A β-catenin survival signal is required for normal lobular development in the mammary gland

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Summary

The Wnt (wingless) family of secreted glycoproteins initiates a signalling pathway implicated in the regulation of both normal mouse mammary gland development and tumorigenesis. Multiple Wnt signals ultimately converge on the multifunctional protein β-catenin to activate the transcription of target genes. Although β-catenin plays a crucial role in canonical Wnt signalling, it also functions in epithelial cell-cell adhesion at the adherens junctions. This study was designed to isolate β-catenin's signalling function from its role in adherence during mouse mammary gland development. A transgenic dominant-negative β-catenin chimera (β-eng), which retains normal protein-binding properties of wild-type β-catenin but lacks its C-terminal signalling domain, was expressed preferentially in the mammary gland. Thus, β-eng inhibits the signalling capacity of endogenous β-catenin, while preserving normal cell-cell adhesion properties. Analysis of the mammary gland in transgenic mice revealed a severe inhibition of lobuloalveolar development and a failure of the mice to nurse their young. Expression of β-eng resulted in an induction of apoptosis both in transgenic mice and in retrovirally transduced HC11 cells. Thus, endogenous β-catenin expression appears to be required to provide a survival signal in mammary epithelial cells, which can be suppressed by transgenic expression of β-eng. Comparison of the timing of transgene expression with the transgenic phenotype suggested a model in which β-catenin's survival signal is required in lobular progenitors that later differentiate into lobuloalveolar clusters. This study illustrates the importance of β-catenin signalling in mammary lobuloalveolar development.

Key words: β-catenin, Wnt, Mammary gland, Apoptosis, Lobular

Introduction

The mammary gland provides a unique model for the study of growth, differentiation, apoptosis and pattern formation involved in development. Many of the properties of growth and differentiation seen in the embryo are mimicked in the mammary gland during ductal and alveolar development, including pattern formation of the ductal tree and inductive interactions that take place between the epithelium and mesenchyme. In addition, local growth factor signalling pathways act in concert with systemic hormones to regulate proliferation and differentiation. Thus, the mammary gland offers a convenient model for studying various aspects of signal transduction and vertebrate development postnatally in an organ that is not essential for viability (analagous to the *Drosophila* eye).

Distinct developmental stages, defined primarily by morphology, but also by differential gene expression, exist in the mammary gland. At birth, the mammary gland is composed of a few rudimentary epithelial ducts surrounded by the fat pad. On sexual maturity, the ductal epithelium begins to grow out into the fat pad, creating branched tree-like ductal structures that extend to the edges of the fat pad, while maintaining extensive interductal space. Systemic hormonal changes, as well as localized gene expression associated with pregnancy, trigger additional branching and lobuloalveolar growth of the epithelium to fill in the interductal space. Lobuloalveolar development continues throughout pregnancy and lactation, at which time the fat pad is virtually filled with polarized epithelium. The lobuloalveolar clusters differentiate into milk-producing units that secrete milk proteins and lipids into the lumen during lactation. On weaning, the mammary lobuloalveolar cells undergo apoptosis during involution, returning to a state that is morphologically, but not genetically, similar to the virgin gland (reviewed by Daniel and Silberstein, 1987).

The *Wnt* (*wingless*) genes, first identified as mouse mammary oncogenes (Nusse and Varmus, 1982), encode a family of secreted glycoproteins that have been well characterized for their roles in vertebrate and invertebrate development (reviewed by Huelsken and Birchmeier, 2001). However, a complete functional study of the Wnt genes in the mammary gland has been hindered by the multiplicity of expression of numerous Wnt family members and their essential role in early embryonic development. The Wnts are involved in many aspects of vertebrate embryonic development, including axis formation in *Xenopus*, myogenesis and neural induction (reviewed by Moon et al., 1997). Germline deletion of most Wnts results in early embryonic lethality (McMahon and Bradley, 1990), so analysis of the role of specific Wnts in postnatal mammary gland

development has not been evaluated. However, a role for Wnt-4 has been suggested by rescue of Wnt-4-null mammary epithelial cells (MECs) by transplantation into the cleared fat pads of wild-type recipients (Brisken et al., 2000). In these studies, a delay in lobuloalveolar development was observed at mid-pregnancy, but by day 1 of lactation no differences were observed between the outgrowths of wild-type and Wnt-4 null MECs, presumably because of compensation by other family members. This problem of Wnt redundancy has been addressed in the current study by examining the requirement for β-catenin-mediated signalling as a convergence point for the canonical Wnt signal transduction pathway.

β-Catenin, the vertebrate orthologue of *Drosophila* Armadillo (McCrea et al., 1991; Peifer et al., 1991), is a multifunctional protein, characterized by a stretch of arm repeats that are the sites of multiple protein-protein interactions (Huber et al., 1997; Peifer et al., 1994). β-Catenin binds to E-cadherin at the adherens junctions, modulates cadherin-dependent cell-cell adhesion (Barth et al., 1997; Steinberg and McNutt, 1999) and links the cadherin/catenin complex to the cortical actin cytoskeleton through the binding of α-catenin (Herrenknecht et al., 1991; Nagafuchi and Tsukita, 1994). Additionally, β-catenin plays a crucial role in the canonical Wnt signalling cascade. The intracellular Wnt signal is propagated from the membrane through Dishevelled (Yanagawa et al., 1995) to downregulate glycogen synthase kinase-3β (GSK-3β) and subsequently disrupt a protein complex that includes GSK-3β, adenomatous polyposis coli (APC), axin, and members of the ubiquitination/proteasome pathway (Easwaran et al., 1999a; Ikeda et al., 1998; Kikuchi, 1999; Kishida et al., 1998; Rubinfeld et al., 1996; Salomon et al., 1997). Disruption of this complex prevents the GSK-3β-dependent phosphorylation of β-catenin on specific N-terminal serine and threonine residues, and thus protects β-catenin protein from degradation through ubiquitin-mediated proteolysis (Easwaran et al., 1999a; Rubinfeld et al., 1996). The stabilized β-catenin protein can then be transported to the nucleus where it forms complexes with members of the T-cell factor (TCF)/Lef family of HMG-box transcription factors (Behrens et al., 1996; Huber et al., 1996; Molenaar et al., 1996). Together, β-catenin and TCF proteins comprise a bipartite transcripton factor in which TCF supplies the DNA binding moiety and β-catenin provides the transactivation domain (reviewed by Barker et al., 2000). This complex activates the transcription of target genes and, in some cases, relieves the repression activity of TCF alone (reviewed by Bienz, 1998). Thus, β-catenin plays crucial roles in both epithelial cell-cell adhesion, as well as in signal transduction.

Additionally, β-catenin is a point of intersection and integration of several other signalling pathways. For example, the retinoic acid receptor RAR binds to β-catenin in a retinoic acid ligand-dependent manner, not only sequestering β-catenin away from Tcf/Lef and downregulating transcription of their target genes, but also potentially using β-catenin to upregulate genes responsive to retinoic acid signalling (Easwaran et al., 1999b). β-Catenin may also be a site of cross-talk with the transforming growth factor-β (TGF-β) signalling network. One mediator of the TGF-β signal, Smad4, binds to the HMG-box sequence of Lef-1 and forms a complex with β-catenin that binds promoters containing dual recognition sequences. The presence of Smad4 in this transcriptional complex is required for the transactivation of several *Xenopus* Wnt/β-catenin target genes, including *twin*, *siamois* and *nodal-related-3* (Nishita et al., 2000). Therefore, the current study analyses the signalling function of β-catenin, a diverse protein that integrates several molecular signals.

Previous characterization of β-catenin function in vivo in the mammary gland has included gain-of-function studies using either transgenic overexpression of stabilized β-catenin or stabilization of the endogenous β-catenin protein through whey acidic protein (WAP)-Cre-mediated recombination. Transgenic overexpression of β-catenin in the mammary gland results in precocious lobular development in both male and female mice (Imbert et al., 2001), lack of complete involution (Imbert et al., 2001) and mammary gland hyperplasias and adenocarcinomas (Imbert et al., 2001; Michaelson and Leder, 2001). Stabilization of the endogenous β-catenin protein in the mammary gland leads to putative dedifferentiation of the alveolar epithelium and transdifferentiation of these cells into epidermal and pilar structures, suggesting that the suppression of β-catenin signalling is required for proper differentiation into secretory epithelial cells (Miyoshi et al., 2002). Although these phenotypes from gain-of-function experiments probably result from the transactivation of target genes in the β-catenin signalling cascade, the differential contributions of β-catenin in normal lobuloalveolar development through its role in adhesion versus signalling have not been directly addressed.

Previous studies in *Xenopus* have successfully isolated β-catenin's signalling function from its role in cell-cell adhesion through the use of a dominant negative mutant, β-eng (Montross et al., 2000). In this mutant, the carboxy-terminal region of β-catenin, the region largely responsible for β-catenin's transactivation activity, has been replaced with the active repressor domain from *Drosophila* Engrailed (Jaynes and O'Farrell, 1991; Smith and Jaynes, 1996). Dorsal overexpression of β-eng in *Xenopus* embryos results in ventralization of the embryos and suppression of Wnt signalling target genes. However, β-eng associates and functions normally with members of the cadherin complex, as shown by immunoprecipation and cellular adhesion assays. Thus, β-eng successfully represses endogenous β-catenin signalling without perturbing its cell-cell adhesion function (Montross et al., 2000).

In an effort to directly analyse the role of β-catenin signalling in the developing mammary gland, β-eng was expressed as a transgene preferentially in the mouse mammary gland. Both in vivo transgenic models and in vitro cell culture experiments revealed that, in response to β-eng expression, apoptosis was induced in mammary epithelial cells and lobuloalveolar development of the mammary gland was severely compromised. Thus, these experiments have shown that β-catenin signalling provides a survival signal in mammary precursor cells that is required for normal lobuloalveolar development.

Materials and Methods

Transfection of HC11 cells

An adenovirus-based transfection method was used to introduce DNA into the HC11 cells as previously described (Allgood et al., 1997). A total of 2 μg of DNA was transfected into each 6-well dish, in various combinations of stabilized β-catenin, β-eng, E-cadherin-luciferase

reporter, RSV-β-gal and empty vector. Activity of the E-cadherin-luciferase reporter construct (kindly provided by Ronald Morton, Baylor College of Medicine, TX) was measured as an indication of β-catenin signalling activity. Luciferase reporter activity was normalized against β-galactosidase activity to control for transfection efficiency.

Transgene construction

The rat WAP promoter fragment from –949 to +1 and the WAP 5′ UTR from +1 to +33 (Li et al., 1994) was cloned into the KCR vector (kindly provided by Franco DeMayo, Baylor College of Medicine), which contains a rabbit β-globin intron and bovine growth hormone polyadenylation sequence. The β-eng mutant was excised from the pcDNA3 vector using *Pme*I and cloned into the blunted *Eco*RI site of WAP-KCR between the intronic and polyadenylation sequences (WBK construct). In a similar manner, β-eng was cloned into the mouse mammary tumour virus (MMTV)-KCR vector (kindly provided by Steven Chua in the laboratory of Sophia Tsai, Baylor College of Medicine), which contains a 2.3 kb fragment of the MMTV long terminal repeat upstream of the KCR sequences (Muller et al., 1988) (MBK construct). Both transgenic constructs were excised using *Bss*HII, purified by gel electrophoresis, and injected into the fertilized eggs of FVB mice by the Transgenic Core, Baylor College of Medicine, supervised by Franco DeMayo. Four transgenic lines were generated: MBK6322, MBK6323, WBK6414 and WBK6426.

Mammary gland morphology and histology

The use of all animals on this project was within the provisions of the Public Health Service animal welfare policy, the principles of the Guide for the Care and Use of Laboratory Animals and the policies and procedures of Baylor College of Medicine as approved by the Baylor Subcommittee for Animal Use. Mammary glands were removed at specific developmental time-points during pregnancy (days post coitus), as verified by staging the embryos. For each animal, one #4 inguinal gland was cut in half lengthwise, and each piece was spread on waxed paper and fixed in fresh 4% paraformaldehyde on ice for 2 hours. One half of each fixed #4 inguinal gland was whole-mounted and stained with hematoxylin as previously described (Williams and Daniel, 1983); the other half was paraffin-embedded and 5 μm sections were stained with hematoxylin and eosin. The remaining mammary glands were harvested and flash frozen for RNA and protein extraction. At least three animals were analysed per developmental time-point.

Immunohistochemical analysis of transgene expression was accomplished as follows: following antigen retrieval as described previously (Seagroves et al., 2000), endogenous peroxidase activity was quenched by soaking slides in 3% H_2O_2 in MeOH for 5 minutes at room temperature. Sections were then incubated overnight in Mouse on Mouse (MOM) block (Vector Laboratories). Primary monoclonal antibody raised against the myc epitope (clone 9E10) was applied to sections for 4 hours, followed by horseradish peroxidase (HRP)-conjugated goat-anti-mouse secondary antibody (Jackson Laboratories) for 1 hour. The peroxidase reaction was developed using the 3,3′-diaminobenzidine substrate in the DAB system (Vector Laboratories), and sections were counterstained with methyl green.

Proliferation and apoptosis assays

Proliferation assays were performed as described previously by monitoring the incorporation of bromodeoxyuridine (BrdU) injected 2 hours before sacrifice (Seagroves et al., 1998). Proliferating cells were quantitated as the number of FITC-labelled (i.e. BrdU-incorporated) cells out of the total DAPI-stained nuclei.

Fixed, paraffin-embedded glands were sectioned and analysed for apoptosis by immunofluorescent terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) as described previously (Humphreys et al., 1996). For both proliferation and apoptosis assays, only luminal epithelial cells were included in these counts, as this is the expected location of transgene expression (Li et al., 1994). At least 2000 cells from two to three animals were counted for each group at each time-point.

Retroviral infection and analysis of HC11 cells

Stabilized β-catenin (Montross et al., 2000) and β-eng constructs were cloned into the pS2 retroviral backbone (kindly provided by Aguilar-Cordova, Baylor College of Medicine) (Faustinella et al., 1994). 293T cells (ATCC) grown in Dulbecco's modified Eagles' medium (JRH Biosciences) supplemented with 10% fetal bovine serum (JRH Biosciences), 2 mM glutamine (Sigma), and 0.05 mg/ml gentamycin (Sigma) were used as packaging cells by transiently transfecting pS2-β-cat or pS2-β-eng with pCL-Eco construct (Imgenex Corp.). Transfection was accomplished using FuGene (Roche) according to the manufacturer's guidelines.

HC11 cells were plated on serum-coated glass coverslips (Fisher) in 100 mm tissue culture plates. Forty-eight hours after transfection, medium was collected from transfected 293T cells, filtered through 0.22 μm syringe filter, and applied to HC11 cells in a 1:1 ratio (1 plate 293T to 1 plate HC11). HC11 cells were spun at 3,000 ***g*** in a Marathon 6K clinical centrifuge (Fisher Scientific) on a swinging platform rotor for 30 minutes. Retroviral medium was removed from HC11 cells and replaced with RPMI (JRH Biosciences) supplemented with 10% fetal bovine serum, 2 mM glutamine (Sigma), 0.05 mg/ml gentamycin (Sigma), 5 μg/ml insulin (Sigma), and 0.01 μg/ml epidermal growth factor (Invitrogen). HC11 cells were grown for 48 hours after infection before harvesting. Coverslips were removed and fixed as described below, and the remaining cells on the plate were scraped into Hanks' Balanced Salt Solution (HBSS; JRH Biosciences), pelleted and flash frozen.

HC11 cells grown on coverslips and infected with pS2-β-cat or pS2-β-eng were fixed in fresh 4% paraformaldehyde for 30 minutes at 4°C, rinsed in PBS, and washed in PEM buffer (80 mM PIPES, 1 mM $MgCl_2$, and 1 mM EGTA). Cells were quenched of background autofluorescence by incubation with 1 mg/ml $NaBH_4$ in PEM buffer for 5 minutes and then permeabilized with PEM buffer supplemented with 0.2% Triton X-100 for 20 minutes. TUNEL analysis was performed by incubating cells for 45 minutes at 37°C with 1 mM ChromaTide™ Alexa Fluor® 488-5-dUTP (Molecular Probes) and components of the TdT Terminal Transferase Kit (Roche) according to manufacturer's instructions. Cells were then blocked overnight in TBS + 1% Tween supplemented with 5% dry milk and 1% bovine serum albumin (Sigma). Primary monoclonal antibody raised against the myc epitope (clone 9E10) was diluted 1:100 in blocking solution and incubated on cells for 1 hour. Secondary antibody, goat-anti-mouse conjugated to Texas Red (Molecular Probes), was diluted 1:600 in blocking solution and incubated with cells for 1 hour. Cells were then washed in TBST and mounted on slides with Vectashield containing DAPI (Vector Laboratories). The number of Alexa-488-positive cells out of total DAPI-stained nuclei was determined, and at least 10,000 cells per group were counted.

Western blotting and antibodies

HC11 cells were infected with β-catenin or β-eng, scraped and flash frozen, and western blot analysis was performed as described previously (Montross et al., 2000; Welm et al., 2002). Antibodies were used at the following dilutions: 9E10 anti-myc antibody at 1:1000, rabbit-anti-β-catenin antibody (raised against the N-terminal region of β-catenin) (McCrea et al., 1993) at 1:2,000, rabbit-anti-pAKT (Cell Signaling) at 1:500, rabbit-anti-AKT (Cell Signaling) at 1:500, goat-anti-mouse-HRP (Jackson Laboratories), and goat-anti-rabbit-HRP (Jackson Laboratories). Quantitation was performed by densitometric

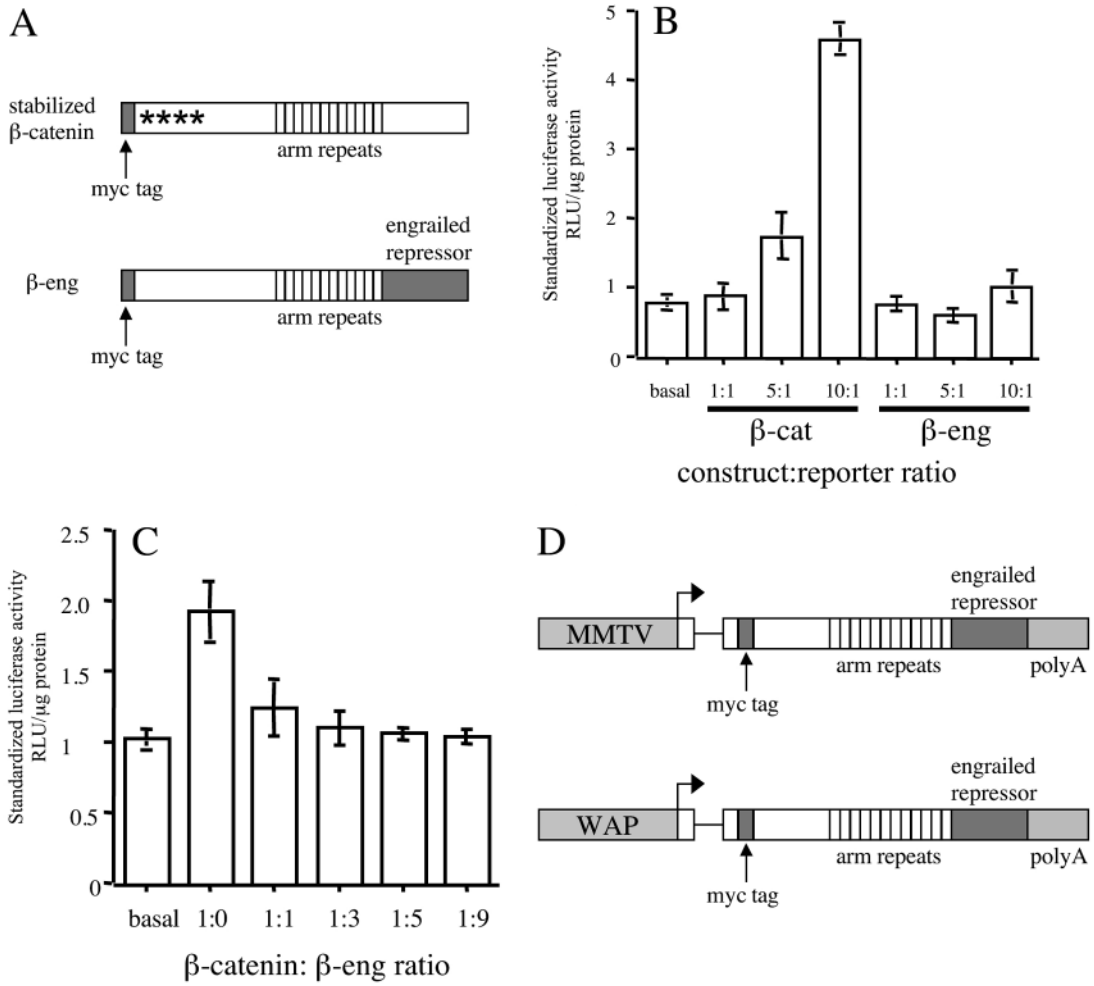


Fig. 1. β-Catenin and β-eng constructs and activity in HC11 cells. (A) Stabilized β-catenin (*, S→A, T→A mutations in the N-terminal domain) and β-eng constructs. The C-terminal domain of β-catenin is replaced with the N-terminal repressor domain of *Drosophila* Engrailed to create β-eng. These constructs were tested for β-catenin signalling activity in HC11 mammary epithelial cells (B-C). Combinations of E-cadherin-luciferase reporter, either stabilized β-catenin or β-eng, and empty plasmid DNA were mixed to equivalent amounts of DNA and transfected into HC11 cells (B). These data show that stabilized β-catenin upregulates transcription at the E-cadherin promoter, but β-eng does not. Additionally, constant amounts of the E-cadherin reporter and stabilized β-catenin were transfected into HC11 cells with increasing amounts of β-eng (C). The activity of the reporter construct shows that the β-catenin-mediated activation of E-cadherin transcription is effectively competed by β-eng in stoichometric ratios with β-catenin. The β-eng chimera was cloned into two mammary-specific transgenic expression vectors, driven by the MMTV long terminal repeat or the WAP promoter (D). Both constructs contain six tandem myc tags, an intron 5′ to the β-eng construct and a growth hormone polyA sequence.

scanning of western blots using a Molecular Dynamics densitometer with the ImageQuant software.

RT-PCR analysis of CD44 and ITF-2 mRNAs

mRNA was extracted from cells using RNAzol (Tel-Test) according to the manufacturer's instructions, and RT-PCR was performed on 500 ng RNA per reaction using the SuperScript One-Step RT-PCR kit (Invitrogen). Twenty-two cycles of PCR were performed under the following conditions: 94°C for 30 seconds, 56°C for 60 seconds, 72°C for 60 seconds. Primers sequences were as follows: CD44-2077: 5′tggatccgaattagctg, CD44-2434: 5′ggcactacaccccaatcttc, ITF2-F1: 5′ccaccccaagacccttacag, ITF2-R1: 5′gctccttgaaagcctcgttg, L19F: 5′ctgaaggtcaaagggaatgtg; L19R: 5′ggacagagtcttgatgatctc. Aliquots were removed from the PCR reaction after completion of 18, 20 and 22 cycles. Each reaction was accompanied by a counterpart reaction with no reverse transcriptase to control for genomic DNA contamination. Quantitation of PCR product bands in ethidium bromide-containing agarose gel was accomplished using Kodak 1D 3.5 USB imaging software.

Results

Although the signalling activities of stabilized β-catenin and β-eng (Fig. 1A) have been clearly shown in other species and cell types (Montross et al., 2000), experiments were designed to verify their activities in mammary epithelial cells. It has been reported that regulation of E-cadherin expression by β-catenin may occur at the transcriptional level (Huber et al., 1996). Therefore, the E-cadherin promoter driving luciferase was used as a reporter of β-catenin signalling, offering a less artificial measure of signalling activity than the previously used concatamerized TCF-binding site reporters. Various

combinations of the E-cadherin reporter construct, stabilized β-catenin and β-eng were transfected into HC11 cells. Fig. 1B shows that the stabilized β-catenin construct induces transcription at the E-cadherin promoter, but an equivalent amount of the β-eng construct did not affect promoter activity. Additionally, HC11 cells were transfected with a constant amount of the stabilized β-catenin construct and increasing amounts of β-eng. Stoichometric amounts of β-eng (compared with stabilized β-catenin) are sufficient to reduce reporter activity to basal levels (Fig. 1C). In addition, analysis of confluent HC11 cells expressing either construct using indirect immunofluoresence revealed the expected localization of stabilized β-catenin, β-eng and E-cadherin primarily at the adherens junctions (data not shown). These data, coupled with previous data in *Xenopus* (Montross et al., 2000), suggest that β-eng functions normally in cell-cell adhesion, and expression of β-eng does not affect the adhesion function of wild-type β-catenin in HC11 cells. Therefore, β-eng efficiently competes with β-catenin for signalling activity in HC11 cells and can be further used as a dominant-negative construct in mammary epithelial cells and transgenic mouse models.

Generation of transgenic mice

To study the effects of β-catenin signalling specifically on mammary gland development, transgenic mice were generated expressing β-eng under two mammary-specific promoters (Fig. 1D). The β-eng construct is composed of the amino-terminal region and armadillo repeats of *Xenopus* β-catenin fused to the active repressor domain of *Drosophila* Engrailed (Montross et al., 2000). This dominant negative β-catenin construct was cloned into mammary-specific expression vectors containing the MMTV long terminal repeat or the WAP promoter (Campbell et al., 1984; Li et al., 1994), both of which have been used extensively to drive mammary-specific transgene expression during pregnancy and lactation (Li et al., 1994; Ma et al., 1999; Muller et al., 1990; Muller et al., 1988; Zahnow et al., 2001). Both constructs included an intron isolated from the rabbit β-globin gene cloned 5′ to the β-eng insert and a polyA sequence isolated from bovine growth hormone. Previous experiments indicated the necessity of placing the globin intron 5′ to the large (3.3 kb) cDNA insert, presumably to ensure that the cDNA was recognized as a terminal exon to facilitate transgene expression (data not shown). Thus, these transgenic constructs were designed to express the β-eng mutant preferentially in the mammary gland.

The MMTV-β-eng (MBK) and WAP-β-eng (WBK) transgene constructs were microinjected into embryos, and five independent founder lines were identified by PCR screening of genomic DNA (data not shown). One of the five lines did not transmit the transgene to its progeny, so the remaining four lines were characterized for expression and phenotype. Two of these four lines expressed β-eng under the MMTV-LTR, whereas two carried the WAP-driven transgene. After morphological characterization of these four lines, the conclusion was drawn that all four lines displayed the phenotype described below, regardless of the promoter driving expression or insertion site of the transgene. Therefore, for simplicity, further description of these mice will refer to transgenic or wild type, regardless of the transgenic line.

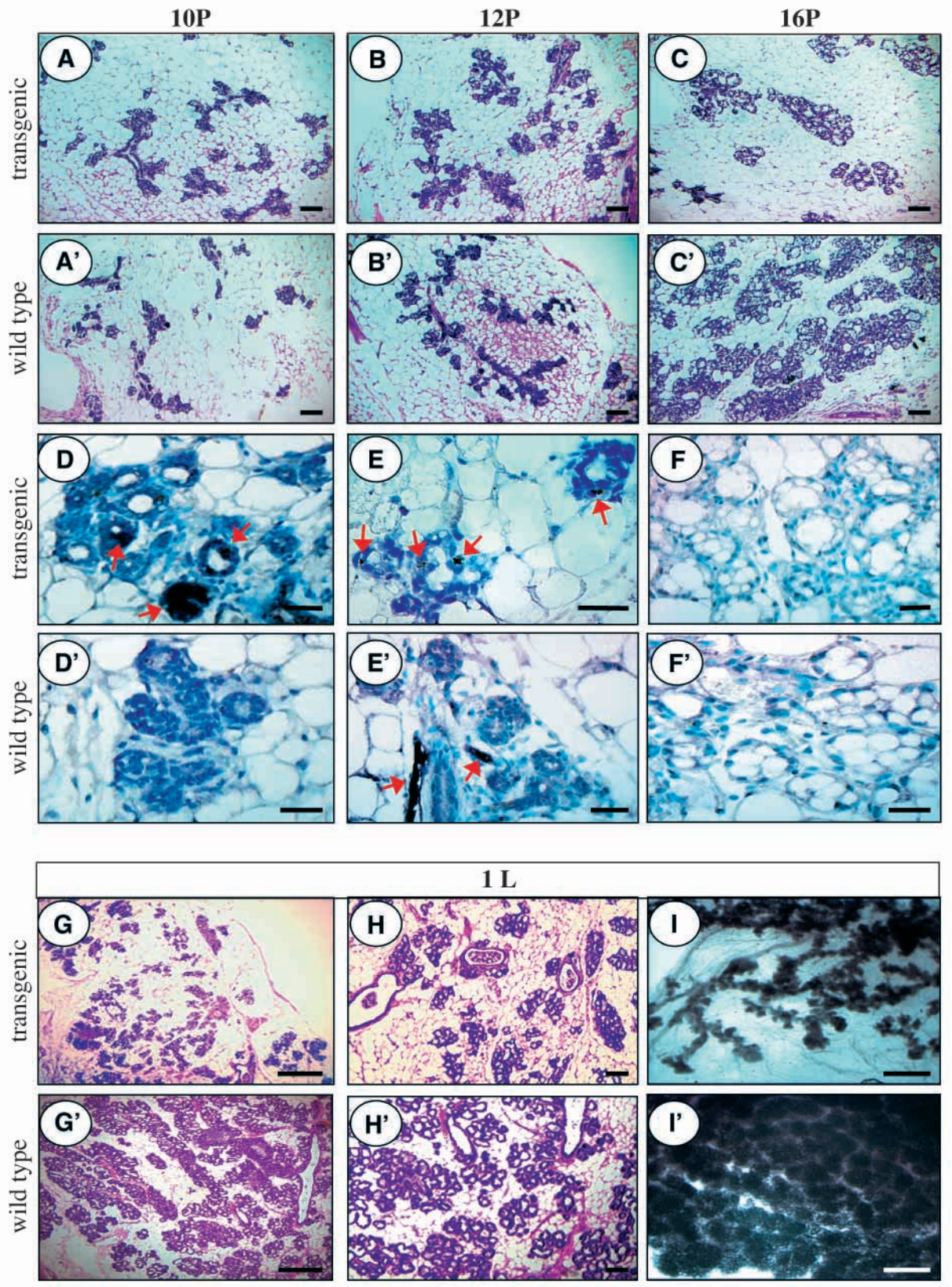
Decreased lobuloalveolar development in β-eng transgenic mammary glands

Mammary gland morphology was analysed in detail in the transgenic and wild-type mice during mid-pregnancy and lactation and compared with transgene expression (Fig. 2). Although the overall reduction in lobuloalveolar development was similar in all four MMTV- and WAP-driven transgenic lines, the extensive characterization of developmental expression pattern and phenotype in the two WAP-driven transgenic lines is described. At day 10 of pregnancy (10P) (Fig. 2A,A') and 12P (Fig. 2B,B'), transgenic mammary epithelium (Fig. 2A,B) was morphologically indistinguishable from wild-type littermates (Fig. 2A',B'). By 16P (Fig. 2C,C'), a reduction in lobuloalveolar clusters could be detected in transgenic glands (Fig. 2C) compared with wild-type littermates (Fig. 2C').

Expression of the WAP-driven transgene is expected to markedly increase, beginning at around 10P with the increase in lactogenic hormones and extending through day 10 of lactation (Bayna and Rosen, 1990; Dale et al., 1992). Using an antibody against the myc-epitope tag in the transgenic construct, WAP-driven-β-eng expression was analysed at 10P (Fig. 2D,D'), 12P (Fig. 2E,E') and 16P (Fig. 2F,F'). β-eng transgene expression was detected at 10P (Fig. 2D) in lobular epithelium of the transgenic mice, but not in wild-type littermates (Fig. 2D'). In addition, expression was nonuniform, similar to the pattern of other transgenes driven from the same promoter (Li et al., 1994); some lobular clusters contained a few expressing cells, whereas some clusters failed to express the transgene. Overall, it was estimated that less than 50% of the lobular epithelial cells expressed the transgene at 10P. At 12P, transgene expression could be detected in transgenic mice (Fig. 2E), but only in a punctate pattern associated with fragmented, apoptotic bodies that had largely been cleared from the gland. No such antibody-reactive cellular debris was detected in wild-type littermates (Fig. 2E'), as the only signal detected was an artefact, resulting from antibody trapping in blood vessels (arrows). By 16P, no transgene expression could be detected in the mammary glands of transgenic (Fig. 2F) or wild-type mice (Fig. 2F').

At day 1 of lactation (Fig. 2G-L (transgenic), Fig. G'-L' (wild type)), the reduction in overall epithelial content was marked in all four transgenic lines, as illustrated at low magnification (Fig. 2G,G'), higher magnification (Fig. 2H,H') and strikingly by whole-mount hematoxylin staining (Fig. 2I,I'). Higher magnification (Fig. 2H,H') showed that the existing epithelium appeared morphologically normal, with organized, albeit fewer, alveolar clusters. Transgenic females from all four lines also failed to support their litters; all pups died within 12 hours after birth with no milk in their stomachs, but survived and developed normally when fostered by a wild-type female (data not shown) Additionally, multiple rounds of pregnancy failed to rescue this phenotype, as transgenic females continued to be unable to support a litter (data not shown). Thus, expression of β-eng in the mammary gland during pregnancy markedly inhibited lobuloalveolar development, such that insufficient milk was produced to nurture the offspring.

Interestingly, transgene expression is detected for only a small window of time (10P-12P), during which the morphology of the gland appears normal. By day 16 of



pregnancy, there appears a distinguishable reduction in the overall amount of epithelium in the transgenic gland (Fig. 2C,C'), and higher magnification of the 16P transgenic gland reveals that persisting epithelium appears morphologically normal, with properly organized alveolar clusters, yet does not express the transgene (Fig. 2F,F'). The lower magnification images shown in Fig. 2G-I compared with their wild-type littermates shown in Fig. 2G'-I' illustrate that this overall

Fig. 2. Morphology of the mammary gland and transgenic expression. Mammary glands from transgenic mice (A-I) and wild-type littermates (A'-I') isolated at day 10 (A,A',D,D'), day 13 (B,B',E,E') and day 16 of pregnancy (C,C',F,F'), and day 1 of lactation (G-I,G'-I') from lines WBK6414 and WBK6426. Haematoxylin and eosin staining of tissue sections (A-C,A'-C', G,H,G'H') and whole-mount haematoxylin staining (I,I') revealed reduced lobuloalveolar development of the mammary glands of transgenic mice compared with wild-type littermates. Immunohistochemistry using an antibody against the myc epitope tag in the transgene construct (antibody signal shown in black) (D-F, D'-F') reveals transgene expression at 10P (D, arrows) and 12P (E, arrows), but not at 16P (F). Note fragmented, apoptotic cells associated with transgene expression at 12P (E, arrows). Nontransgenic littermates (D'-F') show no specific antibody signal, and arrows in E' illustrate nonspecific antibody trapping in blood vessels. Bars, 25 μm (D-F,D'-F'); 100 μm (A-C,A'-C',H,H'); 500 μm (G,G',I,I').

reduction in epithelium is amplified through lactation. This unusual temporal relationship of the expected transgene expression (10P through lactation), detected transgene expression (10-12P) and morphological phenotype (16P-lactation) will be discussed later in the manuscript.

Reduced proliferation and increased apoptosis in β-eng transgenic mice

In an effort to understand the factors contributing to the lack of lobular structures, proliferation and apoptosis were analysed in the mammary glands from transgenic mice and their paired wild-type littermates (Fig. 3) during the time of early transgene expression. Bromodeoxyuridine (BrdU) incorporation was analysed as a measure of MECs entering S phase in wild-type (Fig. 3A) and transgenic (Fig. 3B) mice at days 10-13 of pregnancy. Mid-pregnancy is a time of extensive proliferation in the normal mammary epithelium, and levels of proliferation were decreased approximately twofold in the MECs of β-eng transgenic compared with wild-type mice during this time of development (Fig. 3C). Apoptosis during this time of extensive growth and differentiation of the mammary gland is usually barely detectable. TUNEL analysis of wild-type (Fig. 3D) and transgenic (Fig. 3E) epithelium revealed approximately a fivefold increase in apoptosis in the transgenic animals (Fig. 3F). The cells undergoing apoptosis were localized to specific lobuloalveolar clusters (Fig. 3E), rather than distributed sporadically around the gland, and these clusters correlated with regions of transgene expression (data not shown). These data suggest that the lack of lobular epithelium observed later in development resulted from a small (twofold) decrease in proliferation, as well as a marked (fivefold) increase in apoptosis during mid-pregnancy in the transgenic mice.

As mentioned previously, transgenes driven by the WAP promoter are expected to show increased expression, beginning at around 10P and continuing throughout pregnancy and early lactation. However, transgene expression was only detected from 10-12P in β-eng transgenic mice, at which time the transgene-expressing cells underwent programmed cell death, and the transgene could no longer be detected at later stages of development in the surviving mammary epithelium. This brief window of expression in fewer than 50% of epithelial cells appeared to result in almost immediate apoptosis, which posed a significant challenge for further studies to characterize the mechanisms responsible for these effects. Therefore, a MEC culture model derived from mid-pregnant mice was selected that could be used to further elucidate the role of β-catenin signalling in the mammary gland.

HC11 cells are derived from normal, mid-pregnant MECs (Ball et al., 1988), and, unlike many other MEC lines, they maintain a somewhat 'normal' epithelial phenotype. HC11 cells can be induced with lactogenic hormones to express β-casein. In addition, they occasionally form limited alveolar-like structures when transplanted back into the cleared fat pad, and when grown at confluence, they display clear E-cadherin staining around the cell periphery (Humphreys and Rosen, 1997). Therefore, HC11 cells were selected as an in vitro model system in which to study the effects of β-eng signalling in the mammary gland.

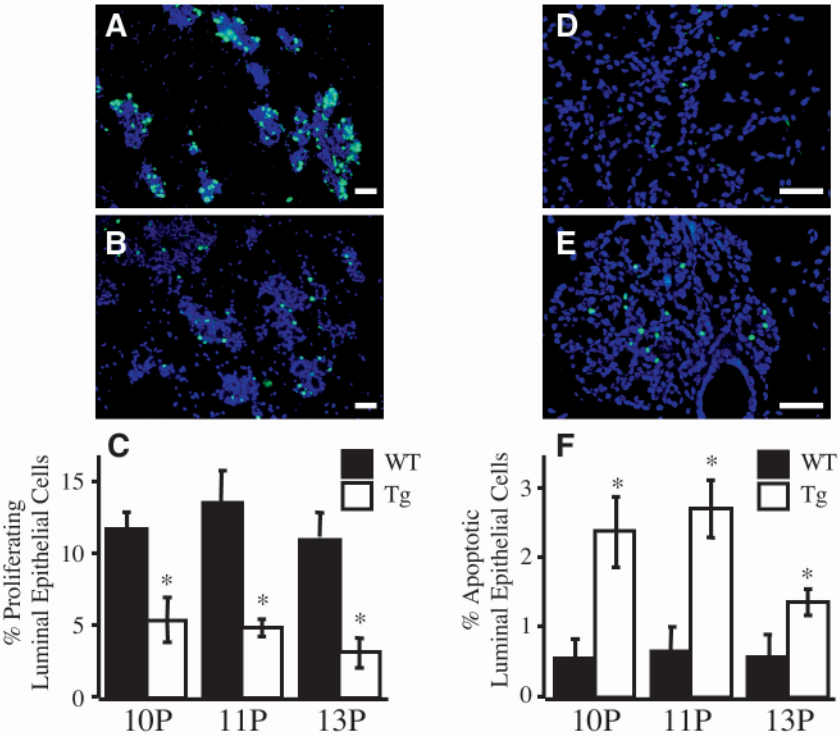


Fig. 3. Proliferation and apoptosis in MECs of β-eng transgenic mice. Immunofluorescent detection of BrdU incorporation in mammary epithelium of wild-type (A) and transgenic (B) mice revealed decreased proliferation in β-eng transgenics (C). Fluorescent TUNEL assay revealed increased apoptosis in MECs from transgenic (E) compared with wild type (D). BrdU and TUNEL signals are represented in green. Nuclei are visualized by DAPI stain (blue). Bars, 100 μm. *P<0.001.

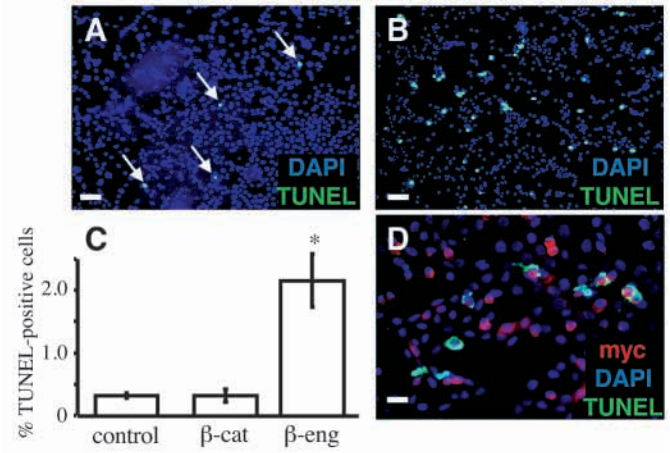


Fig. 4. β-eng expression in HC11 cells induces apoptosis. Fluorescent microscopy revealed apoptotic (TUNEL-positive) cells (green), DAPI-labelled nuclei (blue) and antibody against myc tag (red). Mock-infected HC11 cells show very low levels of apoptosis (A,C), whereas infection of cells with β-eng (B,D) induced apoptosis fivefold (C). Bars, 100 μm (A,B); 25 μm (D). *P<0.001.

β-eng induces apoptosis in HC 11 cells

HC11 cells were retrovirally infected with either stabilized β-catenin (gain-of-function) or β-eng (dominant negative loss-of-function), and apoptosis was analysed by immunofluorescent TUNEL assay 48 hours after infection (Fig. 4). This time-point was selected as the optimum time after infection at which to allow appropriate transgene integration and expression without completely losing the pool of expressing cells to apoptosis. The levels of apoptosis were extremely low in mock-infected cells (Fig. 4A,C) and in β-catenin-infected cells (Fig. 4C), but expression of β-eng in HC11 cells increased the level of apoptosis approximately fivefold (Fig. 4B,C), which is similar to the induction seen in mid-pregnant transgenic glands (Fig. 3). Immunofluorescent detection of the myc epitope in the β-eng construct (Fig. 4D, red) showed that approximately 50% of the HC11 cells were infected. Also, the cells undergoing apoptosis (Fig. 4D, green) colocalized with cells expressing the transgene (Fig. 4D, red). At this fixed time-point, not every infected cell was undergoing programmed cell death, but every dying cell colocalized with β-eng expression (Fig. 4D). Therefore, expression of β-eng induces apoptosis in HC11 mammary epithelial cells, confirming the apoptotic phenotype observed in the mammary glands of β-eng transgenic mice at mid-pregnancy.

It should be noted that HC11 cells, like most established murine cell lines, contain a mutant p53 (Merlo et al., 1993). One might predict that this perturbation of the cells' apoptotic signalling pathway might artificially affect the response to β-eng signalling in these cells. However, the similar induction of apoptosis observed in the transgenic model suggests that the effects of β-eng signalling in HC11 cells mimic the in vivo situation.

β-eng effect on proliferation in HC11 cells

The effects of β-catenin and β-eng expression on the proliferative response of HC11 cells were analysed by BrdU incorporation as well as by western blot analysis of downstream proliferative genes. First, transgene expression levels were verified by western blot analysis (Fig. 5A); expression of exogenous β-catenin and β-eng were detected in infected cells but not in mock-infected cells, using an anti-myc-tag antibody. A band with lower molecular weight (~105 kDa) was consistently detected in cells infected with β-eng and it may be a degradation product. An anti-β-catenin antibody detected the expression of endogenous β-catenin, which served as an internal loading control for epithelial cell content. In the retrovirally transduced cells, the levels of exogenous β-catenin and β-eng protein appeared to be comparable, but less abundant than endogenous β-catenin. However, this comparison may be somewhat misleading, as much of endogenous β-catenin is sequestered in the adherens junctions and not available in the signalling pool, and the transduction efficiency was only approximately 50%. However, it does appear that a substoichiometric ratio of mutant-to-wild-type protein is sufficient to result in phenotypic effects in MECs, in agreement with the previous results in *Xenopus* (Montross et al., 2000).

Proliferation was measured by immunofluorescent detection of FITC-labelled BrdU incorporation. Quantitation of labelled HC11 cells revealed no change in proliferation in cells expressing β-catenin or β-eng compared with mock-infected cells (Fig. 5B). Likewise, western blot analysis of cyclin D1, a transcriptional target of β-catenin signalling, shows no changes in the levels of cyclin D1 protein in β-catenin- or β-eng-expressing cells compared with mock-infected cells (Fig. 2A).

Interestingly, western blot analysis of mitogen-activated protein kinase (MAPK) signalling revealed markedly increased levels of phospho-MAPK (Erk1 and Erk2) in cells expressing β-eng compared with cells expressing β-catenin or mock-infected cells, whereas overall levels of MAPK remained constant (Fig. 5A). This result presents an apparent contadiction regarding the mechanism of action for β-catenin signalling. Activation of MAPK is usually observed as an early reponse to proliferative signals, but HC11 MECs expressing β-eng do not show increased proliferation; instead, they undergo apoptosis.

Downstream signalling effects of β-eng expression

In an effort to understand the downstream signalling events involved in the induction of apoptosis by β-eng, several potential target genes and signalling pathways were analysed (Fig. 6).

To determine whether the induction of apoptosis in HC11 cells involved a β-eng-mediated decrease in the PKB/AKT survival pathway, an analysis of phosphorylated PKB/AKT in HC11 cells infected with either β-catenin or β-eng was undertaken. No change in the levels of activated AKT compared with mock-infected cells was detected (Fig. 6A,B (arrow)). Thus, the apoptotic pathway activated by β-eng appears to act independently of the PKB/AKT survival pathway.

CD44 and immunoglobulin transcription factor-2 (*ITF-2*) are two genes that have been reported to be transcriptional targets of β-catenin signalling (Kolligs et al., 2002; Wielenga et al., 1999), and their putative roles in mammary gland development are addressed in further detail in the Discussion.

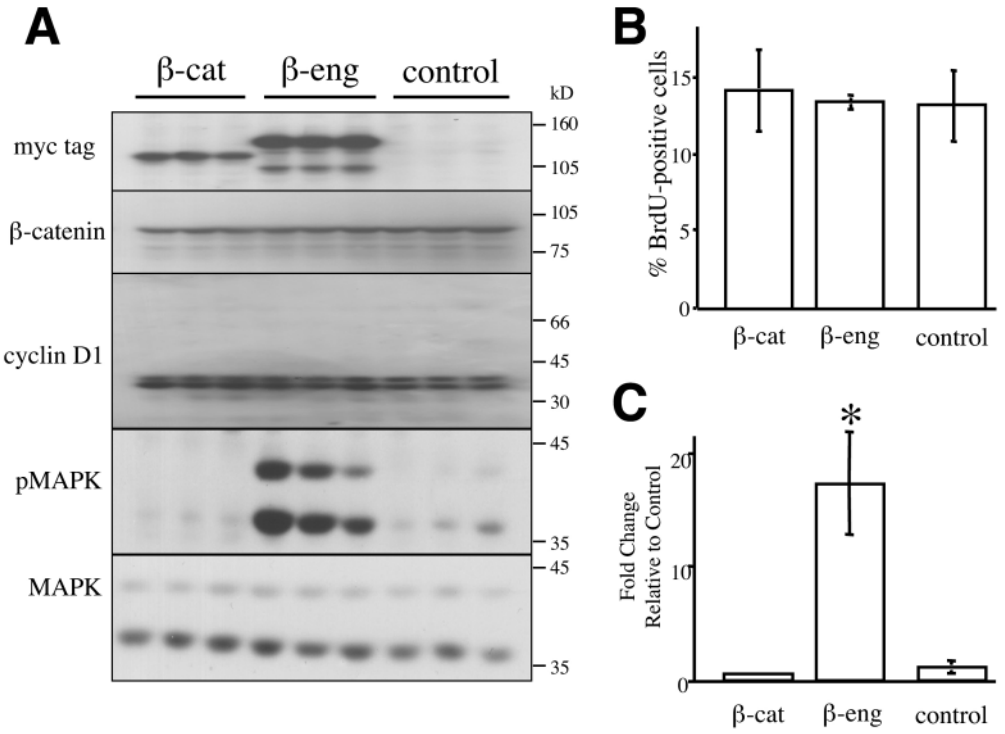


Fig. 5. Effect of β-eng expression on HC11 cell proliferation. Western blot analysis of three replicate HC11 cell cultures infected with β-catenin, β-eng or mock-infected control (A). β-Catenin and β-eng transgene expression in infected cells detected by an antibody against the myc-epitope tag. Anti-β-catenin antibody detected endogenous β-catenin and served as a loading control. Anti-cyclin-D1 antibody detected no change in the level of cyclin D1 protein, whereas activated MAPK was upregulated compared with the total MAPK protein level in response to β-eng expression. Quantitation of pMAPK activation by densitometry (C) revealed a ~15-fold increase in activated MAPK compared with total MAPK. Proliferation in HC11 cells was measured by immunfluorescent detection of FITC-labelled BrdU incorporation. Quantitation of BrdU-labelled cells (B) revealed no change in proliferation in cells infected with β-catenin or β-eng compared with mock-infected cells.

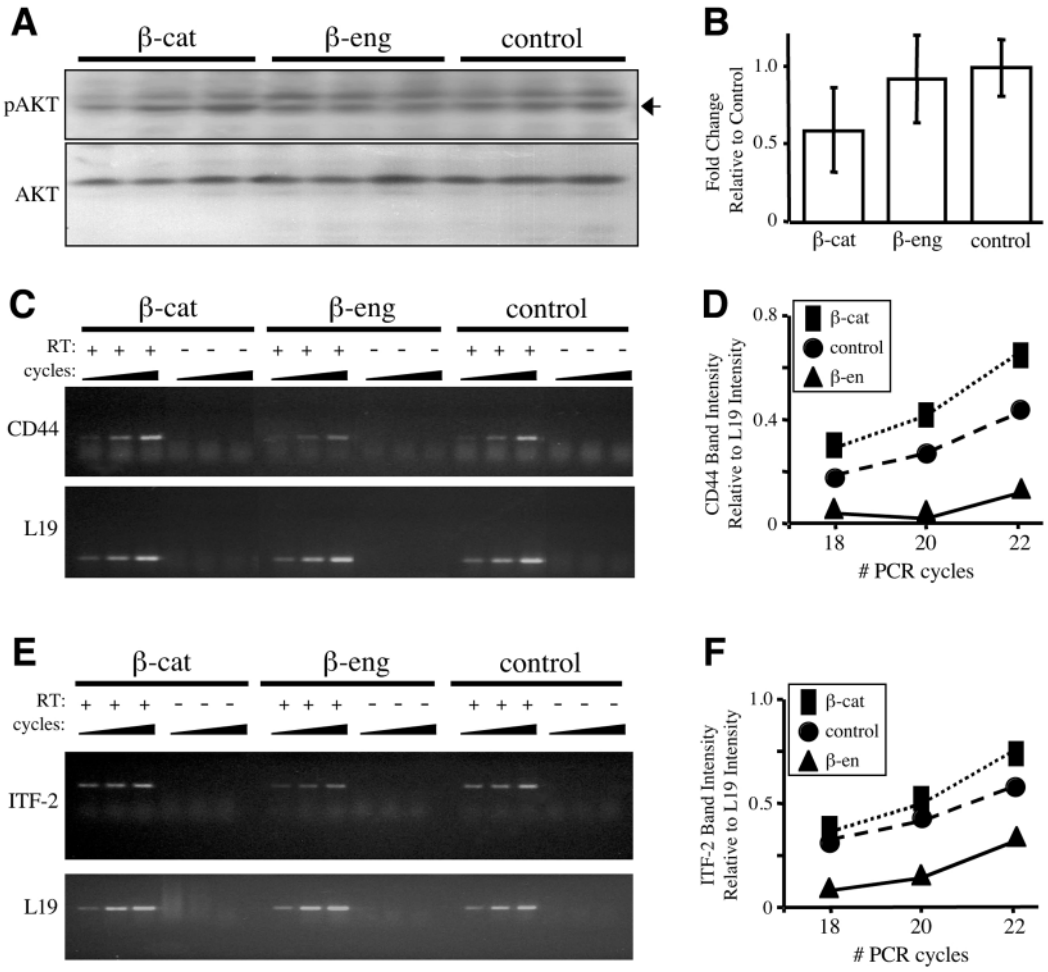


Fig. 6. Downstream signaling of β-eng. Western blot analysis of three replicate HC11 cell cultures infected with β-catenin, β-eng or mock-infected control (A). Phospho-specific AKT antibody revealed no change in activated AKT (A, upper panel) compared with total AKT (A, lower panel). Quantification by densitometry (B) indicated that levels of pAKT relative to total AKT were not significantly changed as a consequence of the exogenous expression of β-catenin or β-eng. Semiquantitative RT-PCR of target genes CD44 (C) and ITF-2 (E) at 18, 20 and 22 cycles of PCR (representative of three separate experiments). *CD44* mRNA levels were increased slightly in HC11 cells infected with exogenous β-catenin, whereas *ITF-2* levels appeared unchanged. However, both *CD44* and *ITF-2* mRNA levels were decreased in cells expressing β-eng (D,F). Quantitation of these results relative to the control L19 RNA showed that expression of β-eng in HC11 cells downregulated the expression of both of these mRNAs by at least twofold.

Their regulation at the RNA level was analysed by semi-quantitative RT-PCR, as sensitivity was crucial for the detection of small changes in these low-expressing genes. Both *CD44* (Fig. 6C,D) and *ITF-2* (Fig. 6E,F) mRNA levels were decreased by expression of β-eng in HC11 cells. It is important to note that these expression results probably underestimate the magnitude of this inhibitory effect, as only 50% of the cells are expressing the retrovirally transduced expression construct, and the cells were not maintained under selection. These experiments show the ability of β-eng to negatively regulate target gene expression and provide some understanding of the downstream events of β-eng signalling that culminate in apoptosis in mammary epithelial cells.

Discussion

This study has shown the importance of β-catenin expression in lobuloalveolar development and that the inhibition of β-catenin signalling in mammary epithelium targets the affected cells for apoptosis. Dominant negative β-eng effectively competes with β-catenin at stoichiometric ratios, so it is likely that a brief pulse of β-eng expression is sufficient to antagonize endogenous β-catenin signal during mid-pregnancy. Therefore, we hypothesize that β-eng (acting as a dominant negative β-catenin) is blocking a survival signal produced by endogenous β-catenin in mammary epithelial lobular progenitors.

The regenerative nature of the mammary gland (throughout the cycle of pregnancy, lactation and involution) implies the presence of stem/progenitor cells. Serial transplantation studies have shown that distinct populations of progenitor cells give rise to ductal and lobular epithelial cells in the mammary gland (Smith, 1996). Importantly, it appears that lobular epithelium, organized into milk-secreting clusters, develops in a clonal manner, as indicated by retroviral tagging experiments and serial transplantation experiments (Kordon and Smith, 1998). Thus, an alteration of gene expression in the lobular progenitor cell will be propagated throughout the lineage of the entire lobuloalveolar cluster.

Given the clonal nature of lobular development, a model can be postulated in which β-catenin signalling is crucial for normal lobular development. In this model, β-catenin provides a survival signal in the lobular progenitors, which allows these cells to divide and differentiate into lobuloalveolar clusters. When the β-catenin signal is perturbed, in this case by the expression of β-eng, the survival signal is lost, and the expressing cells undergo apoptosis. The loss of these few progenitor cells does not have an immediately dramatic effect on the morphological appearance of the gland (i.e. no gross phenotype at 10P-12P). However, as development continues and lobular clusters begin to expand, the lack of lobuloalveolar clusters that would have originated from transgene-expressing precursors, becomes strikingly evident. Transgene expression cannot be detected at this late developmental time-point, because, of course, all expressing precursors have undergone programmed cell death. The seemingly incongruous expression pattern and morphological phenotype data of the β-eng transgenic mice actually support this model of β-catenin-dependent survival of lobular mammary precursor cells.

Comparison of the current data with previous studies supports a model in which a β-catenin survival signal is required in lobuloalveolar progenitor cells. Although several related mammary-specific transgenic or knockout models also inhibit lactation in the mammary gland (Fantl et al., 1995; Hsu et al., 2001; Sicinski et al., 1995), there are subtle differences in phenotype, which lend themselves to alternative interpretations. CyclinD1$^{-/-}$ mice and transgenic MMTV-axin mice have similar phenotypes with a reduced number of alveoli, and the existing alveoli are collapsed and not properly distended with lipids and milk proteins (Fantl et al., 1995; Hsu et al., 2001). The cyclinD1$^{-/-}$ phenotype clearly results from decreased proliferation during development, but the transgenic axin phenotype seems to depend on a combination of decreased proliferation (i.e. reduced cyclinD1 protein level), as well as increased apoptosis. However, the penetrance of this apoptotic response was only partial, as transgene expression could continue to be detected throughout lactation (Hsu et al., 2001). The current study, however, shows no survival of transgene-expressing cells after day 12 of pregnancy, yet the lack of lobular development manifests itself later in development. Thus, expression of β-eng early in the development of lobuloalveolar progenitor cells results in almost immediate apoptosis (supported by the HC11 data in which β-eng induced apoptosis within 48 hours of infection), whereas the surrounding, nontransgenic progenitors develop normally into fully distended lobular clusters.

The potential role of β-catenin in the maintenance of mammary stem/progenitor cells is supported by data from other tissue types. Recent studies have illustrated the requirement for β-catenin signalling in the proper specification and differentiation of stem cells in the skin and hair (DasGupta et al., 2002; Huelsken et al., 2001; Merrill et al., 2001). In the liver, differentiation of hepatic stem cells is associated with the downregulation of Wnt/β-catenin signalling and the repression of target gene transcription (Plescia et al., 2001), and the epithelial stem cell compartment in the small intestine is completely depleted in Tcf-4$^{-/-}$ mice (Korinek et al., 1998). The overexpression of β-catenin in haematopoietic stem cells (HSCs) increases the pool of functional (transplantable) HSCs, and this activity is inhibited by expression of axin, a negative regulator of the β-catenin pathway (Reya et al., 2001). These data suggest that β-catenin signalling may play an important role in inhibiting differentiation and specifying stem-cell identity.

A recent study using extended labelling of mammary epithelial cells in vivo identified a parity-induced population of cells that survived involution and expanded during subsequent pregnancies (Wagner et al., 2002). Although 90% of lobular epithelial cells undergo apoptosis during involution, these cells survive, representing a constant population of putative progenitor cells. This study also shows that this population of progenitor cells can be targeted using the WAP promoter, further validating our model of WAP-driven β-eng expression in lobular progenitor cells. The analysis of β-catenin signalling in this persistant population could offer exciting new insights into the role of β-catenin in stem/progenitor cell maintenance.

Several recent observations suggest that the well-established oncogenic potential of β-catenin signalling may function through an anti-apoptotic mechanism in a variety of tissue types (Carmeliet et al., 1999; Chen et al., 2001; Hsu et al., 2001; Su et al., 2002; You et al., 2002). However, none of these studies has identified the mechanism by which β-catenin's survival signal is propagated. The data presented here

regarding the independence of the PKB/AKT signalling pathway from β-catenin-induced survival in MECs agree with previously published studies indicating that AKT is not involved in the Wnt-induced protection against chemotherapeutic agents in Rat-1 fibroblasts (Chen et al., 2001). In fact, that study concluded that none of the expected apoptotic/survival pathways were apparently involved (i.e. AKT, Janus kinase, nuclear factor-κB), nor were any of the known apoptotic genes misregulated (i.e. Bcl-2 family members, inhibitors of apoptosis (IAP) or Fas) in response to β-catenin signalling (Chen et al., 2001). Thus, these recent studies suggest that β-catenin plays a protective role against apoptosis, but the precise mechanisms regulating such a survival pathway remain to be determined.

The activation of MAPK signalling in this β-eng system provides an interesting puzzle. Why is this traditionally proliferative signal activated in the apoptotic cells expressing β-eng? One potential pathway through which β-catenin might be signalling in this case involves the Bcl-2-related protein Bim, and its potential involvement in anoikis. Bim is a pro-apoptotic BH3-only protein in the subfamily of Bcl-2 proteins that acts upstream to inhibit Bcl-2 survival family members. Cytokine stimulation in haematopoietic cell lines activates the MAPK pathway and subsequently supresses transcription of Bim (Shinjyo et al., 2001). Thus, Bim and its signalling partners could represent a potential mechanism through which β-catenin provides a survival signal to the cell.

The misregulation of the CD44 cell-surface protein in a variety of human carcinomas, including breast carcinomas (reviewed by Naor et al., 1997), and the identification of CD44 as a transcriptional target of β-catenin signalling (Wielenga et al., 1999), provided a potentially important target gene for analysis in this model system. Recent studies by Yu et al. describe the role CD44 plays in activation of ErbB4 signalling via complex formation with matrilysin and heparin-binding epidermal growth factor (HB-EGF) (Yu et al., 2002). In both $CD44^{-/-}$ mice and mice expressing dominant negative ErbB4 in the mammary gland, lactation is impaired (Jones et al., 1999; Yu et al., 2002), similar to the β-eng transgenic phenotype described here. Expression of the β-eng construct downregulated CD44 mRNA expression (Fig. 6D,E), as well as activation of the ErbB4 receptor (data not shown). These data, in conjunction with the lactation phenotype observed in multiple mouse models, suggest that CD44/ErbB4 signalling may be one mechanism through which β-catenin signalling modulates lobular development and lactation in the mammary gland.

However, it is likely that β-catenin signalling influences multiple downstream targets affecting cell survival. ITF-2 is a basic helix-loop-helix transcription factor and a target of β-catenin signalling (Kolligs et al., 2002; Zhai et al., 2002). ITF-2 is inhibited by Id-1, which acts as a dominant-negative inhibitor of basic helix-loop-helix transcription factors, and functions to regulate mammary epithelial cell growth, differentiation and apoptosis (Parrinello et al., 2001). Id-1 induces apoptosis under dense cellular conditions in MECs, and this effect is attenuated by the overexpression of ITF-2 (Parrinello et al., 2001). The downregulation of ITF-2 mRNA by expression of β-eng in conjunction with its apoptotic phenotype provides an additional potential mechanism of β-catenin-mediated cell survival. In this model, β-catenin signalling upregulates ITF-2, which can then effectively compete with its dominant-negative inhibitor Id-1 to sustain a cell survival signal that has yet to be identified.

A recent study in *Drosophila* has challenged the generally accepted view of the mechanism by which β-catenin regulates signal transduction in the nucleus, in part by using a mutant β-catenin construct related to β-eng (Chan and Struhl, 2002). It is important to note that the construct used in those studies was markedly different from β-eng, in that it retained the carboxy-terminal region of β-catenin and contained an extra Gal4 DNA binding domain, which probably accounts for the differences observed by those authors. In addition, previous competition studies using β-eng (Montross et al., 2000) show that β-eng acts in a dominant negative fashion to downregulate canonical signalling targets, such as Siamois. Therefore, β-eng can be used to inhibit β-catenin signalling, regardless of the mechanism of action by which β-catenin functions.

The data presented here provide for the first time unequivocal evidence that β-catenin signalling is crucial for normal mammary lobular development. Although previous studies have shown that overexpression of axin in the mammary gland resulted in increased apoptosis and decreased lobular development (Zhang et al., 1999), axin also activates the c-Jun NH_2-terminal kinase/stress-activated protein kinase (JNK/SAPK) signalling cascade independently of regulating β-catenin degradation. Thus, in this case other signalling events could have contributed to the observed apoptotic phenotype. In $Wnt4^{-/-}$ mice, lobular development was inhibited during early stages of pregnancy, but the defect was rescued later in development, presumably because of compensation by other mammary-expressed Wnt family members (Brisken et al., 2000). Thus, the current study offers the first direct evidence of the requirement of Wnt/β-catenin signaling for normal mammary lobular development, potentially through the maintenance of lobular progenitors.

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