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13. ABSTRACT (Maximum 200 Words)

Many of the molecular events that control normal development of the mammary gland are the same events that, when misregulated, result in cancer. Therefore, the understanding of normal developmental processes in the mammary gland is a crucial first step to rational design of therapeutics which target these systems in breast cancer.

The Wnt family of genes, which were first identified for their role in mouse mammary tumorigenesis, initiate a signaling cascade that manifests in the stabilization of β-catenin protein. The proposed experiments are based on the hypothesis that misregulation of this pathway results in an accumulation of stabilized β-catenin, and genes involved in growth, cell death, and cell invasion are upregulated inappropriately, resulting in tumorigenesis.

The proposed experiments use two complimentary strategies to study β-catenin's direct role in mammary gland development and tumorigenesis, gain- and loss-of-function experiments. Reconstitution experiments using cells expressing stabilized β-catenin and transgenic mice expressing a dominant negative mutant β-catenin (β-cat^{DN}) specifically in the mammary gland provide opposite approaches for study. These two systems will be used to analyze changes in morphology, downstream signaling, and functional differentiation, comparing gain- and loss-of-function of β-catenin.

Many factors implicated in mammary oncogenesis regulate β-catenin, and β-catenin is clearly involved in tumorigenesis in other organ systems, suggesting a role for β-catenin in mammary oncogenesis. The proposed experiments will characterize β-catenin's direct role in mammary gland development and tumorigenesis.

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Introduction

β-catenin's involvement in Wnt and other signaling pathways and its oncogenic potential in other tissues strongly suggest a role for β-catenin in mammary tumorigenesis. This study proposes to examine the direct role of β-catenin in normal mammary gland development and tumorigenesis through gain- and loss-of-function experiments.

Body

Task 1: To study b-catenin's involvement in mammary gland development and carcinogenesis in virgin and pregnant mice using a primary culture reconstitution method

The first Annual Report summarized the difficulties experienced using the mammary reconstitution method. This dilemma prompted a discussion within our group regarding enriching the primary culture for multi-potent progenitor cells. We followed this idea and developed a method for isolating a population of progenitor cells; these experiments are described in Welm BE, Tepera SB, Venezia T, Graubert TA, Rosen JM and Goodell MA, "Sca-1^{pos} Cells in the Mouse Mammary Gland Represent an Enriched Progenitor Cell Population", Developmental Biology, in press (Appendix B).

As the manuscript describes, the mammary gland side population (MG-SP) cells represent the purest population of mammary progenitor cells. However, technical limitations of the method restrict the further characterization of these cells due to very low cell viability. The manuscript also describes an alternative isolation method, using expression of Stem cell antigen-1 (Sca-1) as a marker of progenitor cells. The long-term plan is to retrovirally infect these purified populations with exogenous genes and perform reconstitution experiments. These experiments are ongoing within the lab and will be continued by other students.

Since the mammary reconstitution method has proven to be so problematic, we have pursued alternative methods for studying b-catenin's involvement in virgin and pregnant mammary development. Specifically, stabilized b-catenin is expressed in virgin mammary glands by using an *in vitro* Cre-lox system. Mammary glands are isolated from animals that carry lox sites flanking exon 3 of b-catenin (Catnb $^{loxP\,(ex3)}$). Primary culture cells are isolated from these glands, grown in culture, and infected with adenovirus expressing the Cre recombinase (Adeno-Cre). Upon recombination, the cells and all their future progeny express stabilized b-catenin protein. These cells are then injected into cleared fat pads as previously described, allowed to reconstitute a mammary gland, and analyzed for morphological and molecular phenotype. To facilitate the assessment of the expression of stabilized β-catenin we have generated and utilized heterozygous mice carrying a modified ROSA26 reporter (R26R) construct in combination with the Catnb $^{loxP\,(ex3)}$ allele that express the lacZ gene only after Cre-mediated recombination. Our data shows that Ad-Cre infection of primary mammary epithelial cell culture isolated from mice carrying both the Catnb $^{loxP\,(ex3)}$ and the R26R allele stained positive for β-galactosidase in 75-80% of the primary mammary epithelial cells, indicating that the recombination had occurred in the majority of these cells. PCR analysis of the loxP sites flanking the β-catenin exon 3 fragment from primary mammary epithelial cells indicated that recombination has occurred. RT-PCR analysis confirmed that the primary mammary epithelial cells expressed the mutant β-catenin

mRNA. In addition, by RT-PCR, we have shown that downstream genes such as cyclin D1 and matrilysin (MMP7) are upregulated in infected primary mammary epithelial cells. Finally, we are able to obtain reconstituted mammary glands from Ad-Cre infected primary mammary epithelial cells and are in the process of evaluating them. These experiments are ongoing within the lab and will be continued by other students.

Task 2: To characterize b-catenin's signaling function using a dominant negative transgenic model

Tasks 2a-b: Completed and described in first Annual Summary

Four lines of transgenic mice were developed which express a dominant negative form of b-catenin (b-cat^{DN}) specifically in the mammary gland. Two of these lines express b-cat^{DN} under the Mouse Mammary Tumor Virus (MMTV) promoter, while two lines use the Whey Acidic Protein (WAP) promoter. Both of these promoters express transgene specifically in the mammary gland during pregnancy and lactation, and all four lines have the same morphological phenotype.

Task 2c-d: Completed and described in the following publication.

The experiments performed to characterize β-catenin's signaling function using a dominant negative transgenic model are described in **Stacey B. Tepera, Pierre D. McCrea, and Jeffrey M. Rosen, " A β-catenin survival signal is required for normal lobular development in the mammary gland", Journal of Cell Science (2002) 116, 1137-1149.**

As the manuscript describes, 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 signaling domain, was expressed preferentially in the mammary gland. The chimera β-eng inhibits the signaling 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 lobuloalveoloar 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 lobuloalveolar progenitors that later differentiate into lobuloalveolar clusters. This study illustrates the importance of β-catenin signaling in mammary lobuloalveolar development.

Key Research Accomplishments

- Isolated for the first time a population of functional mammary epithelial stem/progenitor cells
- Initiated experiments using adenoviral-mediated recombination of floxed b-catenin primary culture cells and transplantation *in vivo*
- Established the critical importance of Wnt signaling in normal lobular development

- Confirmed the importance of the Wnt/b-catenin signaling pathway in regulating proliferation as well as its additional function in regulating apoptosis

Reportable Outcomes

Publications:

- Stacey B. Tepera, Pierre D. McCrea, and Jeffrey M. Rosen, " A β-catenin survival signal is required for normal lobular development in the mammary gland", Journal of Cell Science (2002) 116, 1137-1149.
- Welm BE, Tepera SB, Venezia T, Graubert TA, Rosen JM and Goodell MA, "Sca-1^{pos} Cells in the Mouse Mammary Gland Represent an Enriched Progenitor Cell Population", Developmental Biology (2002) 245, 42-56

Poster:

- National Cancer Institute Mouse Models of Human Cancers Consortium, Washington DC, January 2002

Presentations:

- National Cancer Institute Mouse Models of Human Cancers Consortium, Washington DC, January 2002
- Baylor College of Medicine Graduate Student Symposium, October 2001
- 54^{th} MD Anderson Cancer Center Symposium on Fundamental Cancer Research, October 2001

Methods developed:

Isolation and purification of a population of mammary stem/progenitor cells has been accomplished, based on methods and molecular marker expression which are novel to the mammary gland.

Transgenic mice:

Four lines of transgenic mice were established which express b-cat^{DN} under mammary-specific promoters and have severe defects in development and lactation.

Collaborations/funding opportunities:

A valuable collaboration was established with Margaret A. Goodell, Ph.D. and together the group applied for and received a Concept Award from Department of Defense Breast Cancer Research Program.

Conclusions

Experiments that were initiated as a means of improving a method to be used in Task 1, eventually yielded very interesting results independent of the original motivation. The manuscript in press describes the isolation of a population of mammary epithelial cells which are enriched for the ability to reconstitute a mammary gland. This is the first time that functional mammary stem cells have been isolated from the total population of cells in the mammary gland

The transgenic experiments have also yielded very interesting data, including the first conclusive evidence that the Wnt/b-catenin signaling pathway is absolutely required for normal lobular development. Additionally, while much work has focused on b-catenin's involvement in proliferation, we have identified a somewhat unexpected function in regulating apoptosis. These experiments have all contributed to breast cancer research by increasing our understanding of the normal development of the mammary gland, a very important step in understanding the molecular misregulation that leads to cancer.

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Appendix B: Publications

Publications:

- Stacey B. Tepera, Pierre D. McCrea, and Jeffrey M. Rosen, " A β-catenin survival signal is required for normal lobular development in the mammary gland", Journal of Cell Science (2002) 116, 1137-1149.
- Welm BE, Tepera SB, Venezia T, Graubert TA, Rosen JM and Goodell MA, "Sca-1^{pos} Cells in the Mouse Mammary Gland Represent an Enriched Progenitor Cell Population", Developmental Biology (2002) 245, 42-56

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

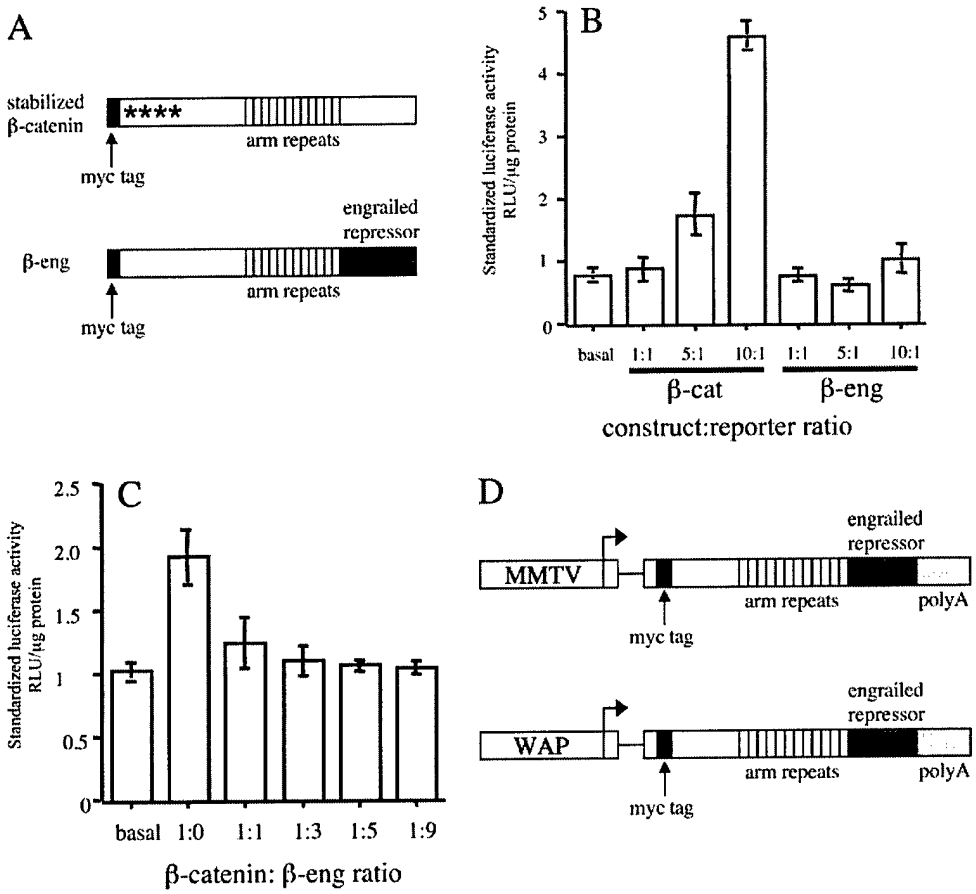


Fig. 1. β-Catenin and β-eng constructs and activity in HC11 cells. (A) Stabilized β-catenin (*, S→A, T→A mutations in the N-terminal domain) and β-eng constructs. The C-terminal domain of β-catenin is replaced with the N-terminal repressor domain of *Drosophila* Engrailed to create β-eng. These constructs 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.

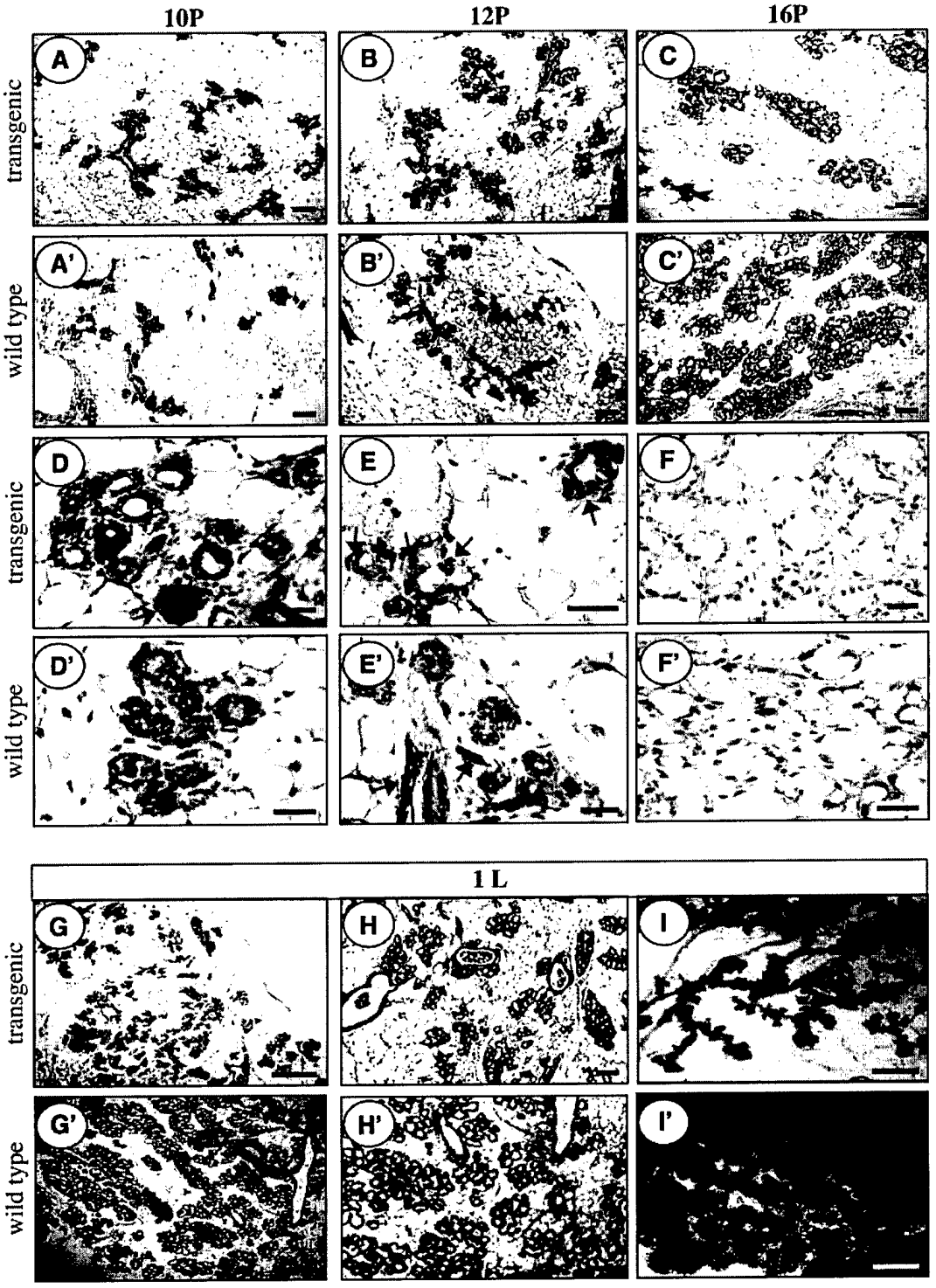
Decreased lobuloalveolar development in β-eng transgenic mammary glands

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.

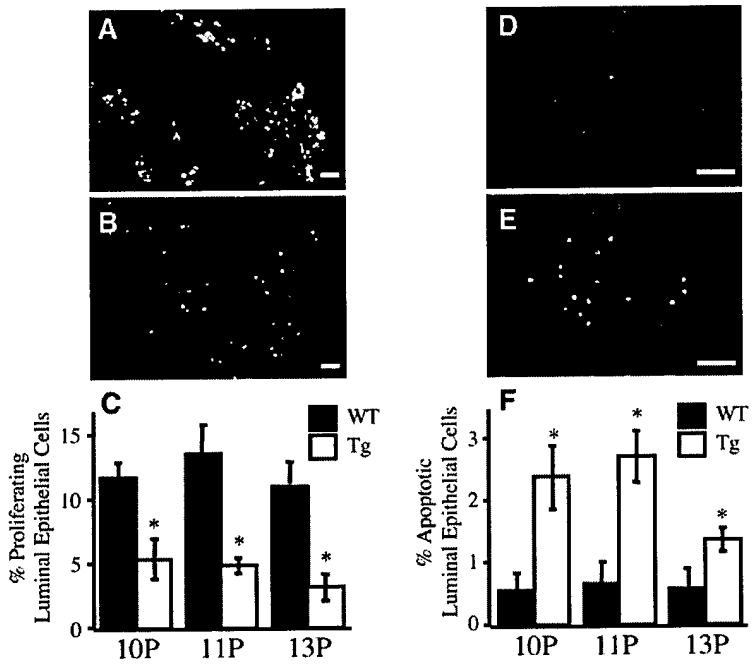


Fig. 3. Proliferation and apoptosis in MECs of β-eng transgenic mice. Immunofluorescent detection of BrdU incorporation in mammary epithelium of wild-type (A) and transgenic (B) mice revealed decreased proliferation in β-eng transgenics (C). Fluorescent TUNEL assay revealed increased apoptosis in MECs 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.

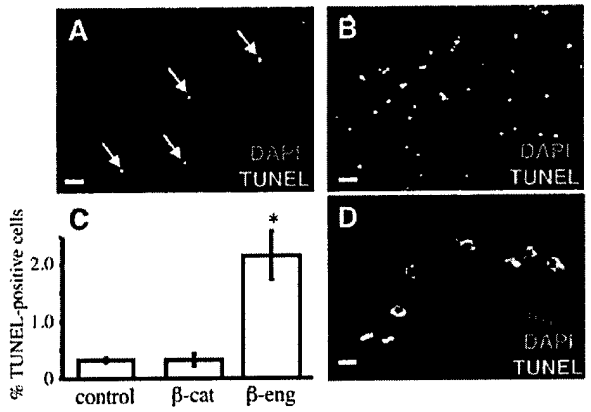


Fig. 4. β-eng expression in HC11 cells induces apoptosis. Fluorescent microscopy revealed apoptotic (TUNEL-positive) cells (green), DAPI-labelled nuclei (blue) and antibody against myc tag (red). Mock-infected HC11 cells show very low levels of apoptosis (A,C), whereas infection of cells with β-eng (B,D)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

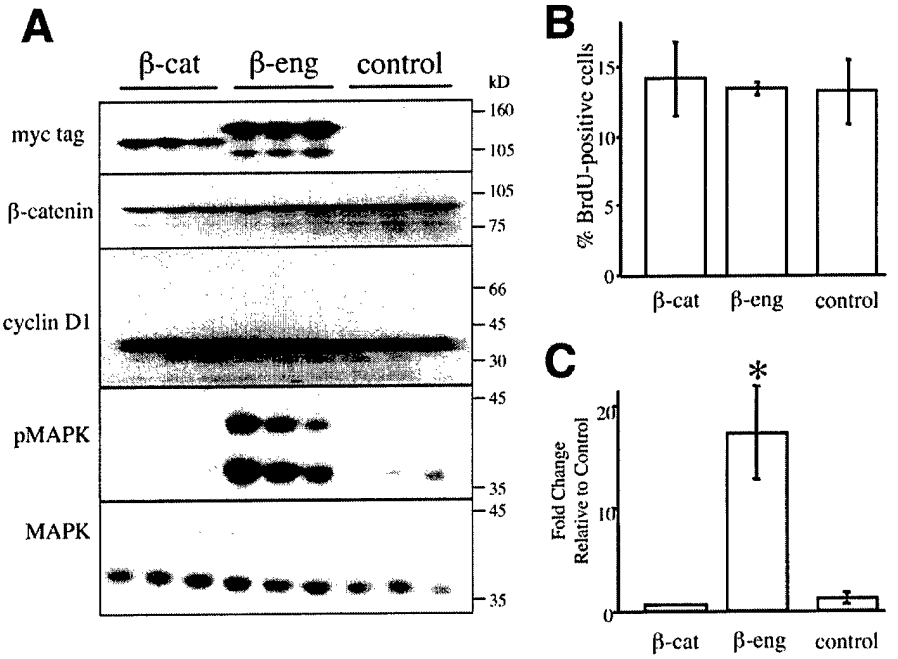


Fig. 5. Effect of β-eng expression on HC11 cell proliferation. Western blot analysis of three replicate HC11 cell cultures infected with β-catenin, β-eng or mock-infected control (A). β-Catenin and β-eng transgene expression in infected cells detected by an antibody against the myc-epitope tag. Anti-β-catenin 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.

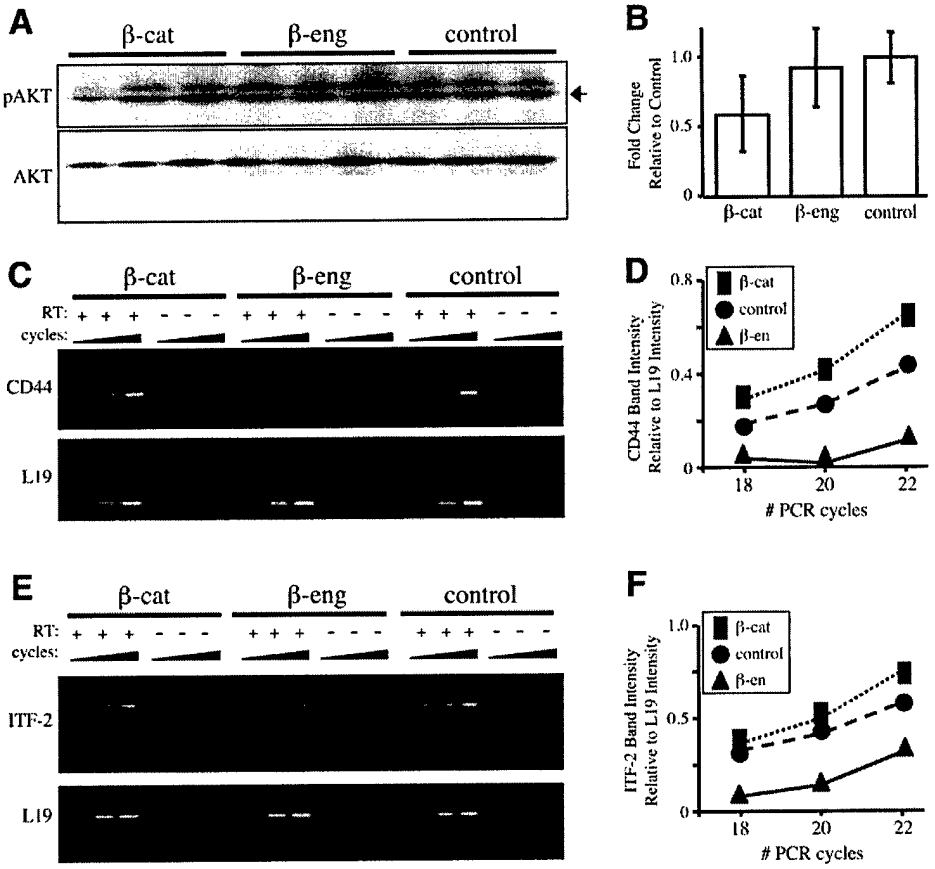


Fig. 6. Downstream signaling of β-eng. Western blot analysis of three replicate HC11 cell cultures infected with β-catenin, β-eng or mock-infected control (A). Phospho-specific AKT antibody revealed no change in activated AKT (A, upper panel) compared with total AKT (A, lower panel). Quantification by 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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Sca-1^{pos} Cells in the Mouse Mammary Gland Represent an Enriched Progenitor Cell Population

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Mammary epithelium can functionally regenerate upon transplantation. This renewal capacity has been classically ascribed to the function of a multipotent mammary gland stem cell population, which has been hypothesized to be a primary target in the etiology of breast cancer. Several complementary approaches were employed in this study to identify and enrich mammary epithelial cells that retain stem cell characteristics. Using long-term BrdU labeling, a population of label retaining cells (LRCs) that lack expression of differentiation markers has been identified. LRCs isolated from mammary primary cultures were enriched for stem cell antigen-1 (Sca-1) and Hoechst dye-effluxing "side population" properties. Sca-1^{pos} cells in the mammary gland were localized to the luminal epithelia by using Sca-1$^{+/GFP}$ mice, were progesterone receptor-negative, and did not bind peanut lectin. Finally, the Sca-1^{pos} population is enriched for functional stem/progenitor cells, as demonstrated by its increased regenerative potential compared with Sca-1^{neg} cells when transplanted into the cleared mammary fat pads of host mice.

Key Words: **mammary; stem cells; BrdU; Sca-1; label retention; progesterone receptor.**

INTRODUCTION

The mammary gland provides a unique model for the study of growth, differentiation, apoptosis, and pattern formation involved in developmental processes. A characteristic of the mammary gland is its ability to postnatally proliferate, terminally differentiate, and involute upon successive cycles of pregnancy, lactation, and involution, respectively. Mammary gland growth and differentiation share many mechanistic similarities with developmental processes occurring during organogenesis in the mouse embryo, including the regulation of patterning through growth factor signaling pathways. Mammary epithelium is also capable of completely and functionally regenerating upon transplantation. This impressive renewal capacity has been ascribed to the function of a multipotent mammary gland stem cell population (for review, see Smith and Chepko, 2001). Thus, the postnatal mammary gland can be utilized for the analysis of developmental pathways not only involved in the regulation of growth and patterning, but also in the determination of cell lineages and differentiation.

The existence of mammary gland stem cells was first demonstrated through classical transplantation studies conducted by DeOme (DeOme *et al.*, 1959). DeOme and colleagues observed that epithelium isolated from several different regions of the mammary gland at any stage of postnatal development was capable of generating functional mammary epithelial outgrowths containing ductal, lobuloalveolar and myoepithelial cells. Subsequent studies by Daniel *et al.* (1968) indicated that mammary epithelium could be serially transplanted, but exhibited senescence following seven or eight transplants. Kordon and Smith (1998) suggested, through the use of retroviral-tagging, that progeny from a single cell can give rise to a complete mammary gland upon transplantation. These data indicate that mammary gland stem cells are distrib-

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uted throughout the gland and exhibit a potent yet limited self-renewal capacity. Furthermore, the mitotic and self-renewal capacities of mammary gland stem cells are thought to make them highly susceptible to mutagenesis, thus making them the primary targets in the etiology of breast cancer.

A stem cell population would be expected to contribute to the cell types in mammary gland development through symmetric cell division (for self-renewal) and asymmetric cell division (to generate progenitors) (Chepko and Smith, 1997). During ductal morphogenesis (3–8 weeks of age in the mouse), the mammary gland is rapidly proliferating and contributing to cell lineages in the developing epithelium. At the terminal ends of the ducts are bulb-like structures called terminal end buds (TEBs). TEBs are the proliferating units of the mammary gland, and as they penetrate the fat pad, their progeny make up the luminal and myoepithelial cell types of the gland. At the distal tip of the TEB exists a layer of highly proliferative cells termed "cap cells" that lack steroid receptors and are thought to represent an undifferentiated, stem/progenitor cell population, giving rise to differentiated luminal and myoepithelial cells in the subtending ducts (Daniel and Silberstein, 1987; Williams and Daniel, 1983). In the mature animal, the mammary gland remains in a quiescent state until the onset of pregnancy. During this period, stem cells are thought to be scattered throughout the gland and contribute to the maintenance of the ductal epithelial network.

Several morphologically distinct cell populations have been identified in the mammary gland. These cell types are distinguished based on DNA condensation, size, shape, location, cytoplasmic granularity, and nuclear morphology by using electron microscopy. These distinctions have been used to detect a population of cells termed "small light cells" (SLC), which make up about 3% of the total epithelial component in rat mammary glands. SLCs exhibit morphological characteristics of putative undifferentiated, division-competent mammary gland stem cells (Chepko and Smith, 1997; Smith and Chepko, 2001). These studies demonstrated that the mammary gland consists of a heterogenous population of epithelial cells in different stages of differentiation. However, few molecular markers have been identified that are expressed during mammary gland differentiation, and the analysis of mammary epithelial cell lineages has been limited. Furthermore, the lack of cell surface lineage markers in the mammary gland has resulted in the inability to characterize the ability of purified epithelial cell populations to regenerate mammary gland outgrowths.

This study describes for the first time the enrichment of a cell population within primary mouse mammary epithelial cell (MEC) cultures that displays multipotent mammary gland progenitor/stem cell characteristics. Bromodeoxyuridine (BrdU) label retention, FACS analysis, and transplantation methods have been combined to enrich and characterize these cells and to determine their functional capacity for regeneration. In addition, these studies have increased our understanding of the largely unknown differentiation lineage during virgin mammary gland development.

MATERIALS AND METHODS

BrdU Label Retention

Alzet minipumps #2002 (Durect Corp., Cupertino, CA) were filled with 200 μl of 60 mg/ml BrdU (Sigma #B-5002) (designed to release 0.5 μl/h for 14 days). Three-week-old C57B6 female mice (Taconic, Germantown, NY) were anesthetized with Avertin, and a minipump was implanted interscapularly into each animal. Fourteen days later, the animals were anesthetized, and the pumps were removed. This label retention experiment was conducted 4 times, using 19–24 mice in each experiment. Inguinal glands from mice were collected and BrdU was analyzed by immunofluorescence at week 0 (4 glands), week 9 (8 glands), and intervening weeks (2 glands each). After the 9-week chase period, lymph nodes were removed from #4 inguinal glands, and the glands were digested into mammary epithelial primary culture suspension (DeOme *et al.*, 1959; Pullan and Streuli, 1996). The cells were sorted by FACS for the MG-SP population and by magnetic column for Sca-1 expression (as described below). These sorted cells were then spun onto glass slides, fixed with cold acetone for 10 min, and washed with PBS. Label-retaining cells on these slides were identified by submerging in 0.07 N NaOH for 2 min, washing in PBS, pH 8.5, staining with anti-BrdU-FITC (Becton Dickinson #347583) for 1 h, and counterstaining with DAPI (Vector #H-1200). The number of $BrdU^{pos}$ cells in each of these populations was counted and graphed.

Immunofluorescence Localization

Glands were fixed in 4% paraformaldehyde for 2 h on ice, embedded in paraffin, and cut into 5-μm sections. After deparaffinization, antigen retrieval was performed as previously described (Seagroves *et al.*, 2000). Sections were incubated with primary antibody overnight at room temperature in a humidified chamber, washed in PBS, and incubated with secondary antibody for 1 h at room temperature. Sections were then washed for 1 h in PBS and mounted in mounting medium containing DAPI (Vector #H-1200). Primary antibodies used were: mouse monoclonal anti-BrdU-FITC (Becton-Dickinson #347583), rabbit-anti-human PR (Dako #A0098), mouse monoclonal anti-human cytokeratin 18 (Progen #61028, clone Ks 18.04), and rabbit-anti-mouse cytokeratin 14 (Covance #PRB-155P). Secondary antibodies used were: goat-anti-rabbit-Texas Red (Molecular Probes #T6391) and goat-anti-mouse-Texas Red (Molecular Probes #T6390).

SP Sorting

All 10 mammary glands were dissected out of mature virgin B6;129S-Gtrosa26 mice (Jackson Labs), and the epithelial cell fraction was isolated as described previously (Pullan and Streuli, 1996). Cells were plated on plastic tissue culture dishes in growth medium consisting of F12 (Gibco BRL) supplemented with insulin (5 μg/ml; Sigma), hydrocortisone (1 μg/ml; Sigma), epidermal growth factor (10 ng/ml; Gibco BRL), penicillin/streptomycin (100 μg/ml; Gibco BRL), gentamycin (50 μg/ml; Sigma), and fetal bovine serum (10%; JRH Biosciences). After 72 h in culture, the cells were

trypsinized with 0.05% trypsin/0.02% EDTA (JRH Biosciences), washed twice in Hanks' Balanced Salt Solution (HBSS; Gibco BRL), and stained with a final concentration of 5 μg/ml Hoechst-33342 in Dulbecco's modified Eagle's medium (DMEM) with 2% FBS at 37°C for 90 min as described previously (Goodell *et al.*, 1996). Sca-1 expression in the MG-SP cells was analyzed by staining the cells with anti-Sca-1-PE antibody (BD Pharmingen #553336, clone E13-161.7) for 15 min on ice following the Hoescht-33342 treatment. Analysis and sorting were performed on a triple laser MoFlo (Cytomation, Fort Collins, CO). The Hoechst dye was excited at 350 nm and its fluorescence was measured at two wavelengths, 450/20 BP filter Blue and 675 EFLP optical filter Red, as described previously (Goodell *et al.*, 1996).

SP Cell Injections

Collected SP cells were washed with HBSS, and 2.5×10^4 or 7.5×10^4 cells were mixed with 2×10^5 unsorted wild-type C57BL/6 cells per injection site. The inguinal glands of 21-day-old Rag-$1^{-/-}$ females (Jackson Labs) were cleared of endogenous epithelium as previously described (DeOme *et al.*, 1959). Cells were injected into the cleared fat pads of these mice by using a 50-μl Hamilton syringe in a blind method. Cells were allowed to grow out for 6 weeks, and then the animals were bred to induce lobuloalveolar development. At day 10–16 of pregnancy, glands were surgically removed and stained for lac-Z expression as previously described (Rijnkels and Rosen, 2001).

Sca-1 Magnetic Sorting

Enrichment of Sca-1-expressing cells was achieved by sorting cells using the MACS system (Miltenyi Biotec, Sunnyvale, CA). Whole primary culture cells isolated from mature virgin C57BL6 mice (Harlan Sprague Dawley) were incubated with biotinylated anti-Sca-1 antibody (PharMingin #553334) for 10 min on ice, washed in $DMEM^+$ [DMEM with 2% fetal bovine serum (JRH Biosciences #12106-500M) and 10 mM Hepes], incubated with streptavidin-conjugated microbeads (Miltenyi Biotec #130-048-101) for 5 min on ice, incubated with streptavidin-PE (Molecular Probes #S-866) for 5 min on ice, washed with $DMEM^+$, and loaded onto a MACS column (Miltenyi #130-041-306). The column was set up in a Miltenyi magnet so the magnetized microbeads and all cells that adhered to them would be retained on the column. The flow through was collected as the Sca-1^{neg} fraction, and the Sca-1^{pos} cells were eluted from the column by removing the column from the magnetic field and washing with $DMEM^+$. For the injection experiments, this process was repeated; each fraction was loaded on new columns, and the purity of the Sca-1 enrichment and viability of the cells (by propidium iodide staining) was analyzed on a FACSCAN (Becton Dickinson, Sunnyvale, CA).

Sca-1-Bead Sorted Cell Injections

Mammary epithelial primary cultures were isolated from #4 and #5 mammary glands (without the lymph node) of mature virgin C57BL6 mice (Harlan Sprague Dawley) as described above. Cells were sorted on the MACS system and Sca-1^{pos} and Sca-1^{neg} fractions were collected and analyzed for purity and viability (by propidium iodide staining). Recipient C57BL6 females at 21 days of age were cleared of endogenous mammary epithelium and either 10,000 or 50,000 viable Sca-1^{pos} or Sca-1^{neg} cells were injected into each cleared fat pad in a blind method. After 4 weeks, a subset of the animals were bred, and at day 8–10 of pregnancy the injected fat pads were surgically removed and fixed in 4% paraformaldehyde for 2 h on ice. Whole mounts were prepared as described previously (Williams and Daniel, 1983), and images were captured by using an Olympus dissecting microscope and Sony video camera (#DXC-151A).

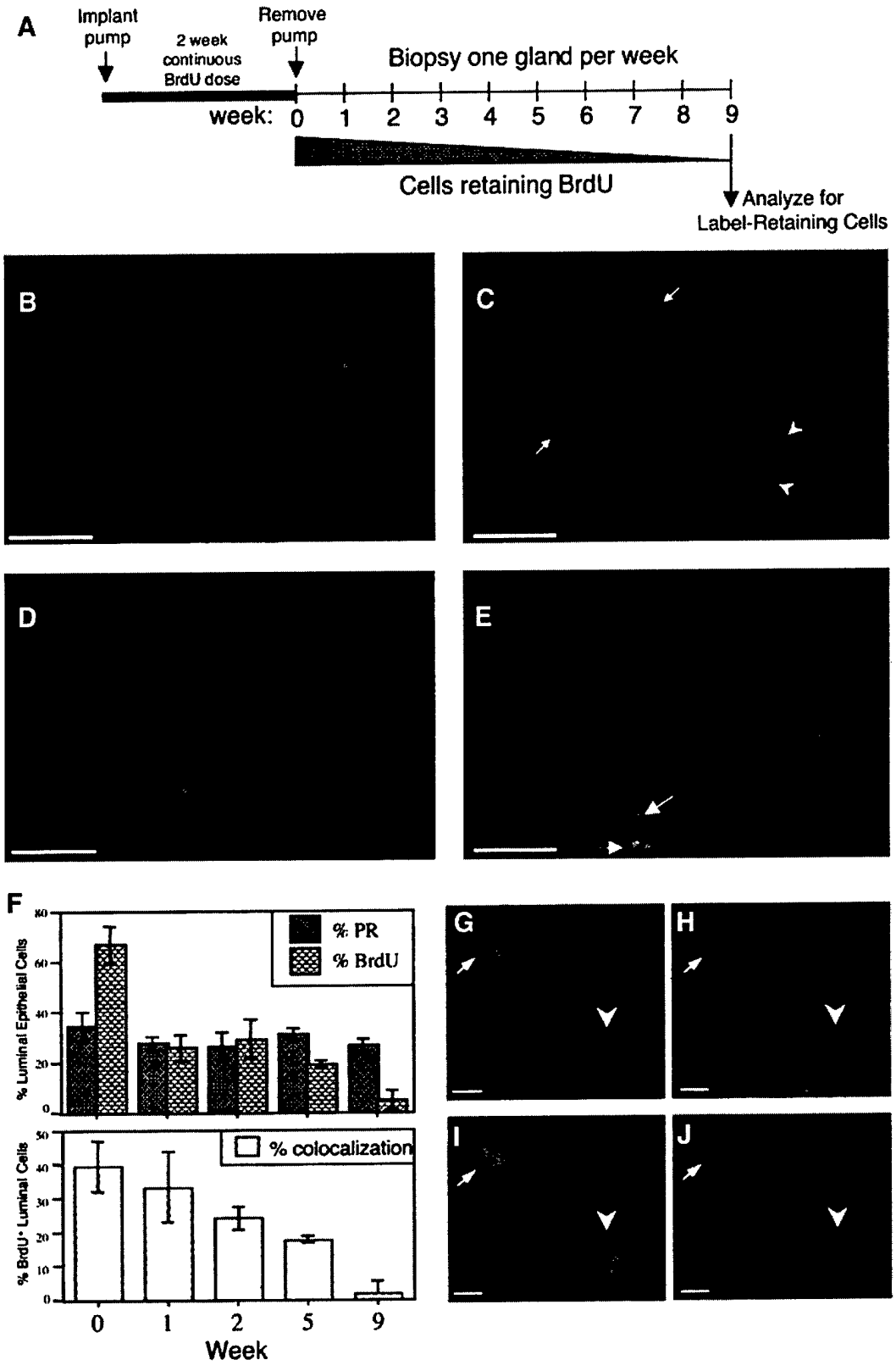
Sca-$1^{+/GFP}$ Transplants

Mammary glands were removed from mature Sca-$1^{+/GFP}$ mice and cut into 2- to 3-mm pieces. One piece of tissue was transplanted into each cleared fat pad of 21-day-old Rag-$1^{-/-}$ females (Harlan Sprague Dawley). After 8 weeks, the fat pads containing transplanted tissue and subsequent outgrowths were harvested and fixed as described below.

Sca-$1^{+/GFP}$ Fluorescence

Transplanted mammary outgrowths and intact Sca-$1^{+/GFP}$ mammary glands were surgically removed, fixed in 2% paraformaldehyde for 2 h, frozen in Tissue Freezing Medium (Triangle Biomedical Sciences #H-TFM) on dry ice, and sectioned in 60-μm sections. Sections were incubated with Texas Red-X phalloidin (Molecular Probes #T-7471) or rabbit-anti-human PR (Dako #A0098) and anti-rabbit-Texas Red (Molecular Probes #T6391) as described above, except incubation times were increased to 1 h/10 μm of section. Sections were analyzed by using Zeiss 510 laser scanning confocal microscope.

FIG. 1. Long-term BrdU label retention. (A) A schematic representation of the experimental design. Three-week-old female mice received a continuous dose of BrdU for 2 weeks via a subcutaneously implanted pump. Upon removal of the pump, mammary gland biopsies were taken each week during the 9-week chase period. After 9 weeks, mammary glands were analyzed for LRCs, a small population of quiescent epithelial cells. (B–E) Immunofluorescence analysis of BrdU-labeled cells at weeks 0 (B, D) and 9 (C, E). Luminal epithelium was extensively labeled with BrdU at week 0 (B), but only about 5% of luminal epithelium retain this label in week 9 (C). Double immunofluorescence analysis (BrdU-FITC and PR-Texas Red) revealed colocalization of $BrdU^{pos}$ and PR^{pos} cells at week 0 (D), but very little colocalization by week 9 (E, arrows). Quantitation of PR^{pos} and $BrdU^{pos}$ cells throughout the chase period (F) showed constant numbers of PR^{pos} cells, decreasing numbers of $BrdU^{pos}$ cells, and decreasing colocalization of PR and BrdU. (G–J) Colocalization of LRCs with cytokeratin 14- and cytokeratin 18-expressing cells after 9-week chase [BrdU-FITC (G), K14-Texas Red and K18-Texas Red (H), BrdU and K14/18 (I), and DAPI (J)]. Two distinct populations of LRCs expressed K14/K18 (arrowheads) and did not express these markers of differentiation (arrows), suggesting LRCs represent a spectrum of differentiated cells. Scale bars represent 100 (B–E) and 10 μm (G–J).



Sca-1$^{+/GFP}$ Sorted Cell Injections

Mammary epithelial primary cultures were isolated from inguinal and pelvic mammary glands (without the lymph node) of mature virgin C57BL6/129 Sca-1$^{+/GFP}$ mice as described above. Cells were sorted on a Beckman Coulter Altra FACS machine with an argon laser tuned to 488 nm. GFP fluorescence was measured at 525 nm BP filter, GFP^{pos} and GFP^{neg} fractions were collected in DMEM with 2% FBS, and data were analyzed by using Expo 32 software.

Between 1,000 and 10,000 viable Sca-1^{pos} or Sca-1^{neg} cells were injected into each cleared fat pad of Rag-1$^{-/-}$ females as described above. After 6 weeks, the transplanted fat pads were surgically removed and whole mounts were prepared as described above.

RESULTS

BrdU Label Retention in Mammary Epithelial Cells

In an effort to identify quiescent stem cells in the mammary gland, a BrdU label-retention approach was employed that has been used successfully to identify stem cells in the skin (Lavker and Sun, 1982), cornea (Cotsarelis *et al.*, 1989), and hair follicle (Cotsarelis *et al.*, 1990; Taylor *et al.*, 2000). BrdU was administered continuously to mice undergoing ductal morphogenesis when stem cells are proliferating and should, therefore, incorporate the thymidine analog into their DNA. The labeling period was followed by a 9-week chase period during which BrdU retention was monitored via immunofluorescence using a FITC-conjugated anti-BrdU antibody. During the chase, ductal proliferation will continue until the ducts reach the edge of the mammary fat pad. At the end of ductal morphogenesis, the mammary gland will remain relatively quiescent, and stem cells are expected to exhibit lower proliferative and apoptotic indices than more differentiated cell types. Under these conditions, quiescent stem cells should retain BrdU while the proliferating and terminally differentiated cell types will lose the label either following mitosis or after undergoing apoptosis.

Specifically, a continuous dose of BrdU was administered to 3-week-old female mice for 14 days by means of an interscapularly implanted Alzet minipump (Fig. 1A). After the 2-week BrdU dose, minipumps were removed and mammary gland biopsies were taken every week for 9 weeks to monitor label-retaining cells. Approximately 70% of MECs were labeled during the 2-week dose of BrdU (Figs. 1B and 1F). At the end of the 9-week chase period, less than 5% of luminal epithelial cells retained BrdU (Figs. 1C and 1F), and these cells were termed label-retaining cells (LRCs). After the chase period, LRCs ranged in intensity from brightly labeled (C, arrows) to dimly labeled (C, arrowheads), suggesting that LRCs represent a spectrum of cells that have undergone varying numbers of cell divisions.

To characterize the differentiation status of LRCs in the mammary gland, MECs were costained for expression of BrdU and either keratin (K)14/18 or the progesterone receptor (PR). After the 2-week dose with BrdU, approximately 40% of BrdU^{pos} cells also expressed PR (Figs. 1D and 1F). Throughout the chase period the number of colocalizing cells steadily decreased to approximately 1.5% after 9 weeks (Figs. 1E and 1F). The total percentage of PR^{pos} cells did not significantly change during this time period, which is consistent with previous reports (Seagroves *et al.*, 2000). A population of LRCs was detected which did not express K-14 or -18, markers of the myo- and luminal epithelium, respectively (Figs. 1G–1J; arrows). Additionally, a subpopulation of label-retaining luminal cells did express K14/18 (arrowheads), which correlates with a model in which label retaining cells represent a spectrum of quiescent stem cells and early differentiating cells. Thus, these data demonstrate that a population of LRCs does not express common mammary gland differentiation markers, suggesting they are maintained in a less differentiated state.

MG-SP Cells in the Mammary Gland

The efficient efflux of the fluorescent dye Hoechst-33342 has been demonstrated previously to be a mechanistic characteristic of pluripotent hematopoietic stem cells (Goodell *et al.*, 1996). In those experiments, bone marrow cells were treated with Hoechst dye and then analyzed by FACS at two different emission wavelengths. A small, distinct population of bone marrow termed "side population" (SP) cells effluxes the Hoechst dye. Further, these SP cells contain the entire hematopoietic potential of whole bone marrow, establishing their functional capacity as hematopoietic stem cells (Goodell *et al.*, 1996). SP cells with multipotent stem cell characteristics also have been identified in other regenerative tissues, such as muscle (Gussoni *et al.*, 1999; Jackson *et al.*, 1999) and liver (G. Wulf, M.A.G., unpublished observations). Additionally, SP cells from bone marrow and muscle are enriched for the expression of stem cell antigen-1 (Sca-1), a cell surface protein shown to be a marker of pluripotent hematopoietic stem cells (Goodell *et al.*, 1996; Jackson *et al.*, 1999; Spangrude *et al.*, 1988).

A similar SP cell staining protocol was employed to isolate potential mammary gland stem cells from primary MEC cultures. Mammary glands were isolated from mice and manually and enzymatically digested as previously described (DeOme *et al.*, 1959; Pullan and Streuli, 1996). These cells were then stained with Hoechst dye and analyzed by FACS. Similar to bone marrow, the mammary gland contained a distinct population of Hoechst-effluxing SP cells, referred to as MG-SP cells (Fig. 2A). The MG-SP population represented approximately 2–3% of the total population of epithelial cells in the mammary gland, which is consistent with previous estimates of the percentage of stem cells in the mammary gland (Chepko and Smith, 1997). Likewise, the same previous report predicted mammary stem cells to be small with little cellular complexity; forward- and side-scatter plots confirmed MG-SP cells also have these characteristics (data not shown). Treatment with verapamil, a multidrug transporter inhibitor shown to

eliminate the SP population in bone marrow, also reduced the MG-SP population by fourfold (data not shown). Thus, the mammary gland contains a population of cells which efflux Hoechst dye in a manner similar to that described previously for hematopoietic and muscle stem cells.

Analysis of MG-SP Cells

Since Sca-1 is expressed on functional hematopoietic stem cells, it was hypothesized that Sca-1 might also be expressed by the MG-SP cells. Sca-1 is a GPI-anchored protein frequently utilized in murine hematopoietic stem cell enrichment strategies (Spangrude *et al.*, 1988). It is encoded by Ly-6A/E, a member of the Ly-6 superfamily of highly homologous genes conserved from humans to snake (Mao *et al.*, 1996; Ploug and Ellis, 1994). Sca-1 is expressed on some differentiated cell types, including in the kidney and brain, and on T cells, where it has been implicated as a costimulatory molecule (Malek *et al.*, 1986; Miles *et al.*, 1997; Stanford *et al.*, 1997; van de Rijn *et al.*, 1989). Since Sca-1 is expressed on both bone marrow- and muscle-derived SP cells (Goodell *et al.*, 1996; Gussoni *et al.*, 1999; Jackson *et al.*, 1999), its expression on MG-SP cells was characterized.

Primary MECs were incubated with Hoechst dye, stained with an anti-Sca-1 antibody, and then analyzed by FACS. About 20% of the total population of the MECs and 75% of the MG-SP cells were found to be Sca-1^{pos} (Figs. 2B and 2C). These data suggest that the MG-SP population is enriched in Sca-1^{pos} cells.

To minimize the possibility of contamination from blood and lymphatic cells (since Sca-1 is expressed on some hematapoietic cells), only inguinal and pelvic mammary glands that were devoid of lymph nodes were used to make primary cultures. Expression of hematapoietic cell markers was analyzed in the MG-SP and Sca-1^{pos} populations to determine whether these cells resulted from blood contamination. MG-SP cells did not express c-Kit or CD45, markers of hematopoietic stem cells and peripheral blood (Fig. 2C; and data not shown). In addition, the Sca-1^{pos} population in total MEC culture was found to be largely CD45- and lineage marker-negative (Fig. 2D; and data not shown). Similar MG-SP and Sca-1^{pos} populations were also isolated from primary MECs after culturing cells for 5 days with daily media changes (data not shown), indicating that the MG-SP and Sca-1^{pos} cells were epithelial in origin and were not derived from blood contamination.

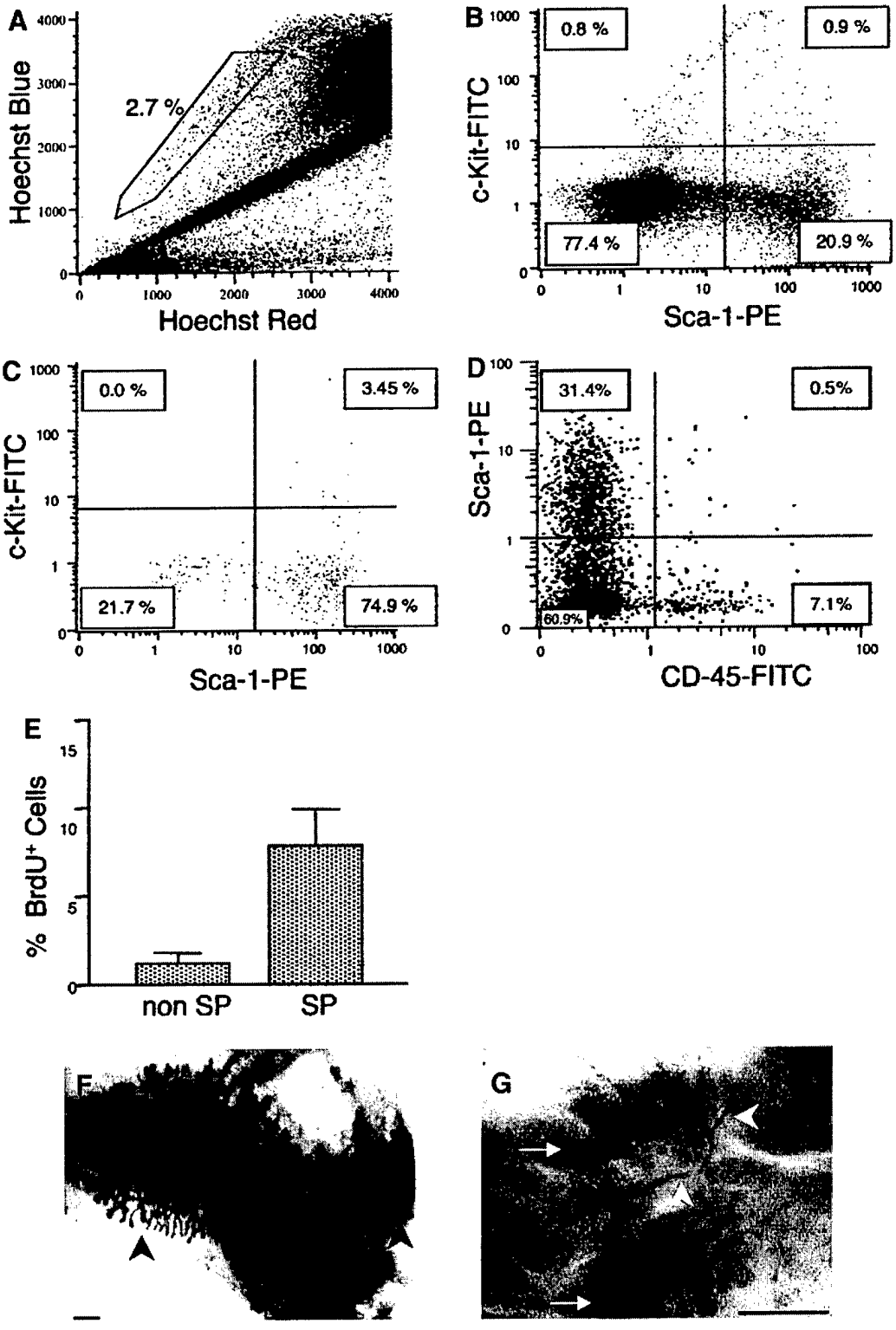
Label Retention in SP Cells

To determine the proliferative nature of the MG-SP cells, the presence of LRCs was analyzed in this population. Primary MECs were isolated from mice that received the long BrdU label followed by the 9-week chase. These cells were then FACS sorted for MG-SP cells, and BrdU^{pos} cells were determined by immunofluorescence. A fourfold increase in LRCs in the MG-SP population was detected as compared to the non-SP population (Fig. 2E). These data suggest that MG-SP cells proliferate during ductal morphogenesis, but remain either more quiescent or less apoptotic than the non-SP population in the mature mouse mammary gland, consistent with their candidacy as mammary gland stem cells.

Outgrowth Potential of Purified MG-SP Cells

To determine whether MG-SP cells are capable of repopulating the mammary gland, MG-SP cells were isolated and transplanted into cleared mammary fat pads in limiting numbers. In this experiment, MECs were isolated from B6;129S-GtROSA26 (ROSA) donor mice and cultured for 5 days. Cells were cultured in this experiment in an effort to improve their viability through the SP-staining and sorting procedure. Cultured MECs retained a MG-SP profile similar to fresh MECs (data not shown). MG-SP cells from the ROSA primary cultures were then isolated by FACS and limiting numbers of cells were mixed with 2×10^5 wild-type C57BL6 primary MECs. This competitive repopulation protocol was used to ensure the presence of any paracrine interactions between MG-SP and non-MG-SP cells that may be required for proper outgrowth. The mixed cell populations were transplanted into the cleared fat pads of Rag-1$^{-/-}$ recipient mice. Immunocompromised recipients were used to prevent host rejection of cells derived from the ROSA mixed background. Recipient mice were bred after 6 weeks to induce lobuloalveolar development in the mammary gland, and outgrowths were isolated 2 weeks later. Whole mounts of outgrowths were stained with X-gal to

FIG. 2. Mammary gland contains a pluripotent population of MG-SP cells. FACS analysis of Hoechst-33342 exclusion reveals a population of cells which effluxes Hoechst dye (A). These cells represent approximately 3% of total mammary epithelial cells and were termed MG-SP cells. This MG-SP population was enriched for expression of Sca-1 (C) when compared to Sca-1 expression in total MECs (B). Sca-1^{pos} cells isolated from total MEC culture do not express the peripheral blood marker CD45 (D). When MG-SP cells were isolated from glands following BrdU incorporation and a 9-week chase, the MG-SP population was enriched for LRCs fourfold compared with the non-MG-SP cells (E). (F, G) Competitive recombination experiments using MG-SP cells isolated from ROSA mammary glands. MG-SP cells, injected into cleared fat pads of host mice, reconstituted an epithelial outgrowth. X-gal staining (indicating the outgrowth originated from ROSA MG-SP cells) revealed two clonal outgrowths (F, arrowheads). Higher magnification (G) illustrates X-gal staining in both ductal (arrowheads) and alveolar (arrows) cell types, suggesting MG-SP cells maintained the ability to differentiate into multiple cell lineages in the mammary gland. Scale bars represent 1 mm.



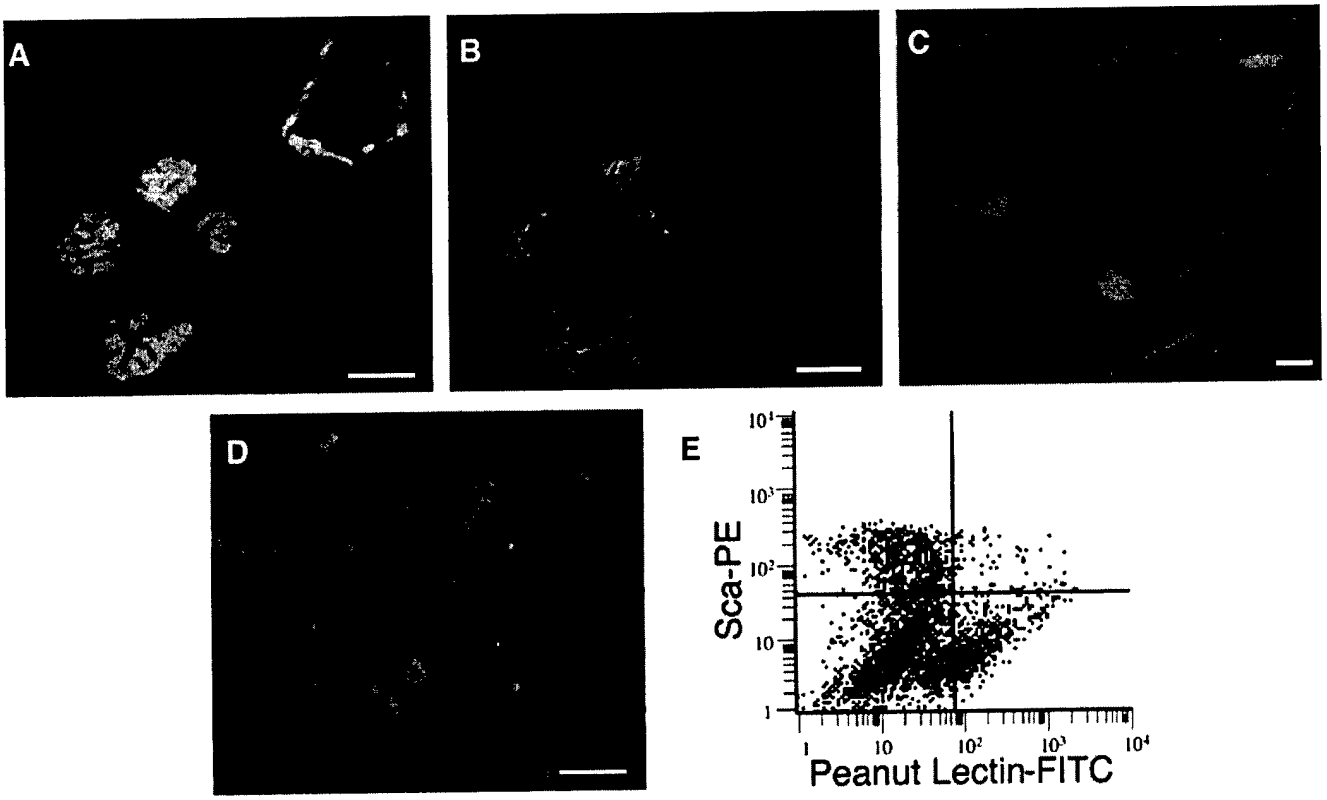


FIG. 3. Expression of Sca-1 in the mammary gland. Mammary glands were removed from Sca-1$^{+/GFP}$ mice, and pieces of gland were transplanted into cleared fat pads of recipient mice. GFP localization was analyzed by immunofluorescence in both endogenous Sca-1$^{+/GFP}$ tissue (C, D) and in transplanted tissue (A, B) (GFP and phalloidin–Texas Red). A confocal composite image (80 × 0.8-μm sections) (A) and a single confocal image (0.8-μm section) (B) from the same region illustrate intense GFP expression at the distal tips of growing ducts, as well as less intense expression in the mature ducts. A cross-section of ductal epithelium shows GFP expression detected sporadically in luminal epithelial cells (C). The abundance of GFP-expressing cells correlates with about 20% Sca-1^{pos} cells detected by FACS analysis (Figs. 2B and 4A). Three-dimensional rotation of this confocal image reveals GFP-expressing cells in contact with the lumen and others located basally. Additionally, GFP immunofluorescence does not colocalize with PR expression in luminal epithelial cells (D). FACS analysis reveals that Sca-1-expressing cells do not bind to peanut lectin, a marker of differentiation in the mammary gland (E). Scale bars indicate 50 (A, B, D) and 10 μm (C).

detect β-galactosidase (β-gal) activity, an indication that such cells originated from the ROSA MG-SP cells. Patches of β-gal-expressing cells could be detected in outgrowths from as few as 2.5×10^4 transplanted ROSA MG-SP cells (data not shown), and robust staining was detected in outgrowths from 7.5×10^4 transplanted ROSA MG-SP cells (Figs. 2F and 2G). In this whole mount, two distinct outgrowths were detected by X-gal staining (Fig. 2F, arrowheads). Higher magnification revealed β-gal-positive cells in both ductal (Fig. 2G, arrowheads) as well as alveolar epithelium (Fig. 2G, arrows). These experiments indicate that purified MG-SP cells have mammary gland outgrowth potential and can contribute to alveolar and ductal epithelial populations.

Previous studies have shown that at least 100,000 total MECs must be injected into the fat pad to reliably form a mammary outgrowth (Smith, 1996). While the current experiments demonstrate outgrowth potential from 25,000 to 75,000 injected MG-SP cells, the combination of proteinase and Hoechst dye treatment and high pressure FACS involved in isolating MG-SP cells resulted in <5% viability (data not shown). Therefore, we estimate that <4,000 viable MG-SP cells were injected and resulted in outgrowth (Figs. 2F and 2G). These results suggest that there was a significant enrichment in outgrowth potential in the MG-SP population as compared to total primary MECs.

Thus, several characteristics of the MG-SP cell population support a stem/progenitor nature of these cells: (1) MG-SP cells comprise a constant 1–3% of the total epithelial population in virgin and pregnant mice; (2) they proliferate when the mammary gland is rapidly establishing the ductal network; (3) they remain quiescent in the mature mouse; and (4) when transplanted, their progeny can de-

velop into ductal and alveolar cell types. However, the low viability of MG-SP cells restricted the use of this approach to further characterize mammary stem cells. Since the MG-SP population was found to be enriched in Sca-1^{pos} cells, Sca-1 expression in primary MECs was investigated as an alternative method to enrich for a population of stem/progenitor cells.

Localization and Characterization of Sca-1^{pos} Cells in the Mammary Epithelium

The localization of Sca-1-expressing cells in the mammary gland was analyzed using a targeted GFP insertion into the Sca-1 locus (Sca-1$^{+/GFP}$). These mice are heterozygous for a mutation in which an EGFP cassette is inserted into the Sca-1 locus, placing it under the regulatory control of the Sca-1 promoter. These mice express GFP in a temporal and spatial manner similar to that observed for Sca-1 in hematopoietic cell lineages, and although this mutation disrupts the endogenous Sca-1 allele, Sca-1 haploinsufficiency has no important consequences for hematopoiesis (T. Graubert, unpublished observations).

Frozen sections of Sca-1$^{+/GFP}$ glands were analyzed by confocal microscopy, and Sca-1-GFP expression was detected scattered throughout the ductal luminal epithelium (Figs. 3A–3D). Pieces of mammary tissue from Sca-1$^{+/GFP}$ donor mice also were transplanted into the cleared fat pads of wild-type recipient mice, and the resulting outgrowths were harvested 8 weeks later. The images shown in Figs. 3A and 3B were derived from these outgrowths of transplanted Sca-1$^{+/GFP}$ tissue. Even following proliferation and differentiation resulting from transplantation, Sca-1-GFP is expressed in the same pattern in these outgrowths as endogenous Sca-1-GFP. Interestingly, more intense GFP expression was detected specifically at the distal tips of growing ducts (Figs 3A and 3B), with decreased expression also detected scattered throughout mature ducts (Figs. 3A–3C). Presumably, this difference in expression level may be attributed to the long half-life of GFP, which may be 1–2 days. Thus, these observations of Sca-GFP expression (intense at the distal tips of growing ducts and scattered sporadically throughout the luminal epithelium) are consistent with the expected distribution of stem or progenitor cells, as predicted from transplantation experiments (Kordon and Smith, 1998).

In an effort to characterize the differentiation status of these Sca-GFP cells, expression of PR and interaction of these cells with peanut lectin were analyzed (Figs. 3D and 3E). Immunofluorescence experiments revealed that Sca-1-GFP and PR expression observed in mature ducts did not colocalize in the same cells (Fig. 3D). Likewise, FACS analysis of these Sca-1-GFP^{pos} cells showed essentially no overlap with cells binding peanut lectin (Fig. 3E), a marker of differentiation in the mammary gland which appears to interact with MUC4 on mammary epithelial cells (Li *et al.*, 2001; Rudland, 1992). These data suggest that the Sca-1^{pos} cells, even those in the mature duct, represent a population of less differentiated cells.

Sorting Mammary Epithelial Cells Based on Sca-1 Expression

The apparent relationship of MG-SP, LRCs, and Sca-1^{pos} cells suggested that it might be possible to isolate mammary stem cells with improved viability using their Sca-1^{pos} status as a criterion for selection (Fig. 4). Accordingly, an immunoaffinity technique was employed using an anti-Sca-1 antibody conjugated to microbeads via a biotin/streptavidin interaction. Freshly prepared primary MECs, isolated from inguinal mammary glands void of lymph nodes (to reduce muscle and lymphocyte contamination), were incubated with biotinylated anti-Sca-1 antibody and streptavidin-conjugated beads. These were then applied to a column in a magnetic field. The Sca-1^{pos} cells that adhered to the magnetic column were washed and then eluted by removing the column from the magnetic field. FACS analysis verified that this method depleted Sca-1^{pos} cells in the flow-through fraction but enriched for a Sca-1^{pos} population in the eluate (Figs. 4A–4C). The bound cell fraction (Fig. 4C) exhibited a 3.5-fold increase in Sca-1^{pos} cells, while the depleted population (Fig. 4B) displayed a 3.5-fold decrease in Sca-1^{pos} cells, as compared to the starting MEC population (Fig. 4A).

To determine the presence of LRCs in the Sca-1^{pos} population, primary MECs were isolated from mice following long-term BrdU labeling and a 9-week chase period. These cells were sorted based on Sca-1 expression by using the magnetic cell sorting technique. Following purification, Sca-1^{pos} and Sca-1^{neg} cells were cytospun onto coverslips, stained for BrdU immunofluorescence, and manually counted. Approximately 19% of purified Sca-1^{pos} cells retained BrdU after the 9-week chase as compared with 7–8% of the cells in the Sca-1-depleted population (Fig. 4D), a greater than twofold enrichment. These data were confirmed by BrdU immunofluorescence and FACS analysis of Sca-1-enriched and Sca-1-depleted populations (data not shown). Thus, Sca-1^{pos} cells isolated from mouse mammary gland primary cultures contain an enriched population of growth quiescent cells, a characteristic consistent with their role as putative stem/progenitor cells.

These data demonstrate that both the MG-SP and Sca-1^{pos} populations contain a subpopulation of quiescent cells (LRCs) with low turn-over rates. However, since the MG-SP cells contain a fourfold enrichment in LRCs while Sca-1^{pos} cells are only enriched twofold, this suggests that the MG-SP cells are less proliferative than the Sca-1^{pos} cells. One possible model to explain these results is that Sca-1 is expressed in a broader population of cells that contain subpopulations of both MG-SP and LRCs (see Fig. 6). This subpopulation of Sca-1^{pos} /mg-SP/LRC cells may represent a less proliferative, more primitive population of progenitor/stem cells.

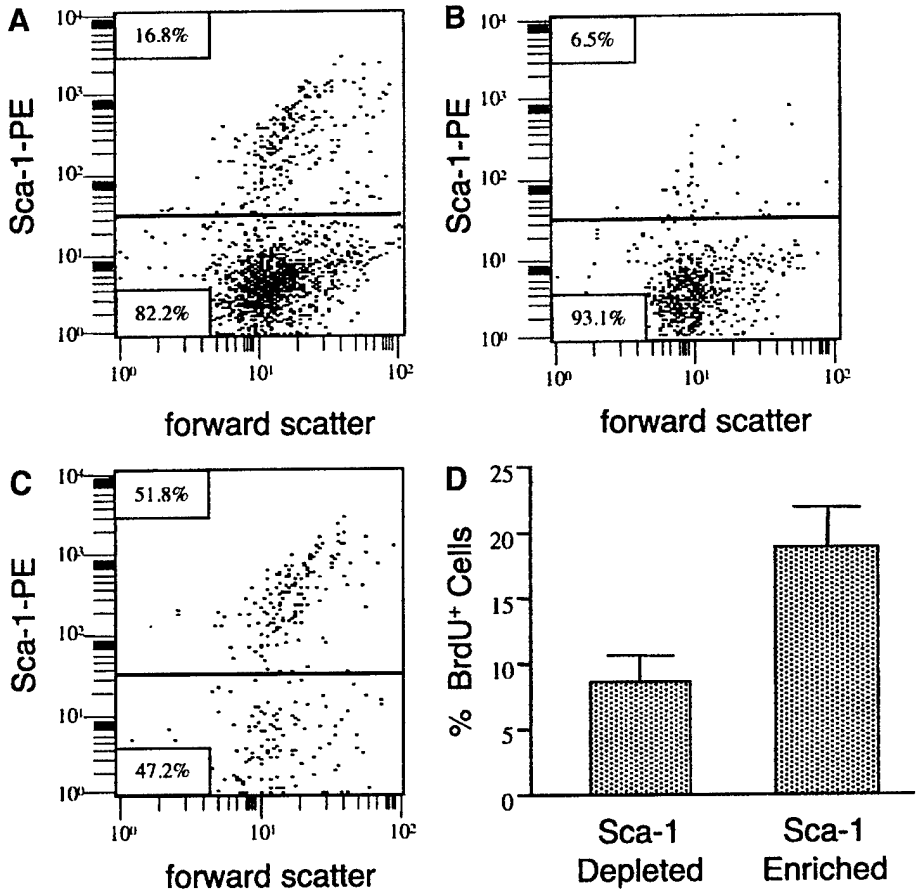


FIG. 4. Enriching Sca-1^{pos} cells by magnetic sorting. Sca-1^{pos} cells were isolated from total mammary epithelial cells by immunosorting using biotinylated anti-Sca-1 antibodies and streptavidin-conjugated microbeads. The results of a single round of enrichment are illustrated in (A–C): whole mammary epithelial cell fraction (A), Sca-1-depleted fraction (B), and Sca-1-enriched fraction (C). When these fractions were analyzed for LRCs, the Sca-1-enriched fraction contained a twofold enrichment of LRCs compared with the Sca-1-depleted fraction (D).

Outgrowth Potential of Sca-1-Enriched Cells

To characterize the regenerative potential of Sca-1^{pos} cells, transplantation experiments into cleared fat pads of syngeneic host mice were again employed by using the Sca-1-enriched and Sca-1-depleted populations of freshly prepared primary MECs (Fig. 5A). Cells were sorted using two rounds of magnetic enrichment, and purity reached 86% Sca-1^{pos} and 92% Sca-1^{neg} cells in the enriched and depleted fractions, respectively. Either 10,000 or 50,000 cells were injected into each cleared fat pad, and after 6 weeks the outgrowths were removed from the mice and stained as whole mounts with hematoxylin to visualize the epithelium. The extent of outgrowth was defined as partial outgrowth (ductal structures fill 5–50% of the fat pad; Fig. 5B), full outgrowth (ductal structures fill >50% of the fat pad; Fig. 5C), or no epithelial outgrowth. All Sca-1-enriched injections formed a partial or full outgrowth, while only 4 out of 10 Sca-1-depleted injections formed any outgrowth (Fig. 5A).

To achieve higher purities, a complementary approach was taken, using low pressure FACS sorting of primary MECs isolated from Sca-1$^{+/GFP}$ mice. Using this FACS-based method, the GFP^{pos} population was enriched to >90% positive, while the depleted population was 99% pure (Fig. 5A). Between 1,000 and 10,000 cells were injected into each cleared fat pad, and outgrowths were harvested after 6 weeks. All six injections of GFP^{pos} cells resulted in an outgrowth, including a minimum of only 1,000 or 2,000 cells injected. Of the six GFP^{neg} injection sites, none formed an outgrowth (Fig. 5A). These data complement the Sca-1-bead sorting technique, offering a purer population of Sca-1^{pos} cells and subsequent increased outgrowth potential. Thus, Sca-1^{pos} cells isolated from mammary gland primary cultures appear be enriched for a population of multipotent progenitor cells and contain increased outgrowth potential. Most significantly, depletion of the Sca-1^{pos} cells from a population removes outgrowth potential from that population.

A

Enrichment Method	Sca-1 Positive			Sca-1 Negative		
	Cell No.	Outgrowth	Purity	Cell No.	Outgrowth	Purity
Magnetic Bead Sorting	10,000	Full	86%	10,000	–	92%
	10,000	Partial		10,000	Partial	
	10,000	Partial		10,000	Full	
	10,000	Partial		10,000	–	
	50,000	Full		50,000	–	
	50,000	Full		50,000	Partial	
	50,000	Full		50,000	Full	
	50,000	Full		50,000	–	
	50,000	Partial		50,000	–	
	50,000	Partial		50,000	–	
Sca-1-GFP	1000	Partial	91%	1000	–	99%
	2000	Full		2000	–	
	2000	Partial		2000	–	
	5000	Full		5000	–	
	5000	Partial		5000	–	
	10,000	Full		10,000	–	

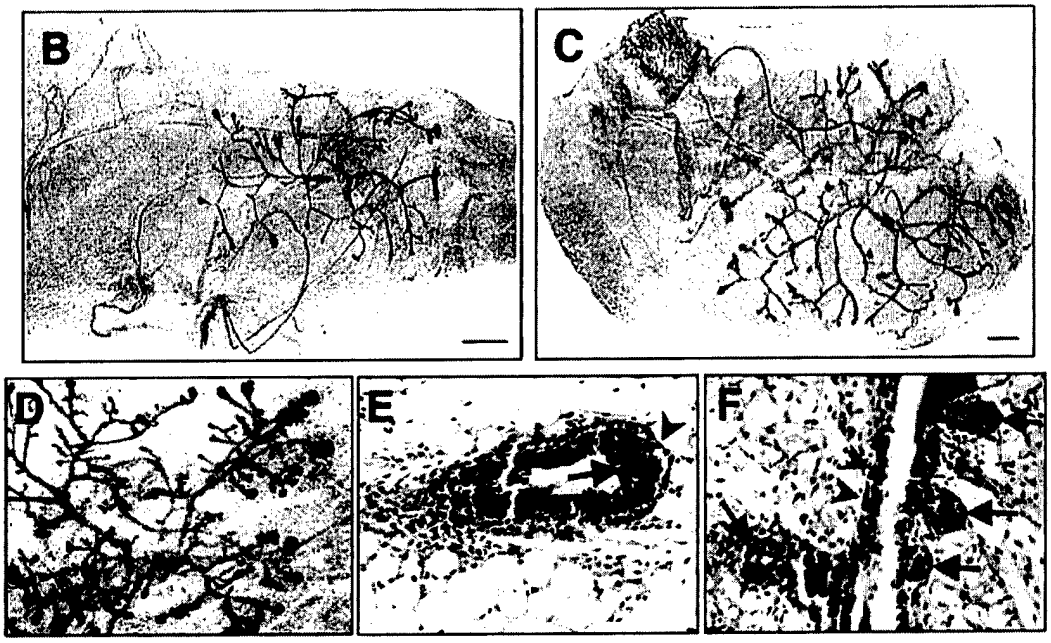


FIG. 5. Outgrowth potential of Sca-1-sorted MECs. Using two methods of sorting, mammary epithelial cells were enriched for or depleted of Sca-1-expressing cells and were injected into cleared fat pads in a standard mammary reconstitution assay. Outgrowths were harvested after 6 weeks and the epithelium in the whole mounts was visualized by hematoxylin (B, C). (A) The sorting methods, purity of the sort, number of cells injected, and the extent of epithelial outgrowth. Injected cells formed either no epithelial structure (denoted as "–"), partial outgrowth, or full outgrowth; partial outgrowth was defined as 5–50% of the fat pad filled with epithelial ductal structures (B), while full outgrowth was defined as >50% of the fat pad filled (C). (D–F) Normal ductal and alveolar development was detected in outgrowths derived from GFP^{pos} cells. Whole mounts (D) and H&E-stained sections (E, F) of outgrowths derived from GFP^{pos} cells showed luminal, myoepithelial (F, arrowheads), and TEBs (E) with normal cap (arrowhead) and body (arrow) cell layers. Alveolar buds were also observed in these outgrowths in early pregnant mice (D, F, arrows). Scale bars indicate 1 mm.

Since the Sca-1-enriched cells produced a morphologically normal outgrowth, it was expected that all three epithelial cells types (luminal, myoepithelial, and alveolar cells) should be present in these outgrowths, as interactions between these cells with their environment is required for normal mammary gland growth and differentiation. To this

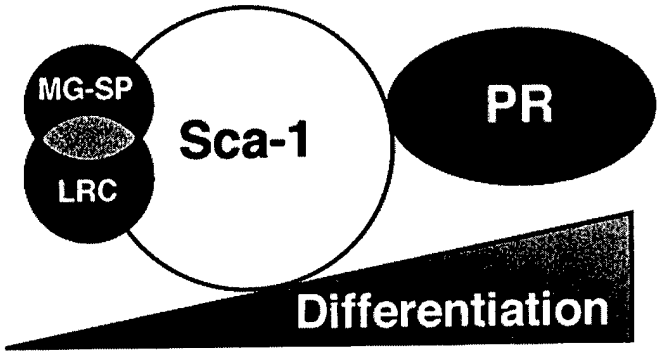


FIG. 6. Model of MEC progenitors. Mammary epithelium can be classified into several distinct cell populations, including MG-SP, Sca-1^{pos}, and PR^{pos} cells. In this model, these populations may represent various stages of epithelial differentiation. LRCs are found primarily in the Sca-1 and MG-SP populations, suggesting that these groups contain within them a quiescent subpopulation. The MG-SP population may represent the most primitive and least differentiated subclass and may contain the multipotent stem cell population.

end, sections of the whole mounted outgrowths were stained with hematoxylin and eosin (H&E) and analyzed by light microscopy to study the morphology of Sca-1-enriched outgrowths. Outgrowths derived from GFP^{pos} cells contained normal luminal and myoepithelial cells as well as TEBs with distinct cap and body cells (Figs. 5D–5F), indicative of normal ductal morphogenesis (Williams and Daniel, 1983). Additionally, outgrowths derived from GFP^{pos} cells formed normal alveolar buds during early pregnancy (Figs. 5D and 5F). These data suggest that Sca-1^{pos} cells isolated from primary MEC culture have the potential to differentiate into multiple mammary epithelial cell types.

DISCUSSION

In this study, several complementary approaches have been employed to identify and, for the first time, enrich mammary epithelial cells that retain stem cell characteristics. Long-term BrdU label retention studies, FACS sorting for an MG-SP population, and Sca-1 enrichment all use differing criteria to identify cohorts of mammary epithelial cells with overlapping stem cell characteristics. These populations of cells collectively represent a spectrum of undifferentiated and differentiated cells with unique molecular and regenerative characteristics.

Differentiation in the Mammary Gland

Cell lineage and early differentiation markers are largely uncharacterized in the mammary gland, unlike many other model systems of development. While decades of work have analyzed milk protein production as a marker of functional differentiation during pregnancy and lactation, few markers of differentiation during virgin development exist and these remain controversial. Here, the identification of Sca-1 as a marker of undifferentiated cells contributes not only to our understanding of stem/progenitor cells in the mammary gland, but also contributes to the body of knowledge regarding differentiation in the mammary gland.

In this study, two populations of cells, identified independently, share characteristics of progenitor/stem cells and remain less differentiated than their neighbors. Neither the LRCs nor the Sca-1^{pos} cells express PR, which has been shown to be expressed in nonproliferating, differentiated mammary epithelial cells (Brisken *et al.*, 1998; Russo *et al.*, 1999; Seagroves *et al.*, 2000). In the current study, however, administration of BrdU for 2 weeks identified >40% of cells that divided during the labeling period also expressed PR, but after the chase period, <5% of BrdU^{pos} cells expressed PR. Taken together, these data suggest that PR-expressing cells do not undergo proliferation, yet cells that have undergone proliferation are capable of eventually expressing PR. Although the exact lineage of differentiation in the mammary gland is unknown and controversial, we propose that proliferating progenitor cells can give rise to more differentiated cells which then exit the cell cycle and remain quiescent.

Mammary Epithelial Sca-1^{pos} Cells Have Increased Outgrowth Potential

The Sca-1$^{+/GFP}$ outgrowths demonstrated that a highly pure population of Sca-1^{pos} cells retained outgrowth potential (100%) compared with a Sca-1-depleted population (0%). In this experiment, as few as 1,000 Sca-1^{pos} cells were necessary to form an outgrowth. Since theoretically, only one stem cell is required to reconstitute an entire gland, injecting 1,000–10,000 cells may not appear to be a significant enrichment. However, unlike hematopoietic cells, which survive naturally in suspension, mammary epithelial cells exist in tissue organization and require paracrine interactions associated with cell–cell adhesion. Therefore, a single mammary stem cell injected into a fat pad would undoubtedly fail to grow out due to the lack of requisite signals and support provided from neighboring epithelial cells. Previous studies have shown that >100,000 total cells must be injected into the fat pad to produce a mammary outgrowth in about 60% of transplant sites (Smith, 1996). Thus, these experiments demonstrate two points: (1) when injecting a highly purified population of Sca-1^{pos} cells, 10–100× fewer cells (compared to whole cell population) can be used to achieve consistent outgrowth, and more importantly, (2) depletion of the Sca-1^{pos} population removes outgrowth potential.

Smith and colleagues have demonstrated that at least three multipotent cell populations with distinct outgrowth potential exist in the mammary gland. These three multipotent cell populations include cells capable of (1) complete mammary gland outgrowth, (2) ductal morphogenesis, or (3)

lobuloalveolar development (Chepko and Smith, 1997). The current study found that Sca-1^{pos} cells retain the ability to contribute progeny to all cell types required to produce a normal epithelial outgrowth. However, the Sca-1^{pos} population could represent a homogenous, multipotent population or a heterogeneous population of more committed progenitor cells. Further dilution experiments will be necessary to distinguish between these possibilities, by determining clonality of the outgrowths, and by analyzing senescence in serial transplants.

Relationship of Mammary Epithelial Cells

Several decades of mammary gland research have demonstrated the regenerative capacity of the mammary gland, verifying the presence and potency of mammary gland stem and progenitor cells (Daniel *et al.*, 1968; DeOme *et al.*, 1959; Hoshino and Gardner, 1967). Stem cells in the mammary gland are expected to contribute to all mammary gland cell types including luminal, myoepithelial and alveolar. The progeny from mammary gland stem cells should not only maintain the ductal networks in a mature animal but also produce precursor cells capable of differentiation. This implies that the mammary gland consists of a mixed population of cells within a spectrum of differentiation states. Data presented here demonstrate the presence of several populations in the mammary gland with different regenerative capabilities and molecular markers that characterize these populations. Mammary gland Sca-1^{pos} cells make up about 20% of the total epithelial population, have increased outgrowth potential, are enriched in LRCs and MG-SP cells and are PR negative (Fig. 6). Sca-1^{neg} cells make up about 80% of the epithelial population, have few LRCs, have decreased outgrowth potential and express differentiation markers PR and K18.

From these results, at least three cell populations in the mammary gland can be defined: PR^{pos}, Sca-1^{pos}, and MG-SP cells. These results suggest that Sca-1^{pos} and MG-SP cells are not mutually exclusive and represent overlapping populations (Fig. 6). The PR^{pos} cells (and peanut lectin/MUC 4 cells), however, are excluded from the Sca-1^{pos} and presumably the MG-SP populations. The label retention technique demonstrated that the MG-SP and Sca-1^{pos} cells proliferate during ductal development, but are quiescent in the mature animal. Characteristics of these populations make them potential candidates for mammary epithelial stem/progenitor cells.

Confocal microscopy revealed some of the GFP-positive cells appeared to contact both the basement membrane and the lumen. Interestingly, previous EM studies have shown that the small light cells (SLC) have limited contacts with the lumen and are primarily basally located (Chepko and Smith, 1997). Since Sca-1^{pos} cells consistently comprised about 20% of the total population of the mammary epithelium and exhibited contact with both the lumen and basement membrane, it is unlikely that these cells exclusively represent the SLC population. Rather, it is possible that the SLCs comprise a subpopulation of the larger Sca-1^{pos} population; likely candidates for the SLC subpopulation are the LRC/mg-SP cells. Recent identification of the functional ABC transporter (brcp1/ABCG2) in SP cells (Zhou *et al.*, 2001) may now provide an alternative method by which to isolate MG-SP cells, avoiding the toxicity problems experienced with the Hoechst dye-staining and sorting protocol.

In the mammary gland, MG-SP cells represent a morphologically homogenous population of small cells with low cytoplasmic to nuclear ratios and decreased forward and side scatter profiles (data not shown), suggesting that these cells do not retain specialized secretory or metabolic function. The low percentage of these cells found in the mammary gland and their distinct morphological properties make MG-SP cells prime candidates for a primitive stem cell population in the mammary gland. However, further analysis of the capacity of MG-SP cells to clonally expand into complete mammary glands upon transplantation will be required to further define the stem cell qualities of this population

Stem Cells and Breast Cancer

The unique replicative capacity and clonal expansion of stem and progenitor cells makes these cells susceptible to transformation; it is likely that mutations occurring during stem cell proliferation may by perpetuated throughout the mammary gland, potentially leading to carcinogenesis. This model is supported by several lines of research: (1) in rat DMBA breast cancer models, the targets of carcinogen-induced transformation are the TEBs (the prospective site of stem cells during ductal morphogenesis), and (2) stem cell markers have been shown to be upregulated in human breast cancer (Dulbecco *et al.*, 1986; Russo *et al.*, 1982; Smith and Chepko, 2001; Smith *et al.*, 1990). Previous studies have found that Sca-1 is upregulated in carcinoma cell lines (including mammary lines), and higher levels of Sca-1 correlated with more aggressive, tumorigenic cell lines (Cohn *et al.*, 1997; Katz *et al.*, 1994; Treister *et al.*, 1998). Preliminary data also suggest that mammary epithelial tumor cell lines yielding more aggressive tumors express higher levels of Sca-1 than less aggressive tumor cell lines (B.E.W., S.B.T., J. Rosen, D. Medina, unpublished observations). Therefore, given the suggestion that Sca-1^{pos} cells may be targets of transformation, it will be interesting to further characterize the status of Sca-1 expression in various breast cancer models.

In summary, this study is the first to report the isolation of functional mammary stem/progenitor cells. While the existence of stem cells in the mammary gland has been previously proven by transplantation studies, the isolation of this population has remained elusive until now. The availability of this isolated population should permit the identification of additional markers that can be used to follow cell lineages during normal mammary development and the progression of breast cancer. The purification of this

population can also be used as a tool to efficiently produce genetically modified outgrowths. Finally, studies are in progress to compare the similarities and/or differences between these mammary progenitor cells with those isolated from other tissues, as well as to analyze their reciprocal tissue plasticity.

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