal mammary epithelial cells leads to the upregulation of Myc and cyclin D1 gene transcription, and a shift in p63 variant expression towards the Δ Np63 form.

Δ N57- β -catenin expression in basal mammary epithelial cells induces basal-type hyperplasia in nulliparous females and invasive carcinomas in multiparous mice

Targeted activation of β -catenin signaling in luminal epithelial cells using the MMTV-promoter has been reported to induce mammary tumors (Imbert et al., 2001; Michaelson and Leder, 2001; Miyoshi et al., 2002a; Miyoshi et al., 2002b). To determine whether expression of Δ N57- β -catenin in basal mammary cells could lead to tumor formation, we analyzed mammary glands from nulliparous and multiparous K5- Δ N57- β cat mice aged between 12 and 18 months. In 10 out of 16 transgenic virgin females, mammary gland whole-mount staining and histological analysis revealed large hyperplastic areas comprising polyp-like structures and multiple, focal and diffuse thickenings of the ductal epithelium (Fig. 6A,B). Cells in the hyperplastic areas presented a homogeneous phenotype; they expressed basal cell-type keratins K5 and K14, and were negative for K8, a

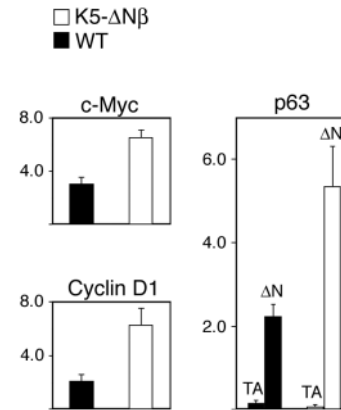


Fig. 5. Real-time RT-PCR analysis of Myc, cyclin D1 and p63 transcript levels in 7.5-day-pregnant mouse mammary glands. Myc, cyclin D1, TAp63 and Δ Np63 transcripts were quantified as described in the Materials and methods. Bars represent the mean values \pm s.e.m. for five wild-type (WT) and five K5- Δ N57- β cat (K5- Δ N β) mice normalized to the *Gapd* mRNA levels. The difference in the Myc, cyclin D1 and Δ Np63 transcript levels between transgenic and wild-type mice was statistically significant ($P < 0.0001$, 0.001 and 0.02, respectively).

luminal cell marker, and for α -SM-actin, a myoepithelial cell marker (Fig. 6D; data not shown). The anti-HA antibody detected the transgene product, Δ N57- β -catenin, in the nuclei of the basal-type (K5/K14-positive) cells in the hyperplastic ducts (Fig. 6C). The BrdU-incorporation assay detected numerous proliferating cells in the basal layer of the observed multilayered structures (not shown). Although an anti-laminin antibody detected the basement membrane around all these hyperplastic structures, the staining was irregular and discontinuous, suggesting degradation or impaired deposition of the ECM (Fig. 6F). In accordance with the basal (K5/K14) keratin expression, most of the cells found in the hyperplastic structures stained positive with an anti-p63-antibody recognizing all variants of the protein.

Lesions observed in the mammary glands of the multiparous females were histologically diverse and included non-invasive ductal hyperplasia, squamous carcinomas similar to those described earlier in the transgenic mice with targeted activation of β -catenin signaling in luminal epithelium (Miyoshi et al., 2002a; Miyoshi et al., 2002b) and invasive carcinomas (Fig. 7). The invasive carcinomas were composed essentially of K5/K14-positive cells and contained a few K8-expressing cells (Fig. 7; data not shown). These tumors were highly proliferative, as revealed by the BrdU incorporation assay (not shown), and contained numerous cyclin D1-positive cells. The nuclear location of cyclin D1 was correlated with nuclear β -catenin staining (Fig. 7). No tumors developed in K5- Δ N57- β cat male mammary rudiments.

Expression of Myc, cyclin D1, Timp3 and p63 genes in the mammary hyperplasia in K5- Δ N57- β cat mice

We used real-time RT-PCR to compare levels of Myc, cyclin D1, Timp3 and p63 variant transcripts in hyperplastic areas from 12- to 18-month-old nulliparous transgenic mouse mammary glands to those in morphologically unaltered areas from the same glands (Fig. 8). Myc transcript levels were 1.5-

to 2-fold higher in the samples from the hyperplastic areas than in the tumor-free specimens (Fig. 8A). Cyclin D1 expression was significantly elevated in one only out of four hyperplastic gland samples (Fig. 8A). Timp3 transcript levels were 1.3- to 1.8-times lower in all the specimens from the hyperplastic transgenic glands (Fig. 8A). The levels of all these transcripts detected in the tumor-free areas did not differ significantly from those found in age-matched nulliparous wild-type mouse glands (not shown).

The alteration of p63 variant expression in K5- Δ N57- β cat glands was more important and therefore, it could be assessed statistically (Fig. 8B). The TAp63 transcript levels were lower in transgenic glands than in wild-type glands (4- and 2.5-fold for tumor-free and hyperplastic areas, $P < 0.0015$ and 0.002 , respectively). On the contrary, the levels of Δ Np63 transcripts were 3.7- and 18.3-fold higher in apparently normal and hyperplastic regions of transgenic glands than in wild-type mammary tissue ($P < 0.003$ and 0.001 , respectively).

Semi-quantitative RT-PCR revealed no significant differences in the MT1-Mmp and Timp1 transcripts levels between wild-type and transgenic glands or between hyperplastic and apparently normal tissue from the transgenic glands (not shown).

Thus, mammary hyperplasia developing in K5- Δ N57- β cat mice was characterized by elevated Myc transcript levels, decreased Timp3 levels and a shift in p63 variant expression towards the Δ N-form.

Discussion

The results of this study demonstrate that activation of β -catenin signaling in basal mammary epithelial cells affects the entire process of mammary gland development, inducing precocious development in pregnancy, persistent proliferation in lactation, accelerated involution, basal-type mammary hyperplasia and invasive carcinoma comprising essentially basal cells.

Transgenic mice were able to feed normal-sized litters, proving that milk production and expulsion were not impaired. Thus, constitutive activation of β -catenin signaling did not perturb the contractile activity of the myoepithelium, and the myoepithelial cells in K5- Δ N57- β cat mice underwent

complete functional differentiation. Mammary hyperplasia comprising undifferentiated basal cells developed focally and rather late in life.

Activation of β -catenin signaling in basal cells affected the proliferation, survival and differentiation of luminal cells, suggesting crosstalk between the two mammary epithelium cell layers. Desmosomes, adherens and gap junctions may provide a means of communication between the myoepithelial

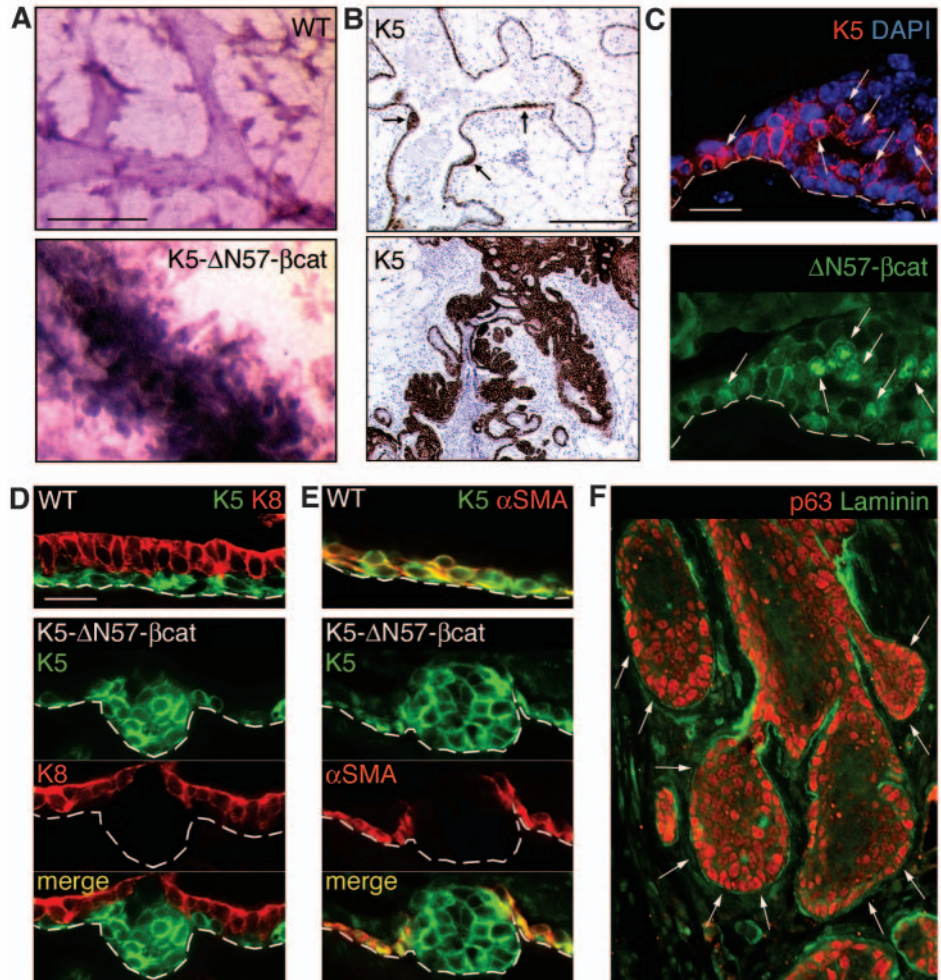


Fig. 6. Basal-type mammary hyperplasia in 12- to 18-month-old K5- Δ N57- β cat mice. (A) Whole-mount staining of 18-month-old wild-type (WT) and K5- Δ N57- β cat nulliparous mouse mammary gland fragments containing large ducts. (B) Sections through two 18-month-old K5- Δ N57- β cat nulliparous mouse mammary glands stained with anti-K5 antibody. Note focal thickenings of ductal epithelium (arrows, upper panel) and large hyperplastic areas (lower panel) containing K5-positive cells. (C) Double indirect immunofluorescence staining of a section through a 16-month-old nulliparous K5- Δ N57- β cat mouse mammary gland containing focal epithelial thickenings with anti-K5 and anti-HA-tag-antibodies revealing transgene product. There are numerous HA-positive nuclei (arrows). (D,E) Serial sections through a wild-type (WT) and a 16-month-old K5- Δ N57- β cat nulliparous mouse mammary gland containing focal epithelial thickenings double stained with anti-K5 and anti-K8 (D) or with anti-K5 and anti- α -SM-actin-antibodies (E). Cells in the hyperplastic areas are K5 positive, but do not express K8 or α -SM-actin. The broken line indicates the position of the basement membrane. (F) Double indirect immunofluorescence staining of a section through a hyperplastic area from a 12-month-old multiparous K5- Δ N57- β cat mouse mammary gland with anti-laminin and anti-p63 antibodies. The staining of the basement membrane with the anti-laminin antibody is discontinuous around hyperplastic ducts filled with p63-expressing cells (arrows). Scale bars: 0.6 mm in A; 0.2 mm in B; 30 μ m in C-E; 50 μ m in F.

and luminal compartments. However, the mechanisms underlying signal transduction from basal to luminal cells remain to be elucidated. The results of this study suggest that myoepithelial cells may influence luminal cell growth and differentiation status by modifying the composition and organization of the ECM and by affecting epithelial-stromal relationships.

Activation of β -catenin signaling in myoepithelial cells affects Mmp and Timp expression

Balanced expression of Mmp and Timp proteins is an important factor in the control of mammary development and tumorigenesis (Wiseman and Werb, 2002). Ectopic expression of Mmp3 in luminal epithelial cells results in precocious lobuloalveolar differentiation, accelerated involution and development of mammary tumors, a phenotype reminiscent of that described in this study (Sternlicht et al., 1999; Sympson et al., 1994; Witty et al., 1995). Similarly, over-expression of MT1-Mmp in luminal cells driven by the MMTV-promoter leads to hyperplasia in virgin mice and adenocarcinoma in multiparous animals (Ha et al., 2001). The expression of Timp1 in the transgenic mouse mammary glands overexpressing Mmp3 prevents excessive ECM degradation and rescues premature apoptosis in involuting glands (Alexander et al., 1996).

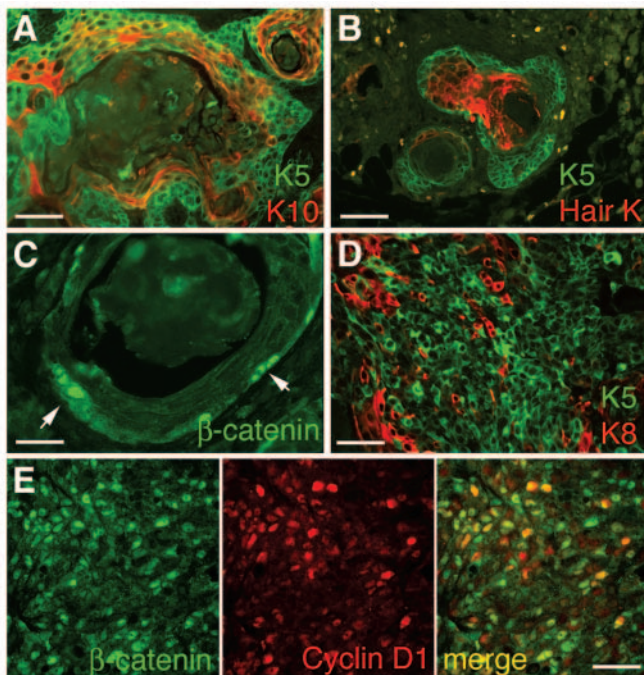


Fig. 7. Mammary lesions in multiparous K5- Δ N57- β cat mice. (A-C) Squamous metaplasia in a 12-month-old K5- Δ N57- β cat mouse mammary gland. Double immunolabeling with anti-K5 and anti-K10 antibodies (A), anti-K5 and anti hair keratin (B), or immunostaining with anti- β -catenin antibody (C). β -catenin-positive nuclei are present (C, arrows). (D) Section through an invasive mammary carcinoma from a 12-month-old K5- Δ N57- β cat mouse stained with anti-K5 and anti-K8 antibodies. Double indirect immunofluorescence. (E) Section through the same tumor stained with anti- β -catenin and anti-cyclin D1 antibodies. Numerous nuclei stain positive with both antibodies. Scale bars: 50 μ m in A,B,D,E; 30 μ m in C.

We found that the expression of Mmps and their inhibitors was altered in K5- Δ N57- β cat mice. An increase in MT1-Mmp expression early in pregnancy was followed by a decrease in Timp1 transcript levels in transgenic mouse glands. MT1-Mmp, Mmp2 and Mmp3 were expressed at higher levels in 3-day-old involuting K5- Δ N57- β cat glands than in their wild-type littermates, whereas Timp1 and Timp3 were expressed at lower levels. These perturbations in the Mmp/Timp expression balance suggest changes in ECM turnover. Resulting alterations in ECM composition and organization can affect morphogenesis, proliferation and survival of mammary epithelial cells, contributing to the abnormal mammary phenotype observed in K5- Δ N57- β cat mouse glands. Thus, myoepithelial cells playing an important role in the control of ECM turnover, and cell-ECM interactions may actively

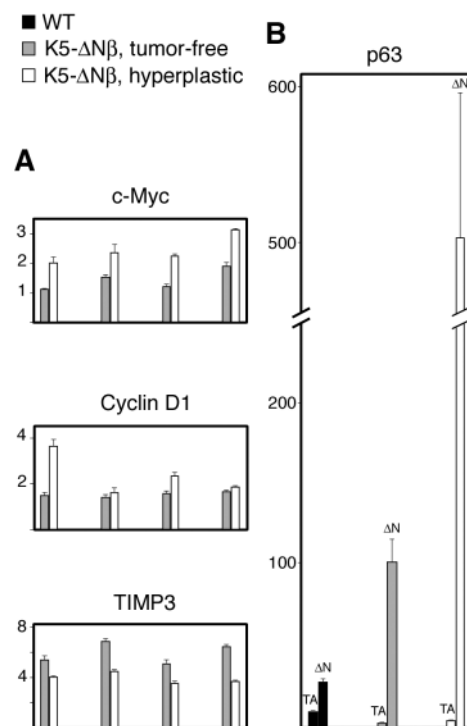


Fig. 8. Levels of Myc, cyclin D1, Timp3 and p63 transcripts in the mammary hyperplasia in K5- Δ N57- β cat mice. (A) Real-time RT-PCR analysis of levels of Myc, cyclin D1 and Timp3 transcripts in hyperplastic and tumor-free tissue samples from 12- to 18-month-old transgenic glands (K5- Δ N β). Bars represent the mean values $\times 10^{-2} \pm$ s.e.m. obtained in two independent experiments performed in duplicate. (B) Real-time RT-PCR analysis of TAp63 and Δ Np63 transcript levels in wild-type and transgenic glands. Bars represent the mean values $\times 10^{-4} \pm$ s.e.m. obtained for RNA samples isolated from hyperplastic and tumor-free areas from four 12- to 18-month-old K5- Δ N57- β cat (K5- Δ N β) and four wild-type (WT) mice. The differences in Δ Np63 transcript levels were statistically significant between wild-type and transgenic tumor-free specimens, wild-type and transgenic hyperplastic tissue, and transgenic tumor-free and transgenic hyperplastic tissue ($P < 0.003$, 0.001 and 0.002, respectively). The differences in TAp63 transcript levels were statistically significant between wild-type and transgenic tumor-free specimens, and between wild-type and transgenic hyperplastic tissue ($P < 0.0015$ and 0.002, respectively). Transcript levels were normalized to the *Gapd* mRNA.

participate in morphogenesis and tissue remodeling during mammary development.

Role of myoepithelial cells in the crosstalk between the mammary epithelium and the stroma in normal glands and during tumorigenesis

Differentiated myoepithelial cells from normal breast or benign myoepithelial lesions were suggested to be 'natural tumor suppressors' (Deugnier et al., 2002b; Lakhani and O'Hare, 2001; Sternlicht et al., 1997). They can induce growth arrest and apoptosis of tumor cells, and inhibit angiogenesis and tumor cell invasion into the stroma. However, the changes in gene expression that occur in the myoepithelial cells in malignant breast tumors can significantly modify their properties so that they enhance the proliferation, migration and invasion of the tumor (Allinen et al., 2004).

Significant changes in the p63 gene expression were observed in K5- Δ N57- β cat mouse glands. Upregulation of Δ Np63 expression was accompanied by a decrease in TAp63 transcript levels in pregnancy and, particularly, in the basal-type mammary hyperplasia observed in the 12- to 18-month-old mice. Ablation of the p63 gene in mice results in an absence of mammary glands and other epidermal appendages (Mills et al., 1999; Yang et al., 1999). However, the functions of the p63 variants, Δ N and TAp63, in mammary epithelium are not known. Δ Np63 was suggested to be associated with proliferation and appears to accumulate in some tumors (Michael and Oren, 2002; Westfall and Pietenpol, 2004).

The shift towards Δ Np63 variant expression observed in K5- Δ N57- β cat mouse glands may have important consequences on crosstalk between the epithelial and the stromal mammary gland components. Whereas TAp63 is inhibitory, Δ Np63 stimulates the expression of factors essential for angiogenesis (Senoo et al., 2002; Wu et al., 2003). Therefore, activation of β -catenin signaling in myoepithelial cells, either in response to physiological stimuli during normal development, or in cancer, may lead to a shift towards Δ Np63 expression and the stimulation of angiogenesis in the adjacent stroma. The decrease in Timp3 expression observed in basal-type mammary hyperplasia might also favor angiogenesis, as Timp3 is an angiogenesis inhibitor (Qi et al., 2003). Therefore, the role of myoepithelial cells in the control of angiogenesis during normal mammary development, as well as in mammary tumors, deserves to be investigated further.

Undifferentiated progenitors with basal characteristics: possible origin of basal-type mammary carcinoma

Transgenic mice expressing Wnt1 or N-terminally truncated β -catenin under the control of the MMTV promoter, in luminal epithelial cells, develop adenocarcinoma containing both luminal and myoepithelial cells, as estimated by cell-type-specific keratin and α -SM-actin expression (Li et al., 2003; Liu et al., 2004). These tumors contain numerous K6-positive cells, and are characterized by elevated expression of Tcf/ β -catenin target genes, cyclin D1 and Myc (Imbert et al., 2001). By contrast, mammary hyperplastic lesions observed in nulliparous K5- Δ N57- β cat mice are composed of K5/K14-positive cells lacking luminal and myoepithelial lineage markers and negative for K6. In addition, these cells express p63, a known marker of the basal cell layer in the stratified

epithelia (Yang et al., 1998). Although Myc expression is elevated in these hyperplastic areas, expression of another Tcf/ β -catenin target gene, cyclin D1, was found to be upregulated in only one out of four tumors. It has been suggested that the mammary tumors found in MMTV-Wnt1 and MMTV- Δ N- β -catenin mice originate from bipotent mammary progenitor cells in the luminal compartment (Li et al., 2003; Liu et al., 2004). Hyperplastic lesions observed in nulliparous K5- Δ N57- β cat mice contained only one cell type – basal, undifferentiated – suggesting that they originate from a different population of mammary progenitors, those possessing basal characteristics. Presumably, these progenitors are able to amplify and remain undifferentiated as long as the K5-promoter is active and Δ N- β -catenin is expressed. The Wnt/ β -catenin signaling pathway has been shown to play a central role in the control of the proliferation/differentiation switch and in tumorigenesis in humans as well as in mouse models. The amplification of undifferentiated progenitors has been reported to occur upon stimulation of this pathway in other tissues (Giles et al., 2003).

Diverse, often invasive, lesions were observed in multiparous transgenic mice. They included squamous metaplasia presenting cutaneous epithelial lineage markers, as described by others (Miyoshi et al., 2002a; Miyoshi et al., 2002b) and invasive carcinoma composed essentially of basal (K5/K14-positive) cells. Although most human breast carcinomas express phenotypic markers suggestive of a luminal origin, cDNA microarray analysis has revealed a distinct subclass of tumors that strongly express the genes encoding basal keratins, ECM proteins, integrins and other markers of basal mammary cells (Perou et al., 2000; Sorlie et al., 2003). These tumors are associated with a poor clinical outcome. Expression of the entire set of basal cell markers by this subclass of breast tumors suggests that they may originate from mammary cell progenitors with the molecular characteristics of basal cells. The K5- Δ N57- β cat transgenic mouse line provides a model with which to study the induction and progression of basal mammary carcinomas.

In conclusion, our study demonstrates that activation of β -catenin signaling in basal mammary epithelial cells (1) affects the growth, survival and differentiation of luminal cells, altering the entire process of the postnatal mammary gland development; and (2) leads to amplification of undifferentiated basal cells, resulting in the development of basal-type non-invasive and invasive mammary tumors. These data suggest that myoepithelial cells play an important role in the control of cell-ECM interactions and in crosstalk between the mammary epithelium and stroma, and indicate that the basal cell layer may harbor undifferentiated progenitors able to give rise to mammary tumors.

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