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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

The mammary gland is made up of several epithelial cell populations including luminal, myoepithelial, stem and progenitor cells. It is not understood how these cell populations or the differentiation pathways that generate them contribute to tumorigenesis. The experiments in this proposal investigate the role of mammary epithelial cell differentiation processes in tumorigenesis. FACS sorted mammary epithelial cells expressing Sca-1exhibit enhanced transplantation potential and may be enriched for stem or progenitor cells[1] . We will further refine the Sca-1 cell population by screening mammary epithelial cell primary cultures by FACS to identify markers that are coexpressed on a subset of Sca-1 cells. Once markers have been identified then the isolated cell populations will be assayed for outgrowth potential and differentiation marker expression. The Sca-1 cell population will also be analyzed in mouse models of tumorigenesis. We are also developing new mouse models that can used to study how genes expressed in mammary Sca-1 cells affect differentiation and tumorigenesis.

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Table of Contents

INTRODUCTION:

The mammary gland is made up of several epithelial cell populations including luminal, myoepithelial, stem and progenitor cells. It is not understood how these cell populations or the differentiation pathways that generate them contribute to tumorigenesis. The experiments in this proposal investigate the role of mammary epithelial cell differentiation processes in tumorigenesis. FACS sorted mammary epithelial cells expressing Sca-1exhibit enhanced transplantation potential and may be enriched for stem or progenitor cells[1]. We will further refine the Sca-1 cell population by screening mammary epithelial cell primary cultures by FACS to identify markers that are coexpressed on a subset of Sca-1 cells. Once markers have been identified then the isolated cell populations will be assayed for outgrowth potential and differentiation marker expression. The Sca-1 cell population will also be analyzed in mouse models of tumorigenesis. We are also developing new mouse models that can used to study how genes expressed in mammary Sca-1 cells affect differentiation and tumorigenesis.

BODY:

Task 1. **Characterize mammary epithelial cell populations in tumors and the normal gland**

a) Identify cell populations by FACS analysis in mammary epithelial primary cultures isolated from normal and hyperplastic mammary glands and from tumors.

Results: Freshly prepared primary mammary epithelial cells (MECs) that express the Sca-1 cell surface marker display enhanced stem cell activity in functional transplantation assays [1]. This task seeks to characterize the expression of the Sca-1 marker in hyperplastic and tumor tissue. We have analyzed several mouse mammary gland tumors and hyperplasias for Sca-1 by FACS and have reported some of this data (see appendix) [2]. The mammary tumor mouse models that we focused on include the MMTV-Neu, -Wnt1, and -Polyoma middle T (PyMT) mice. Primary MECs were isolated from wildtype glands, Neu-induced hyperplasias and tumors, Wnt1 induced hyperplasias and tumors, and PyMT-induced tumors and were FACS analyzed for Sca-1 (Figure 1). Immunohistochemisty of MEC differentiation markers was also performed on the same mouse models in this collaboration. These data suggest that several mouse models of mammary tumorigenesis can be separated into "progenitor" type tumors that display myoepithelial and luminal cell markers as well as Sca-1 and "luminal" type that are restricted for expression of the luminal epithelial cell marker keratin-8 (see appendix).

Another goal of this project is to further refine the Sca-1 cell population by identifying markers that are either coexpressed or absent on Sca-1 MECs. If a cell surface marker can be identified on Sca-1 cells that marks (or is absent) on a fraction of those cells then further enrichment of MEC stem cell activity may be achieved. Results from screening wildtype tissue suggest that CD24 and Sca-1 can be resolved as distinct cell populations containing Sca-1 alone, Sca-1/CD24 double positive, CD24 alone and double negative cell populations (Figure 2). In freshly prepared wildtype mammary glands Sca-1 and CD24 are each expressed on about 30% of MECs. About 30% of the Sca-1 cells and 30% of the CD24 cells are double positive. Using CD24 as a second marker Sca-1 cells can be refined into Sca-1 alone cells representing about 15-20% of total freshly prepared MECs and Sca-1/CD24 double positive cells representing about 5-10% of MECs. Since the MEC Sca-1 population has been shown to have enriched MEC stem cell activity the ability of CD24 to further resolve the Sca-1 population could provide a tool for further enrichment of MEC stem cells. Subtasks *b* and *c* below characterize marker expression and outgrowth potential of the Sca-1 and CD24 sorted cells. Additionally, CD24 is a small GPI-anchored mucin-like protein that can bind to P-selectin expressed on activated endothelial cells and may mediate rolling of breast carcinoma cells along blood vessels [3]. This function may influence breast carcinoma metastasis. Thus, we screened mouse mammary tumor models for Sca-1 and CD24 (figure 3). We found that while Sca-1 expression is dependent on the tumor oncogene CD24 positive cells increase in all mouse tumor models (Figure 3). This is consistent

with an increase in expression of CD24 in human breast carcinomas. Additionally, the percentage of Sca-1/CD24 double positive cells changes between tumors and hyperplasias and between tumor models. For example, in Wnt-1 induced hyperplasias about 70% of CD24 positive cells also express Sca-1, however, in tumors the number of double positive cells decrease to about 50% (Figure 3b).

b) Characterize gene expression in sorted cell populations by RT-PCR and Western blot analyses.
Results: We have examined the expression by immunofluorescence of K14 (myoepithelial marker), K8 (luminal epithelial marker) and K6 (putative progenitor marker) in sorted Sca-1 cells from wildtype (Figure 4) and MMTV-Wnt1 induced hyperplasias and tumors [2] . For most of these experiments MECs were cultured for 5-7 days on plastic, trypsinized and FACS analyzed. Culturing the primary MECs prior to FACS analysis allows the cells to more easily disaggregate and results in increased sorting efficiency. However, culturing prior to sorting also altered cell surface marker profiles when compared with sorting fresh MECs (see Task1 discussion below). We observed that in cultured MECs Sca-1 alone cells were mostly negative for the keratin markers K6, K8 and K14 (Figure 4). However, Sca-1^{high}/CD24 and Sca-1^{low}/CD24 double positive cells were positive for all three markers. The most K6 positive cells were found in the Sca-1^{low}/CD24.

c) Transplant enriched epithelial cell populations into cleared fat pads.
Results: We have transplanted FACS sorted and enriched cell populations from MMTV-PyMT tumors and wildtype mouse mammary glands into the cleared fat pads of syngeneic mice. These data suggest that in cultured MECs the Sca-1 alone cell population is not enriched for stem cell activity or tumorigenic potential. Rather, the Sca-1/CD24 double positive population displays both tumorigenic potential and outgrowth capacity when isolated from MMTV-PyMT and wildtype tissue, respectively (Figures 5 and 6). If K6 is a marker for mammary gland progenitor cells then these data are consistent with Task1b data that the Sca-1/CD24 double positive cells isolated from wildtype tissue contain the most K6 positive cells. Interestingly, in wildtype tissue the Sca-1^{low}/CD24 double positive cells contained about 2.5-times more K6 cells then Sca-1^{high}/CD24 and they also exhibited the best transplantation take-rate (100%) and the most growth in the fat pad (70% of the transplants had full outgrowth). When Sca-1/CD24 cells were transplanted from MMTV-PyMT induced tumors the Sca-1 alone cells displayed very poor tumor growth (Figure 6). Both Sca-1^{high}/CD24 and Sca-1^{low}/CD24 sorted cell population exhibited tumorigenic potential (Figure 5).

d) Analyze Sca-1 localization in Sca-1^{GFP}/ MMTV-Neu and Sca-1^{GFP}/ MMTV-Wnt1 mouse mammary tumors using real-time two-photon confocal imaging.
Results: We have optimized a technique to visualize in real-time cell-cell interactions in the mammary gland of living mice (Figures 7 and 8). A major portion of this task has been to develop methods to anesthetize mice for 4-12 hours while maintaining body temperature, hydration and respiration and develop surgical techniques to expose and immobilize the mammary gland without rupturing blood vessel. Additionally, we optimized microscope settings and optics to perform this technique. This imaging technique provides insight into the cell types that express Sca-1, their location in the tissue and interaction with other cells. We use mice that have EGFP knocked-in to the Sca-1 locus to visualize Sca-1 expressing cells in the mammary gland. Sca-1 is also expressed in the blood vessels and leukocytes so it provides a nice imaging tool to visualize tumor interactions with the vasculature (Figure 7). We have bred MMTV-PyMT and MMTV-Wnt-1 to Sca1-EGFP mice allowing us to visualize Sca-1 positive cells in hyperplasias and tumors. We have decided to use MMTV-PyMT mice instead of MMTV-Neu

(as proposed) since MMTV-PyMT and –Neu both have similar "luminal" tumor phenotypes but PyMT induced tumors arise much faster.

By FACS analyses about 10% of cells isolated from MMTV-PyMT tumors were positive for Sca-1. We were interested in determining if the low number of Sca-1 cells in the PyMT tumors represent a "tumor stem cell" that could give rise to luminal cell types. However, when we imaged Sca1-EGFP/MMTV-PyMT tumors we observed that the tumor epithelium is negative for EGFP (Figure 7). All EGFP positive cells in the PyMT tumor appeared to be either endothelial cells or infiltrating leukocytes. This imaging suggests that the 10% Sca-1 cells found in the PyMT induced tumors most likely represent blood vessels and leukocytes isolated during primary culture preparation of the MECs. By contrast, the MMTV-Wnt-1 induced tumors are highly positive for Sca-1 expression and exhibit expression of EGFP in the tumor epithelium. Using this imaging technique we have been able to confirm that Sca-1 is differently expressed in tumor epithelium and to visualize interactions of tumor cells with infiltrating leukocytes in both MMTV-PyMT and MMTV-Wnt-1 mouse models.

Task 1

Discussion: This task seeks to characterize the Sca-1 marker and identify novel markers in the normal mammary gland and tumors that can be used to characterize mammary epithelial cell progenitors during tumorigenic progression. We have found that several mouse mammary tumor models can be divided into "progenitor" and "luminal" phenotypes. Progenitor tumor phenotypes contain cells that express myoepithelial and luminal cell types and exhibit some degree of organization. In many of these tumors the K14 (myoepithelial) and K8 (luminal) positive cells are structured into distinct concentric layers suggesting organizational signals are maintained. Putative progenitor cell markers such as keratin-6 and Sca-1 are also highly expressed in these tumors and their initial hyperplasias. Interestingly, progenitor type tumors are mainly observed from oncogenes in the Wnt/β-catenin pathway including Wnt-1, Myc, and β-catenin. Furthemore, squamous metaplasia is often observed in regions of these tumors. In contrast, luminal tumor phenotypes are mainly observed with receptor tyrosine kinase pathway oncogenes such as Neu, Ras and PyMT. These tumors are poorly organized and express almost exclusively the K8 luminal epithelial marker. These data suggest that specific oncogenic pathways expand a particular cell type that can give rise to the tumor mass. Since these tumors result from clonal expansion the progenitor tumor phenotypes must be derived from a cell type that can give rise to both luminal and myopeptithelial cells while luminal tumors are somehow restricted in this differentiation. We are interested in determining whether the luminal inducing oncogenes are forcing a specific differentiation pathway while progenitor inducing oncogenes are maintaining MEC stem or progenitor cells. Further studies will determine whether progenitor type hyperplasias have enhanced stem cell activity when assayed by limited dilution transplantation.

We have also identified a new marker, CD24, expressed on a subset of Sca-1 MECs. We have further characterized cells that express this marker in Task1*b* and *c*. We have examined the expression of CD24 and Sca-1 in mouse mammary tumor models and we have performed transplantation and IHC on sorted Sca-1/ CD24 cell populations. Several observations and technical issues have been raised from these experiments that must be addressed in further experiments. The Sca-1 marker does not appear to have restricted expression in primary MECs that are cultured for several days on plastic. The expression of Sca-1 increases from about 20-30% positive total cells in freshly prepared primary MECs to about 80% in MECs cultured on plastic. Furthermore, many immortalized MEC cell lines such as EpH4 and SCP2 cells also express high levels of Sca-1. However, cells that are cultured in Matrigel express Sca-1 in only about 15-20% of epithelial cells suggesting that culturing conditions are critical to maintain a restricted expression pattern for Sca-1 (Figure 9). Interestingly, even primary MECs of PyMT tumors cultured on plastic are highly positive for Sca-1 while by *in vivo* imaging and FACS of

freshly prepared MECs the tumor epithelium is negative for Sca-1. Thus, an increase in Sca-1 expression when culturing on plastic appears to be an artifact of the conditions. The FACS sorting and transplantation experiments performed for Task1 *b* and *c* used MECs cultured on plastic and the results were not expected. The data from Task1 *b* and *c* suggest that cells expressing low levels of Sca-1 and that were positive for CD24 were enriched for K6 expression and correlate with better outgrowth of normal mammary epithelium than cells expressing high levels of Sca-1. The Sca-1 alone cells (CD24 negative) gave very poor outgrowth and were mostly negative for K6, K14 and K8. These data suggest that Sca-1 alone cells most likely are contaminating endothelial cells found in the primary culture preparation. Thus, it appears when cultured on plastic, low levels of Sca-1 correlate with greater stem cell activity and experiments now have to be redesigned to maintain restricted Sca-1 expression. The sorting and transplantation experiments will be repeated using freshly prepared primary MECs to complete this task.

Task 2. **Characterize rtTA expression in Sca-1-rtTA mice.**
Results: Upon testing a rtTA construct we determined that rtTA had high dox-independent transcription activity (Figure 10). We found that in the presence of dox only a 4-fold increase in transcriptional activity was observed mainly due to high background activity. To reduce the dox-independent rtTA activity we subcloned an rtTA-IRES-tTS insert into the Sca-1 targeting plasmid. This construct expresses the rtTA and tTS together under the Sca-1 promoter. tTS is a variant of tTA that contains a KRAB-AB repressor domain in place of a VP16 transaction domain and represses background rtTA transcriptional activity by binding to the TRE in the absence of dox. In the presence of dox tTS comes off the TRE allowing rtTA to bind and activate transcription. We observed a significant reduction of background transcription when using the rtTA-IRES-tTS construct allowing for a 270-fold increase in transcriptional activity over uninduced cells. This increase in dox induced transcriptional activity results from a significant reduction of background transcriptional activity by about 100-fold. Thus, we repeated the transfection of ES cells with the new Sca1-rtTA-IRES-tTS targeting construct and we now have 160 clones that are currently being screened for proper targeting. Based on our collaborators experience that this construct is about 2-3% efficient at generating properly targeted clones in these ES cells we anticipate about 3-4 positive clones will be established. Once we have identified properly targeted ES clones we will perform blastocyst injections and establish mouse lines. Established mouse lines should be generated in about 8 months.

Task 3. **Generate tetracycline responsive (TRE) DsRED -Timer transgenic mice.**
Results: This task seeks to establish a mouse model that can be used to isolate and purify cells during stages of differentiation. However, the DsRED timer displays some cell toxicity due too aggregation and may not produce distinct cell populations that can easily be isolated. Due to these potential limitations we have decided to modify this task and develop a mouse model that can be used to visualize and isolate long surviving/slow turnover cells in the mammary gland. This method is based on data that stem cells have slow turnover in several organs including the mammary gland. Cells that slowly turnover can be identified by using stabilized fluorescent proteins that can be transiently transcribed (pulse) followed by a period when no new protein is generated (chase). During the chase period cells that are actively proliferating or cells that undergo apoptosis will not be labeled leaving a fluorescent quiescent cell population. These cells can be isolated by FACS or visualized in the mammary gland using the imaging techniques described in Task1*c*. To perform these experiments we have obtained the stabilized fluorescent proteins H2B-EGFP and H2B-mRFP, cloned them under the control of the Tet-responsive promoter (TRE) and have generated transgenic mice. H2B-EGFP and H2B-mRFP are histone-2B proteins fused with green and red fluorescent proteins that are highly stabilized. We have also

generated Chicken-actin (CAG)-rtTA-IRES-tTS transgenic mice that express rtTA and tTS in all tissue. These CAG-rtTA-IRES-tTS mice will be bred with the TRE-H2B-EGFP and TRE-H2B-mRFP mice to establish bigenic strains. These bigenic mice will be treated with dox transiently and then chased for 1-4 weeks without dox to allow isolation and visualization of slow turnover cells in the mammary gland. Additionally, once the Sca1-rtTA-IRES-tTS mice have been established we can perform the same experiment using bigenics of Sca1-rtTA-IRES-tTS and TRE-H2B-EGFP or TRE-H2B -mRFP to specifically isolate Sca-1 cells with slow turnover. At this time we have generated CAG-rtTA-IRES-tTS, TRE-H2B-EGFP and TRE-H2B-mRFP and are currently characterizing expression of the transgenes. Bigenic mice of CAG-rtTA-IRES-tTS and TRE-H2B-EGFP or TRE-H2B-mRFP should be established in about 2 moonths for these experiments.

Task 4. **Establish Sca-1^{rtTA} /TRE mouse models.**
Results: We have decided to pursue a retroviral infection and transplantation method to cost effectively study genes in the mammary gland. This method can be used instead of generating bigenic mouse models. For these experiments MEC primary cultures will be generated from CAG-rtTA-IRES-tTS or Sca1- rtTA-IRES-tTS mice and then infected with a self-inactivating retrovirus that drives genes under the TRE promoter. The transduced primary MECs will then be transplanted into syngeneic mice and these host mice will be treated with dox to induce gene activation. There are several advantages of this method particularly the cost and time effectiveness as compared to generating bigenic mice. We have tested several retroviral constructs and have optimized the infection protocol to allow for between 30-70% infection rates of primary cultures and efficient expression of genes upon transplantation (Figure 11). We are currently expanding the CAG-rtTA-IRES-tTS transgenic lines to provide donor MECs for these experiments.

Task 5. **Characterize Differentiation of Sca-1 cells.**
Results: Currently waiting for Sca1- rtTA-IRES-tTS mice.

Task 6. **Investigate the role of Kuzbanian in mammary gland development and differentiation.**
Results: We will be performing these experiments using the retroviral transduction method as described in Task 4 and we are currently expanding CAG-rtTA-IRES-tTS transgenic mice for this approach. We will also use the Sca1- rtTA-IRES-tTS mice once they are developed.

Task 2-6
Discussion: Tasks 2-6 seek to establish mouse models that can be used to study mammary epithelial stem and progenitor cells during development and tumorigenic transformation. All of these tasks rely on the generation of Sca1-rtTA-IRES-tTS knock-in mice and these experiments have been delayed. We have redesigned some proposed methods to make them more technically feasible, cost effective, and innovative to address the same questions as originally proposed. In particular, using the method of retroviral transduction of MECs isolated from our rtTA lines will allow us to study cooperativity between several genes and bypass the need to generate triple transgenic mouse lines. Furthermore, we feel that it was prudent to delay the generation of the Sca1-rtTA-IRES-tTS so that we could create and test the rtTA-IRES-tTS construct for its ability to reduce background rtTA activity as this is essential for the experiments proposed. We have also made progress on alternative approaches including generating CAG-rtTA-IRES-tTS, TRE-H2B-EGFP and TRE-H2B-mRFP transgenic mice that can be used in Tasks 3-6.

KEY RESEARCH ACCOMPLISHMENTS:

-In a collaborative effort analyzed mouse mammary tumor models for MEC progenitor cell markers and published these data.
-Identified that CD24 can mark a subpopulation of Sca-1 cells in the mammary gland and tumors.
-Identified differential expression of keratin markers in sorted Sca-1/CD24 cell populations isolated from cultured normal MECs primary cultures.
-Identified that cultured Sca-1 alone cells have reduced outgrowth potential while enhanced outgrowths were observed in Sca-1/CD24 double positive MECs.
-Generated Sca1-rtTA-IRES-tTS selected ES clones.
-Generated CAG-rtTA-IRES-tTS, TRE-H2B-EGFP and TRE-H2B-mRFP transgenic mice.

REPORTABLE OUTCOMES:

Li Y, **Welm B**, Podsypanina K, Huang S, Chamorro M, Zhang X, Rowlands T, Egeblad M, Cowin P, Werb Z, Tan LK, Rosen JM, Varmus HE.
Evidence that transgenes encoding components of the Wnt signaling pathway preferentially induce mammary cancers from progenitor cells.
Proc Natl Acad Sci U S A. 2003 Dec 23;100(26):15853-8. Epub 2003 Dec 10.

Bryan E. Welm, Mikala Egeblad, Max Krummel and Zena Werb. Real-time imaging of mammary carcinoma progression. AACR-Advances in Beast Cancer Research Conference. (2003)

CONCLUSIONS:

Understanding the etiological events that can give rise to tumors is critical for prevention, early diagnoses and treatment of breast cancer. The studies being performed here will elucidate cell populations that are targeted by specific oncogenic pathways. We have observed that tumors can be divided into progenitor and luminal phenotypes suggesting that oncogenic events can target specific differentiation pathways. These data suggest that diverse mechanisms and cell populations can contribute to tumorigenesis. The results of this work may aid in the development of new therapeutics that target specific cell populations or differentiation pathways. Our research to date has identified CD24 was as a marker expressed on a subset of Sca-1 MECs that can be used to separate cell populations. The significance of identifying markers coexpressed on Sca-1 cells allows us to further enrich the Sca-1 cell population for stem cell activity. By sorting with Sca-1 and CD24 markers we have characterized several MEC populations for differentiation factors and outgrowth potential. However, due to alterations of Sca-1 expression when MECs are cultured on plastic prior to sorting we will repeat these experiments using freshly prepared MECs. Once a highly enriched stem cell population is identified then future studies can address how these cells contribute to mammary tumorigenesis.

We are also in the process of developing several mouse models and methods that can be used for our studies on role of MECs stem cells in tumorigenesis. These mouse models will allow us to enrich for stem cells, observe cell interactions *in vivo* and target gene expression to specific cell populations. We have optimized techniques to visualize fluorescently labeled cells in the mammary glands of living mice. Additionally, we have generated two transgenic lines that express stabilized green and red fluorescent proteins under the control of the tet-responsive promoter. We have also generated and tested a construct that co-expresses tTS with rtTA that significantly reduces rtTA dox-independent transcription. The rtTA-IRES-tTS construct has been used to generate a transgenic line that expresses it ubiquitously and will be used in a knock-in line that expresses it under the Sca-1 regulatory elements. These mice will be useful not only for our studies but should also benefit many studies in the mammary gland biology research community.

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2. Li, Y., et al., *Evidence that transgenes encoding components of the Wnt signaling pathway preferentially induce mammary cancers from progenitor cells.* Proc Natl Acad Sci U S A, 2003. **100**(26): p. 15853-8.
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Meeting Abstract
AACR meeting, Huntington Beach, October 2003

Real-time imaging of mammary carcinoma progression

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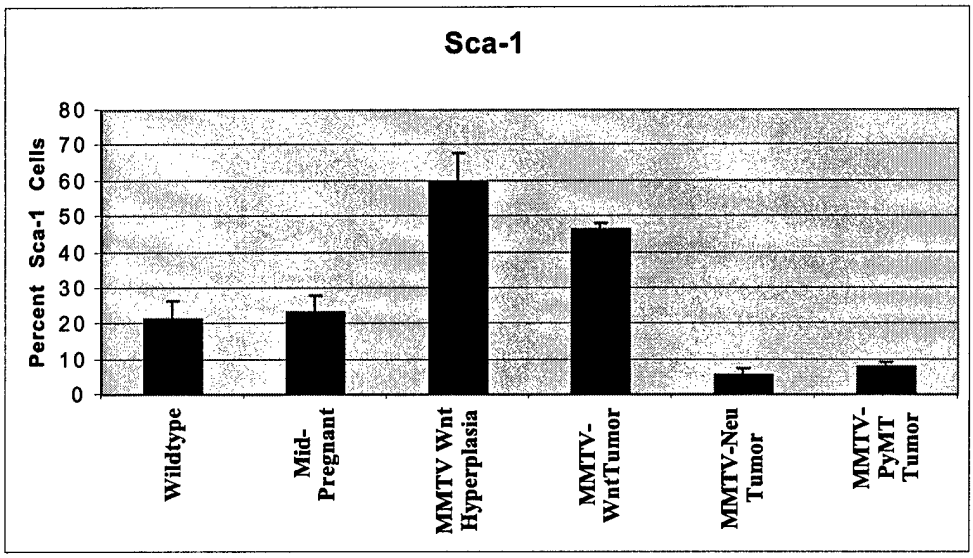
Leukocyte infiltration of the mammary gland is required for mammary gland development. In particular, infiltrating macrophages and neutrophils are necessary for epithelial invasion during ductal morphogenesis. Leukocyte infiltration also potentiates invasion and metastasis during tumorigenesis. For example, mice that lack mature macrophages have reduced metastases when bred into the mouse mammary tumor virus polyoma middle T antigen (MMTV-PyMT) breast cancer mouse model. Thus, leukocytes are important both during normal and pathological invasive processes of the mammary epithelium. However, it is not understood how leukocytes interact with the mammary epithelium during these events. We studied the interaction between leukocytes and epithelial cancer cells during tumor progression by breeding the MMTV-PyMT mice bred into chicken-beta-actin-enhanced green fluorescent protein (CAG-EGFP) or chicken-beta-actin-cyan fluorescent protein (CAG-CFP) and Sca1-EGFP mice. We visualized the behavior of the cancer cells and leukocytes during time periods of up to 4 hours in the living, anaesthetized mice using spinning disk confocal microscopy. The cancer cells expressed higher levels of EGFP in the MMTV-PyMT/CAG-GFP bigenic mice than the surrounding stromal fat cells, thus allowing visualization of cancer cells invading into the mammary fat pad stroma. Furthermore, leukocytes expressing high levels of EGFP were observed at the tumor-stroma interphase, resembling the localization of the leukocyte marker CD45 found by immunohistochemical staining on fixed tumors from MMTV-PyMT mice. These EGFP-high leukocytes were very motile, but stayed in close proximity to the cancer cells during the observation period. In addition, dendritic-like cells that expressed low levels of EGFP were observed migrating inside the tumor tissue using filopodia and lamellipodia-like protrusions. In the MMTV-PyMT/CAG-CFP/ Sca1-EGFP triple transgenic mouse lines, areas of mammary carcinoma were identified based on strong expression levels of CFP. In such areas, EGFP, expressed under the Sca1 promoter, was expressed in blood vessels and in highly motile leukocytes. The leukocytes expressing EGFP under Sca1 promoter had preferred paths of migration, and were observed interacting with blood vessels as well as with each other. In conclusion, using spinning disk confocal microscopy we could image the behavior of cancer cells and leukocytes during mammary carcinoma progression in living mice. This technique will be useful for further studies on interactions between specific cell types and, when combined with various chemical inhibitors, for the importance of specific pathways in cancer cell and leukocyte behavior *in vivo*.

Figure 1.



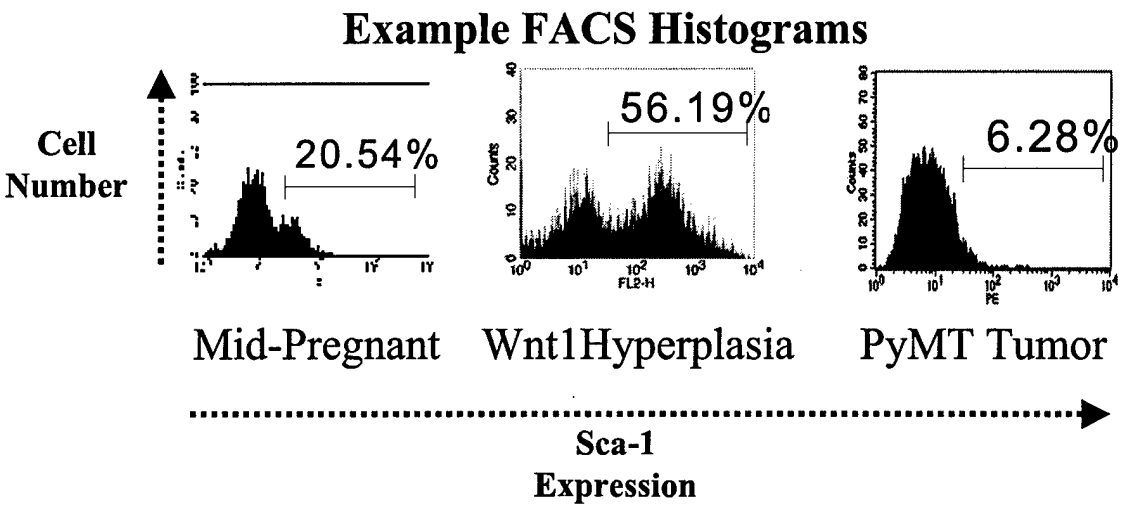


Figure 1: The graph on top shows the percent of MECs expressing Sca-1 in mouse models of tumorigenesis. Sca-1 is normally found in about 20-30% of MECs of tissue isolated from wildtype and pregnant mice. About a two-fold increase in Sca-1 positive cells is observed in hyperplasias and tumors isolated from MMTV-Wnt-1 mice while Sca-1 positive cells are reduced about three-fold in tumors isolated from MMTV-PyMT and MMTV-Neu mice.

Figure 2.

A.

Marker	Detection by FACS in Normal MECs
CD43	-
CD62L	-
CD44	-/+
CD34	-
CD24	+++
CD45	-/+
CD69	-
CD5	-
Thy1.2	-
ECadherin	+
Fall3	+++
CD62P	-
CD133	+
B220	-/+
MMP16	-
MMP14	-
β1 Integrin	++

B. **Example FACS Histogram of Sca-1 and CD24 Cells**

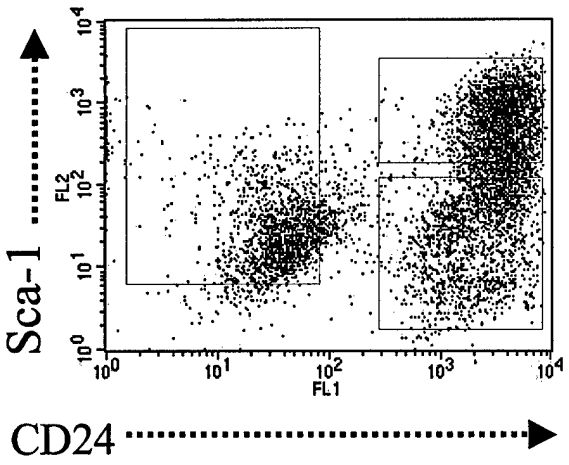
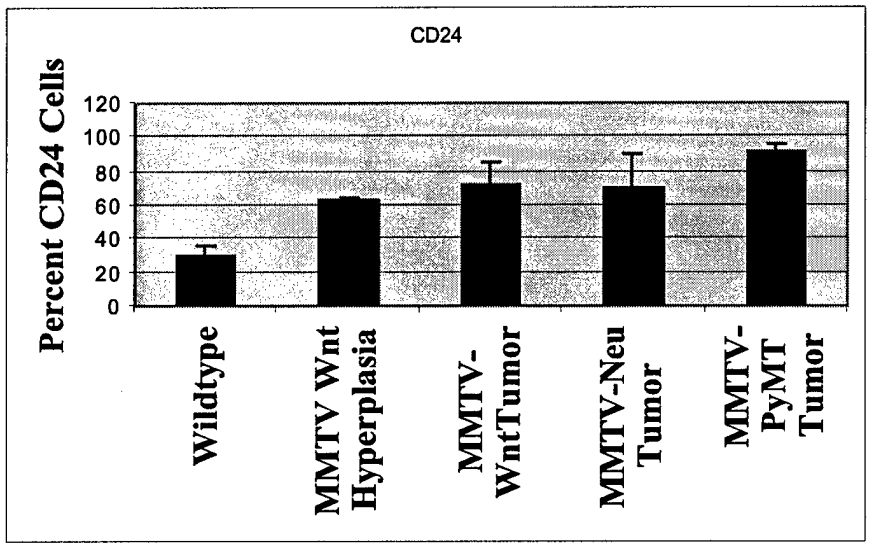


Figure 2: A) Table of cell surface proteins screened by FACS from freshly prepared MECs and their relative expression level. B) This is an example of a FACS histogram showing expression of Sca-1 and CD24 in cultured primary MECs. Nearly all cultured cells express Sca-1 and CD24 however distinct populations can be observed (boxes). The boxes represent cell populations that were further analyzed for outgrowth potential and differentiation markers.

Figure 3.

A.



B.

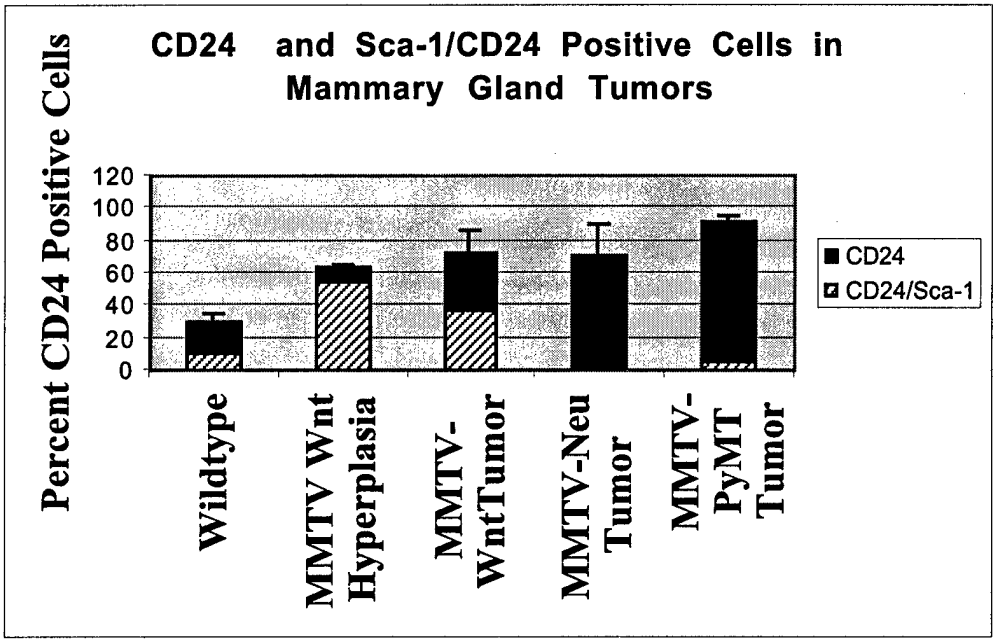


Figure 3 A) This graph shows the percent of freshly prepared MECs that are CD24 positive as measured by FACS. About 25-30% of freshly prepared MECs from normal virgin mammary glands express CD24. The expression of CD24 increases 2-3 fold in all tumors and hyperplasias analyzed. B) This graph shows the percent of CD24 positive cells in tumors with a hatched representation of double positive Sca-1/CD24 cells. Double positive Sca-1/CD24 cells are observed in Wnt hyperplasias and tumors but are mostly absent from PyMT and Neu induced tumors.

Figure 4.

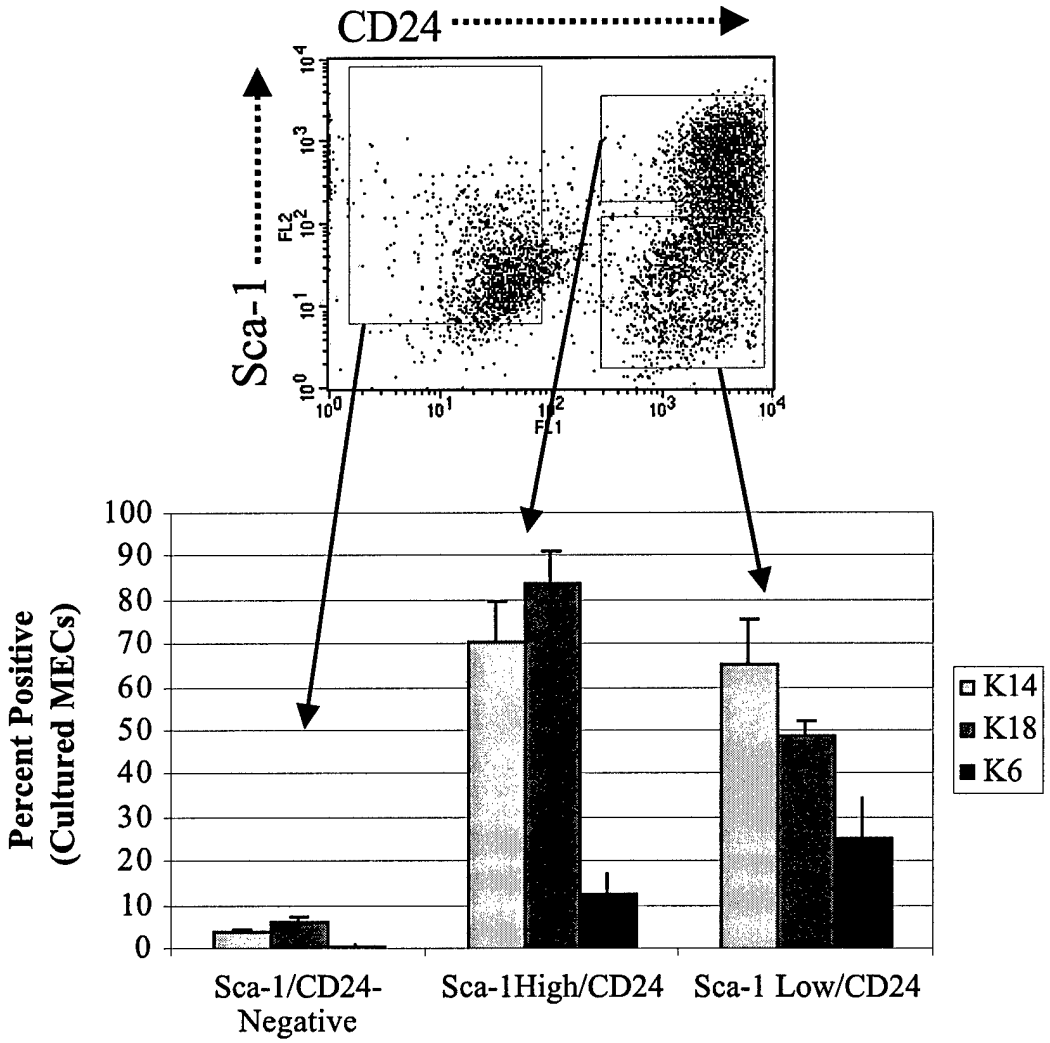


Figure 4 Sorted cell populations from cultured MECs express different keratin differentiation markers. Mammary gland primary culture cells isolated from wildtype mice were placed in culture for five days. Culturing resulted in an increase in the percent of Sca-1 positive cells from 20% on day 0 to about 80% on day 5. Cells were trypsinized from the plate on day five of culturing and sorted into three populations by FACS using Sca-1 and CD24 as markers. The three sorted populations were Sca-1/CD24 ^{negative}, Sca-1^{high}/CD24 and Sca-1^{low}/CD24. Sorted cells were fixed in 4% paraformaldehyde, centrifuged onto coverslips and stained for keratin-14 (K14), -18 (K18) and –6 (K6). Data represents the percent and standard deviation of positive cells in at least four fields representing 1000-5000 total cells.

Figure 5.

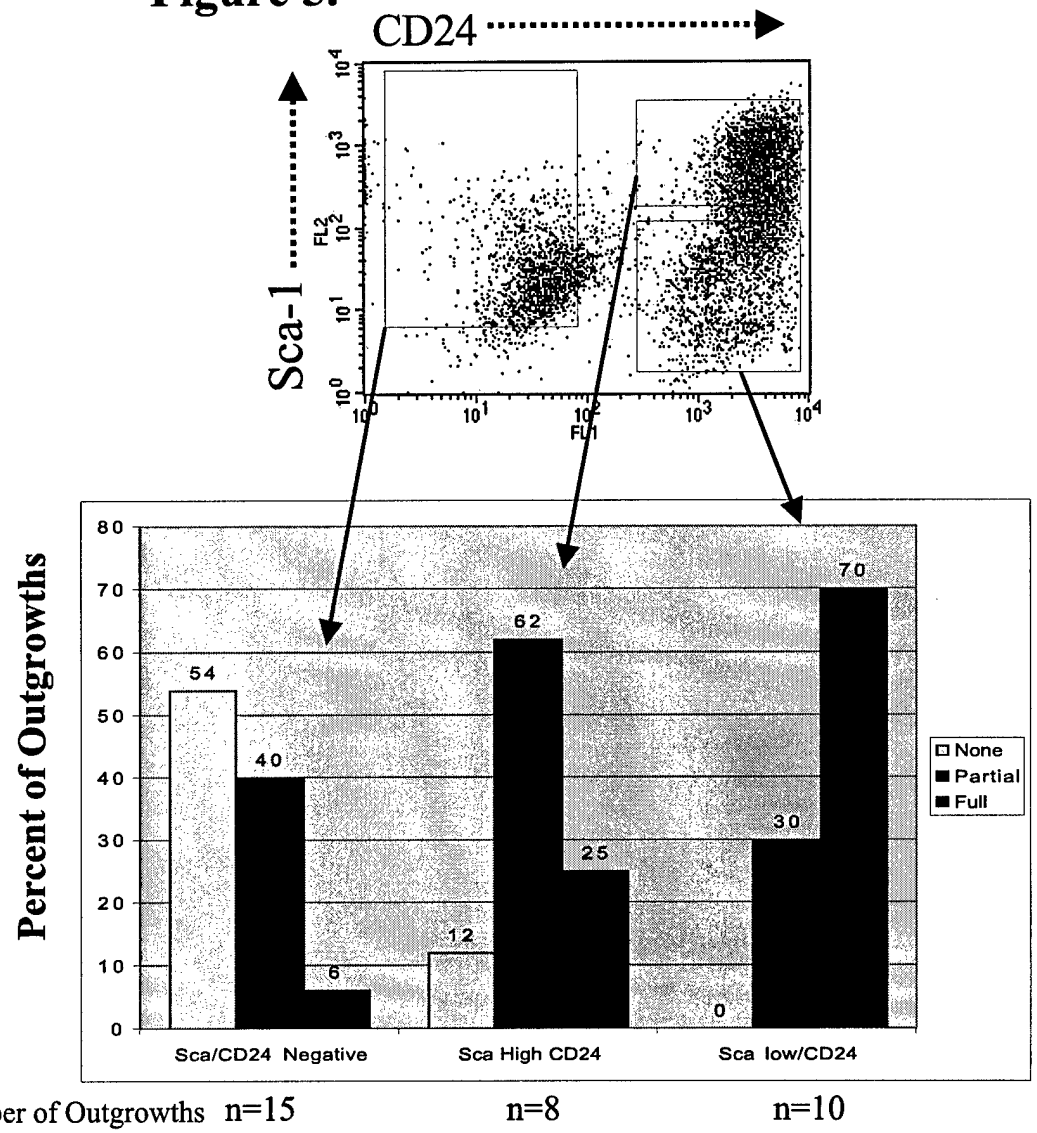


Figure 5 Sorted cell populations from cultured primary MECs exhibit different outgrowth potential. Primary MECs were cultured and sorted for Sca-1 and CD24 and distinct cell populations were transplanted into cleared mammary fat pads. About 10,000 cells were injected into each fat pad. Outgrowths were assessed for the extent they filled the fat pad and given a score of no outgrowth (no morphologically normal mammary gland visible), partial outgrowth (< 50% of the fat pad contains a gland) and full outgrowth (> 50% of the fat pad contains a gland).

Figure 6.

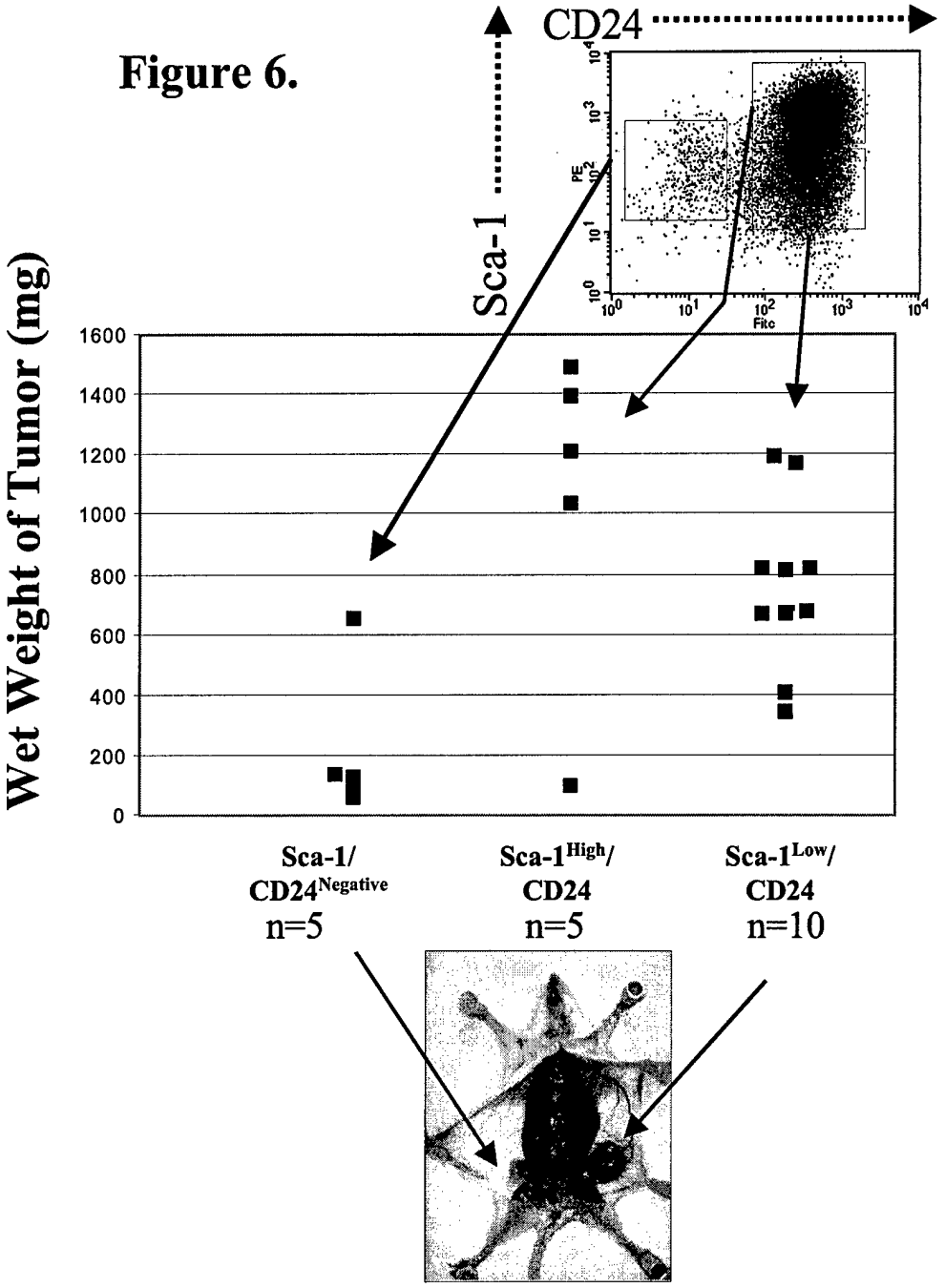


Figure 6 Cell populations isolated from PyMT induced tumors have distinct tumorigenic potential. Tumors from MMTV-PyMT mice were cultured and sorted against Sca-1 and CD24 and transplanted into cleared fat pads. FACS sorted cell populations were isolated from mammary tumors derived from MMTV-polyoma-middle–t (PyMT) transgenic mice. Sorted populations were transplanted into the cleared fat pads of syngeneic host mice to assess their tumorigenic potential after 3-weeks. The scatter blot represents the wet weight of the tumor in mg.

Figure 7.

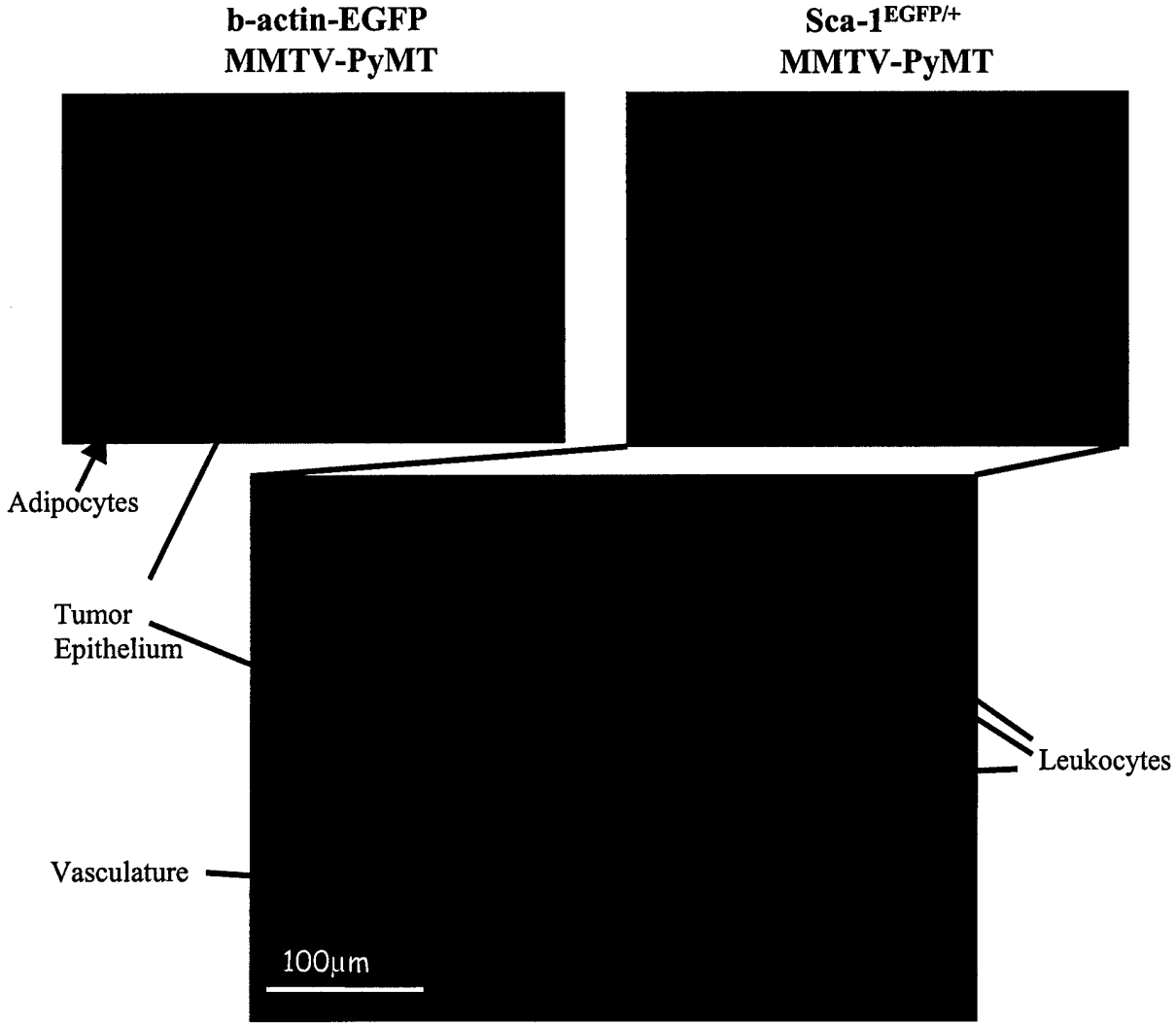


Figure 7 Real-time *in vivo* imaging of the mouse mammary gland can be used to follow cell populations during tumor progression. This figure shows representative still images of movies. The upper left panel is from a bigenic MMTV-PyMT::Chicken actin-EGFP mouse that expresses EGFP ubiquitously. The image shows EGFP fluorescence in a tumor nodule. The upper right panel shows a still image of a tumor in bigenic MMTV-PyMT::Sca1-EGFP mice. These stills show that EGFP is absent from PyMT induced tumors when expressed under the control of Sca-1 regulatory elements. These movies suggest that the 10% of Sca-1 cells in PyMT induced tumors as detected by FACS represents blood and vasculature contamination of the primary culture.

Figure 8. **MMTV-Wnt1::Sca1-EGFP**

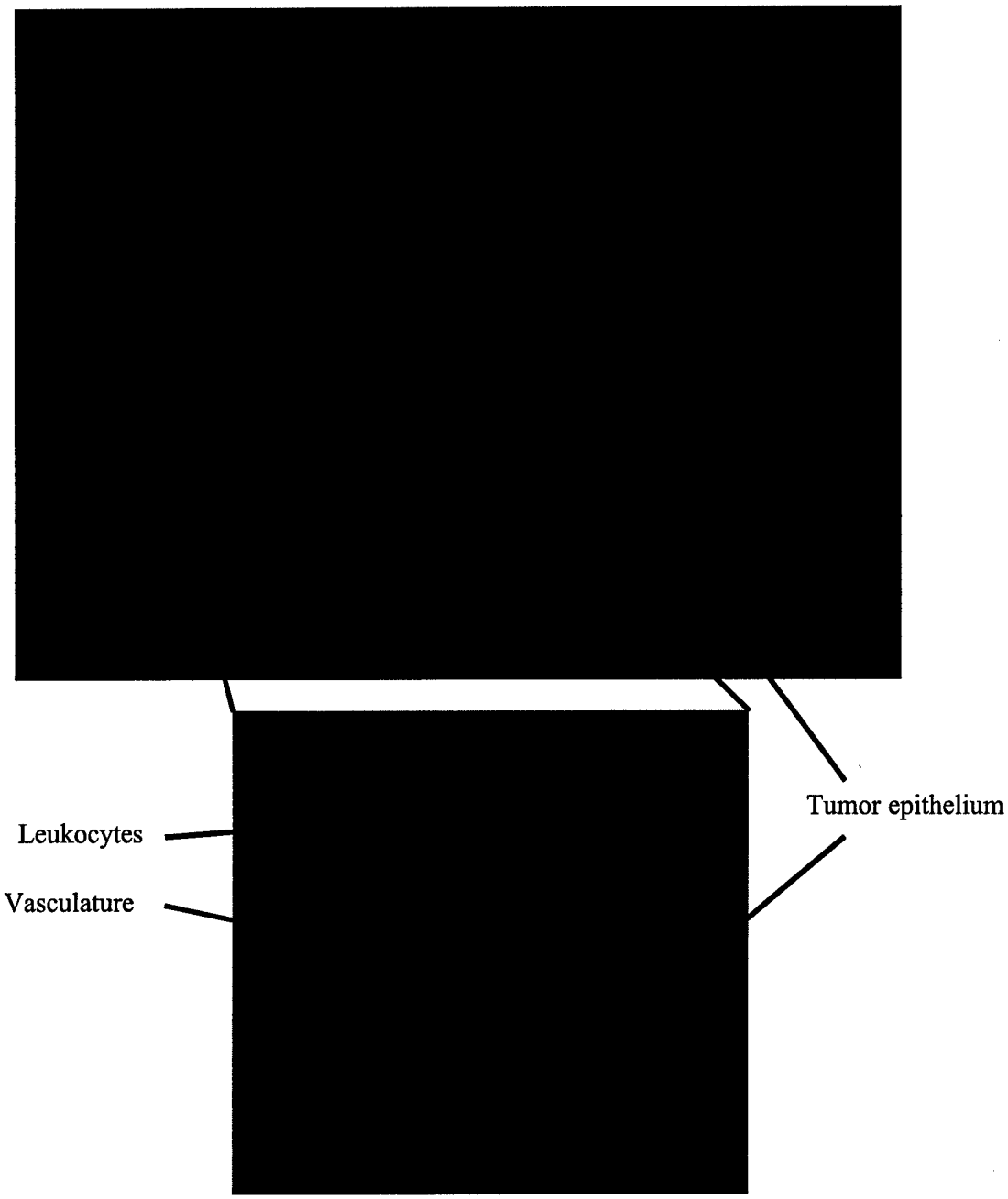


Figure 8 This figure shows still images from a 91 minute movie of Wnt-1 induced tumor epithelium interacting with leukocytes. These movies also show that the Sca-1 regulatory elements can drive expression of EGFP in the tumor epithelium unlike in PyMT induced tumors.

Figure 9.

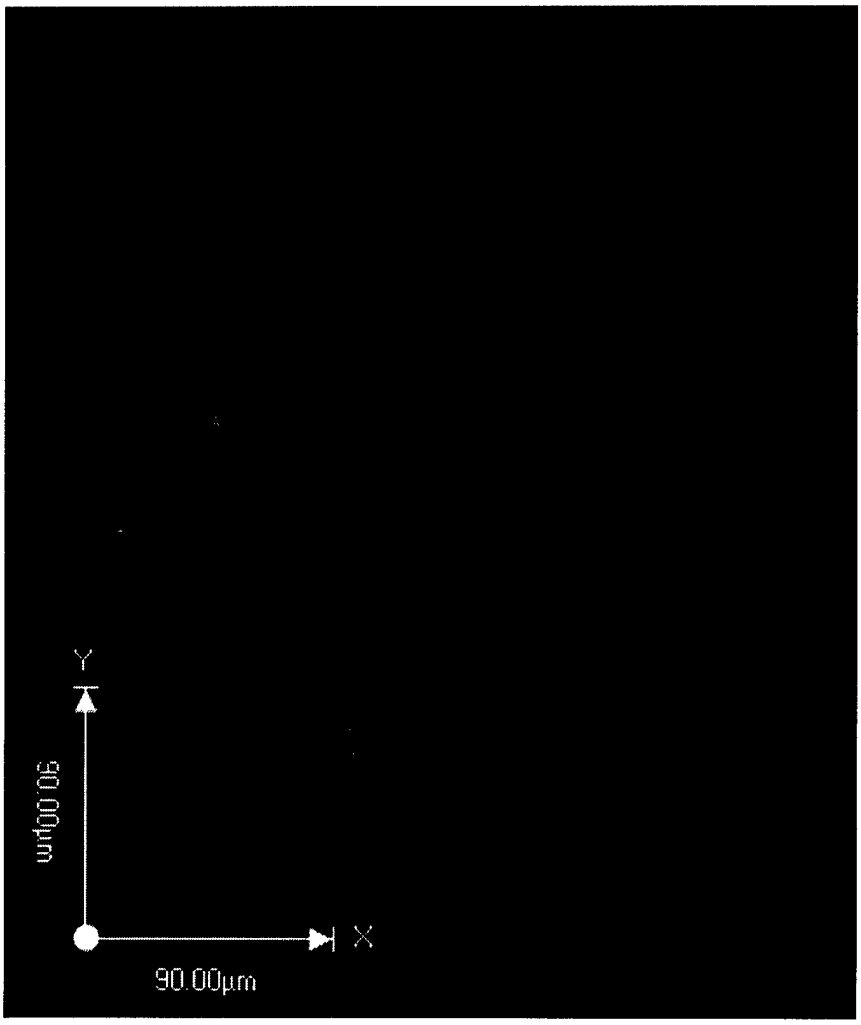
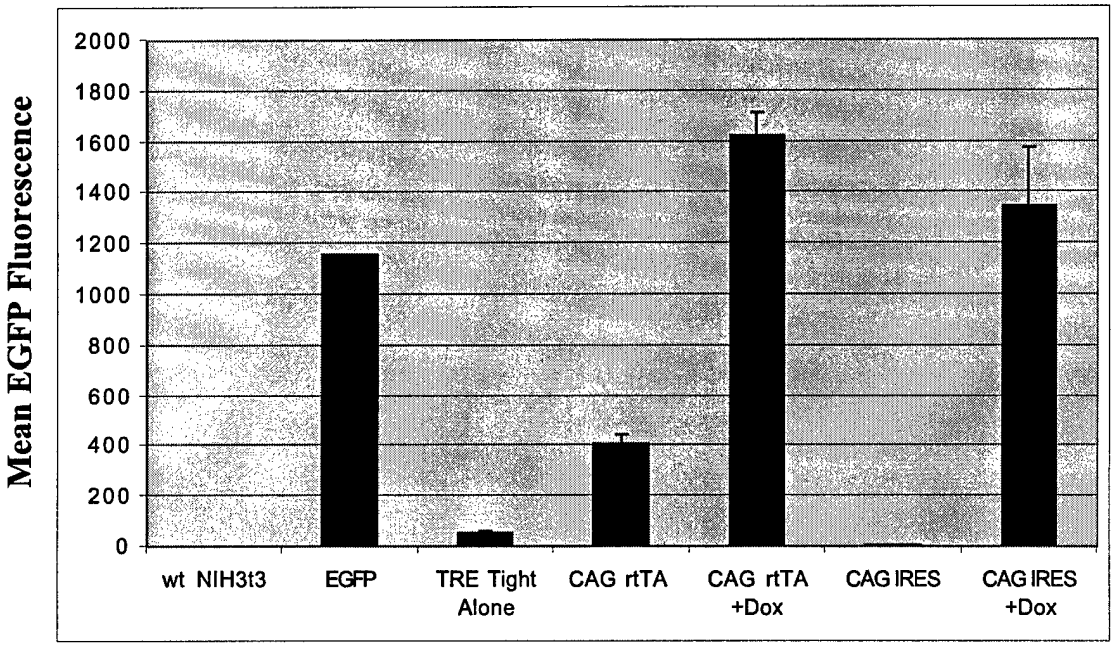


Figure 9 Sca-1 expression remains limited to about 15-20% of the epithelium when MECs are cultured in matrigel. Primary MECs were isolated from Sca1-EGFP::CAG-CFP mice and plated in matrigel. The green represents EGFP expression driven by the Sca-1 regulatory elements and the red represents CFP expression driven by the chicken-actin promoter. (Image was provided by Andrew Ewald)

Figure 10.

rtTA-IRES-tTS Reduces Dox-Independent rtTA Transcriptional Activity



Plasmid							
CMV-EGFP	-	+	-	-	-	-	-
TRE-Tight-EGFP	-	-	+	+	+	+	+
CAG-rtTA	-	-	-	+	+	-	-
CAG-trTA-IRES-tTS	-	-	-	-	-	+	+
					+Dox		+Dox

Figure 10 High dox-independent transcriptional activity by rtTA can be blocked by rtTA-IRES-tTS. NIH 3T3 cells were cotransfected with TRE-tight driving EGFP and chicken-actin driving either rtTA alone or rtTA-IRES-tTS. The presence of tTS not only reduced rtTA-dox independent transcriptional activity by 100-fold it also reduced leaky expression from the TRE-Tight plasmid (in the absence of rtTA) by 10-fold.

Figure 11.

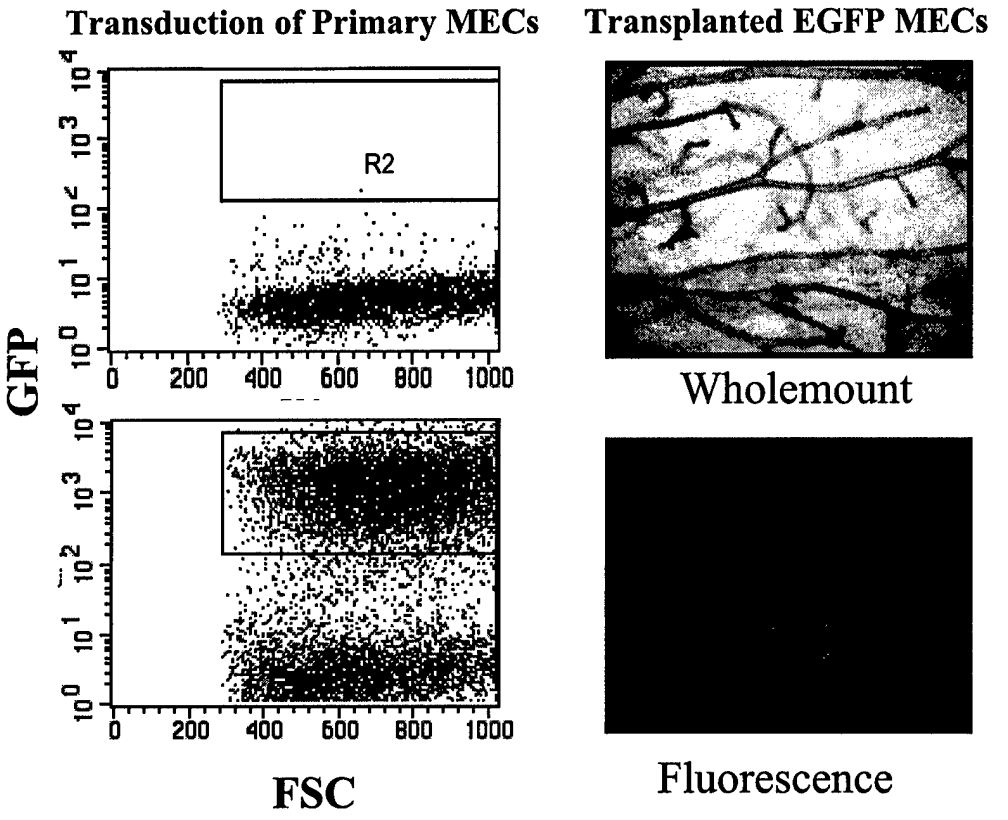


Figure 11 Primary MECs can be efficiently transduced with retroviral constructs and express transduced genes upon transplantation into cleared fat pads. This figures shows FACS histograms (left) of a representative infection of primary MECs with a EGFP containing retrovirus. The top histogram is uninfected and the bottom is infected primary MECs. The panels on right show an outgrowth of transplanted infected MECs wholemount stained with Carmine-Alum Red (top) and EGFP fluorescence imaged from a fluorescent dissecting scope (bottom). (Figures provided by Alana Welm)

Evidence that transgenes encoding components of the Wnt signaling pathway preferentially induce mammary cancers from progenitor cells

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Contributed by Harold E. Varmus, October 21, 2003

Breast cancer is a genetically and clinically heterogeneous disease, and the contributions of different target cells and different oncogenic mutations to this heterogeneity are not well understood. Here we report that mammary tumors induced by components of the Wnt signaling pathway contain heterogeneous cell types and express early developmental markers, in contrast to tumors induced by other signaling elements. Expression of the *Wnt-1* protooncogene in mammary glands of transgenic mice expands a population of epithelial cells expressing progenitor cell markers, keratin 6 and Sca-1; subsequent tumors express these markers and contain luminal epithelial and myoepithelial tumor cells that share a secondary mutation, loss of *Pten*, implying that they arose from a common progenitor. Mammary tumors arising in transgenic mice expressing *β-catenin* and *c-Myc*, downstream components of the canonical Wnt signaling pathway, also contain a significant proportion of myoepithelial cells and cells expressing keratin 6. Progenitor cell markers and myoepithelial cells, however, are lacking in mammary tumors from transgenic mice expressing *Neu*, H-*Ras*, or *polyoma middle T antigen*. These results suggest that mammary stem cells and/or progenitors to mammary luminal epithelial and myoepithelial cells may be the targets for oncogenesis by Wnt-1 signaling elements. Thus, the developmental heterogeneity of different breast cancers is in part a consequence of differential effects of oncogenes on distinct cell types in the breast.

Transgenic (TG) activation of different oncogenic pathways in mouse mammary glands induces tumors with different gene expression profiles and histopathological features (1–8). The mouse mammary tumor virus (MMTV) promoter usually used to regulate these transgenes is expressed in a diverse population of mammary cells (9, 10). Therefore, cells at different developmental stages may undergo tumorigenesis as a result of the activation of different oncogenic pathways.

It has been difficult to define the precise lineage of target cells of breast cancer because of the lack of animal models expressing oncogenes in specific mammary progenitors. However, studies in the epidermis and the hematopoietic system have demonstrated that cancers that arise from stem or progenitor cells usually express markers of the originating cells (11, 12). Thus, the presence of stem or progenitor cell markers in mammary tumors may suggest that tumors arose from immature cells. Two genes, keratin 6 and stem cell antigen 1 (Sca-1), appear to be preferentially expressed in mammary stem and/or progenitor cells. Keratin 6 is expressed in mammary gland anlage at embryonic day 16.5 (ref. 13 and J.M.R., unpublished observations) and in some of the body cells in the terminal end buds (TEBs); but keratin 6 is not found in the highly proliferative cap cells (14, 15) and rarely in cells in the mature ducts and differentiated alveoli (15), consistent with the distribution of progenitor cells. In addition, keratin 6 is associated with the arrested state of differentiation observed in the mammary ducts from *C/EBPβ*-null mice (13). Sca-1, encoded by *Ly-6A/E*, is a GPI-linked protein also found in hematopoietic stem cells (16); it is expressed on the surface of a population of mammary cells enriched for stem cells and present in TEBs (17). Depletion of Sca-1-positive cells results in a loss of functional stem cells in mammary gland reconstitution experiments (17). Although retained in a population of more mature ductal cells, Sca-1 is not observed after cells have further differentiated to express the progesterone receptor (17).

Here we report that keratin 6 and Sca-1 cells are observed in mammary tumors induced by the Wnt-1 signaling pathway, which has been implicated in proliferation and maintenance of undifferentiated cells in several tissue types, including the mammary gland (18–28), but not in tumors induced by oncogenes affecting other pathways. In addition, we provide other evidence to suggest that ectopic activation of the Wnt pathway may target these undifferentiated mammary progenitors for tumorigenesis.

Materials and Methods

Mice. TG mice expressing MMTV-*Wnt-1* (29), MMTV-β-catenin (30), MMTV-c-Myc (31), MMTV-*Neu* (32), MMTV-*H-Ras* (33), or MMTV-*PyMT* (34) have been described, as have mice harboring targeted inactivating mutations of *Pten* (35). MMTV-*Wnt-1* TG mice were a mixture of FVB, SJL, and C57BL/6 strains; *Pten* mutant mice were a mixture of FVB/N, 129, and C57BL/6 strains; all others were in a pure FVB/N background.

Tissue Processing and Immunocytochemistry. Tissues were fixed and processed as described (36). Immunohistochemistry was performed by using Vector ABC and MOM kits (Vector Laboratories) according to the manufacturer's recommendations and as described (35). The following antibodies were used: purified rabbit antibodies against mouse keratin 6 (Covance, Princeton) and Pten (NeoMarkers, Fremont, CA); purified mouse monoclonal antibodies against α-smooth muscle actin (SMA, Dako) and against BrdUrd (BD Biosciences Immunocytometry Systems, product no. 347580); and partially purified rat antibodies against keratin 8 (37, 38), purchased from the Developmental Studies Hybridoma Bank, Iowa City, organized under the auspices of the National Institute of Child Health and Human Development and maintained by the University of Iowa. To label cells in S-phase of cell cycle, BrdUrd (Sigma, product no. B-5002) at 100 μg per gram of body weight in saline was injected i.p. 1 h before mice were killed.

Abbreviations: FACS, fluorescence-activated cell sorting; LOH, loss of heterozygosity; MMTV, mouse mammary tumor virus; SMA, smooth muscle actin; TEB, terminal end bud; TG, transgenic.

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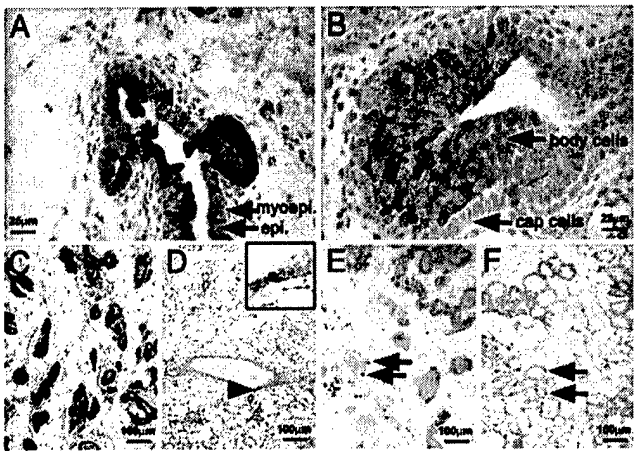

Fig. 1. Keratin 6-positive cells are expanded in hyperplastic mammary glands from MMTV-*Wnt-1* TG mice. Immunohistochemical staining was used to detect keratin 6 (*A–D*) or BrdUrd (*E* and *F*) in mammary gland sections from MMTV-*Wnt-1* TG mice that were 3-week-old (*A*) or 3-month-old (*C* and *E*) virgins, from non-TG females that were 3 weeks old (*B*), 3 months old (*D*), or in mid-pregnancy (*F*). The hyperplastic keratin 6-positive cells projecting into the lumen are indicated by an arrowhead (*A*). The insert in *D* is a 4-fold higher view of the site denoted with an arrowhead. Staining for BrdUrd, as represented by arrows, demonstrates similar levels of proliferation in mammary glands from MMTV-*Wnt-1* TG virgin (*E*) and wild-type mid-pregnancy (*F*) mice. Cell types are indicated by arrows in *A* and *B*; scale bars are shown in each panel.

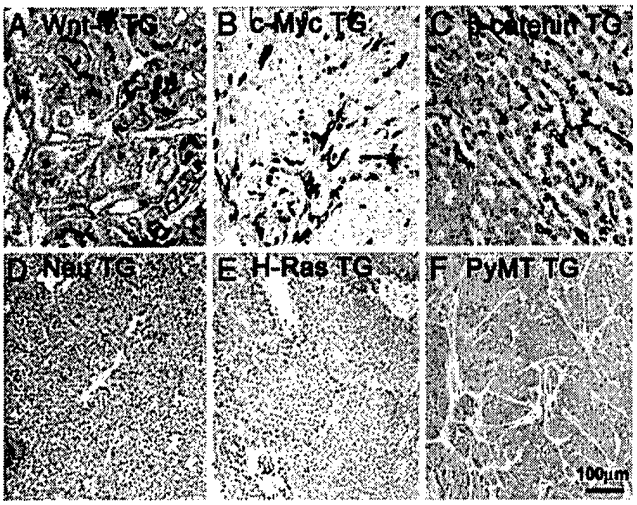

Fig. 2. Keratin 6-positive cells in mammary tumors arising in TG mice expressing components of the Wnt signaling pathway. Keratin 6 was detected by immunohistochemical staining of tumors from mice carrying the indicated MMTV TG. The scale is shown in *F*.

Southern Hybridization and Western Analysis. Tumor DNAs were digested with *Pst*I and processed for Southern blotting by using a [^{32}P]dCTP-labeled probe made from a 0.9-kb template located in intron 5 of *Pten* as described (36). For Western blotting, tumors were ground to powder in liquid nitrogen and lysed by boiling in the sample-loading buffer for acrylamide gel electrophoresis. Protein lysates were resolved in 10% polyacrylamide gels containing SDS and transferred to nitrocellulose membranes. After incubating with rabbit antibodies against Pten (NeoMarkers, 1:1,000) or mouse monoclonal antibodies against γ-tubulin (Sigma, 1:5,000), the specific reaction was visualized by peroxidase-conjugated secondary antibodies (Roche) and a chemiluminescent substrate (SuperSignal, Pierce).

Flow Cytometry. Mammary cells and tumors were isolated by using collagenase as described (17). After incubating with FITC-conjugated rat IgG against Sca-1 (BD Pharmingen, product no. 553336, 10^6 cells per μg), fluorescence-activated cell sorting (FACS) was performed as described (17).

Results

Keratin 6 and Sca-1 Are Expressed in Preneoplastic and Neoplastic Mammary Lesions Induced by Components of the Wnt Signaling Pathway but Not by Neu, Ras, and PyMT. While carrying out experiments designed to identify genes differentially regulated in the evolution of mammary tumors in MMTV-*Wnt-1* TG mice (S.H., unpublished work), we discovered that both *keratin 6* and *Sca-1* are more highly expressed in hyperplastic mammary glands and tumors from MMTV-*Wnt-1* TG mice than in non-TG virgin mammary glands (Fig. 6, which is published as supporting information on the PNAS web site), suggesting that Wnt-1-induced mammary tumors may originate from progenitor cells. To confirm this observation, we used immunohistochemical staining to identify keratin 6-expressing cells. In non-TG mammary glands, keratin 6 was detected in some body cells within the terminal end buds (TEBs) and occasionally in mature luminal epithelial cells (Fig. 1 *B* and *D*), consistent with previous reports (14, 15). However, in MMTV-*Wnt-1* TG mice, we found keratin 6 in a greater number of ductal cells, usually in enlarged ducts, in a heterogeneous pattern (Fig. 1 *A* and *C*). Many of the stained cells are in the luminal layer, but some of them have invaded into the lumen (Fig. 1*A*). Ducts that were not stained were usually smaller in diameter and did not appear to be hyperplastic (Fig. 1*C*). Because transgenes regulated by MMTV are known to be expressed in heterogeneous patterns in the mammary gland (9, 39–42), the keratin 6-negative ducts may not express the MMTV-*Wnt-1* transgene.

The mammary cells in adult MMTV-*Wnt-1* TG mice have a greater rate of proliferation than those in non-TG virgin mice, as measured by immunohistochemical staining for cells labeled with BrdUrd and for cells expressing Ki-67, another proliferation marker (data not shown). It is therefore possible that the expansion of keratin 6-positive cells in the mammary glands of MMTV-*Wnt-1* TG mice may result from an increased proportion of proliferating cells. Indeed, keratin 6 has been associated with hyperproliferation in the suprabasal layer in the skin (43). To determine whether the increased expression of keratin 6 is an oncogene- or proliferation-induced effect in the mammary gland, we compared mammary glands from adult MMTV-*Wnt-1* TG virgins with proliferating mammary glands from mid-pregnancy non-TG mice. Similar numbers of BrdUrd-stained cells (Fig. 1 *E* and *F*) and Ki-67-positive cells (data not shown) were detected in virgin TG and pregnant non-TG mammary glands. However, only a few cells per field were stained with anti-keratin 6 antibodies in the pregnant non-TG mice (data not shown). Taken together, these results suggest that aberrant *Wnt-1* expression arrests differentiation of mammary cells at an early phase and stimulate their proliferation and that proliferation *per se* is not associated with expression of keratin 6.

We also used immunohistochemistry to confirm the microarray results suggesting that keratin 6 is also expressed in mammary tumors from MMTV-*Wnt-1* TG mice. Strong, although not uniform, staining for keratin 6 was observed in Wnt-1-induced tumors (Fig. 2*A*). To determine whether the observed expansion of keratin 6-positive cells is unique to the MMTV-*Wnt-1* TG model, we stained sections of mammary glands and tumors from a number of other TG models. Interestingly, keratin 6-positive cells were abundant in both mammary gland and tumor sections from MMTV-*β-catenin* and MMTV-*c-Myc* TG mice, but keratin

6-positive cells were not detected in premalignant glands or tumors from MMTV-*Neu*, MMTV-*H-Ras*, or MMTV-*PyMT* TG mice (Fig. 2 and Fig. 7, which is published as supporting information on the PNAS web site), implying a transgene-specific effect on the expansion of keratin 6-positive cells with keratin 6 expressed in tumors induced by components of the Wnt signaling pathway. These results confirm that keratin 6 is not a by-product of proliferation, because the frequency of replicating cells is high in mammary tumors from all these TG mice, as evidenced by staining for Ki67 (data not shown).

We used FACS to confirm and extend the microarray data in Fig. 6 showing that Sca-1, a second marker for progenitor cells, was expressed in Wnt-1-induced hyperplasias or tumors. About 50% of the mammary cells from the hyperplastic mammary glands and from mammary tumors from virgin MMTV-*Wnt-1* TG mice were positive for Sca-1 (Fig. 3 *A* and *B*). In contrast, ≈20% of mammary cells from non-TG virgin or pregnant mice were positive, consistent with our earlier report that Sca-1 antibody labels progenitor cells, in addition to a subset of partially differentiated cells (17). Furthermore, <10% of cells from MMTV-*Neu* or MMTV-*PyMT* TG mice were positive. To determine whether keratin 6 and Sca-1 are expressed in the same cells, we stained mammary cells prepared from hyperplastic mammary glands and tumors from MMTV-*Wnt-1* TG mice with anti-Sca-1 antibodies, separated Sca-1-positive and Sca-1-negative cells by FACS, and stained them with antibodies against keratin 6 (Fig. 3*C*). Approximately 10% of Sca-1-positive cells were positive for keratin 6 (data not shown), whereas keratin 6 was not detected in Sca-1-negative cells.

Myoepithelial and Luminal Epithelial Tumor Cells Coexist in MMTV-*Wnt-1* TG Mice but Not in *Neu*, *H-Ras*, or *PyMT* TG Mice. Tumors arising from stem or progenitor cells may show mixed lineage differentiation (11). To investigate whether tumors from MMTV-*Wnt-1* TG mice may contain transformed myoepithelial cells (the normal type of which constitutes the outer layer of normal mammary ducts) in addition to transformed luminal epithelial cells (which comprise the majority of human and transgene-induced murine mammary carcinomas), we stained their sections for both keratin 8, a marker for luminal epithelial cells (44), and α-SMA, which is expressed in myoepithelial cells and pericytes in non-TG mammary glands (ref. 45 and data not shown). The number of α-SMA-positive cells was high, approximately equal to the number of keratin 8-positive cells (Fig. 4). Keratin 14, another marker for myoepithelial cells (46), was also found in the α-SMA-positive subset of cells (data not shown). These results suggest that the Wnt-1-induced tumors are composed of two predominant cellular components, luminal epithelial and myoepithelial cells, consistent with the findings of Rosner *et al.* (2) and Cui and Donehower (49).

Because the α-SMA-positive cells are pleomorphic, hyperproliferative, disorganized, and present in large quantities (Fig. 4 *A* and *C*), they are probably neoplastic cells rather then benign cells recruited to the tumor mass. The presence of myoepithelial cells was also observed in mammary tumors from MMTV-*β-catenin* or MMTV-*c-Myc* TG mice, but not in those from MMTV-*Neu*, MMTV-*H-ras*, or MMTV-*PyMT* TG mice (Fig. 8, which is published as supporting information on the PNAS web site), similar to the report by Rosner *et al.* (2). However, in contrast to the plump abnormal myoepithelial cells detected in tumors from MMTV-*Wnt-1* TG mice, the myoepithelial cells in β-catenin- and c-Myc-induced tumors were more elongated and well organized, forming a single layer around the keratin 8-positive tumor cells, as in normal ducts.

Loss of *Pten* Occurs in Both Luminal Epithelial and Myoepithelial Cells in Wnt-1-Induced Mammary Tumors. In previous studies, we found that the wild-type allele of *Pten* was undetectable in the majority

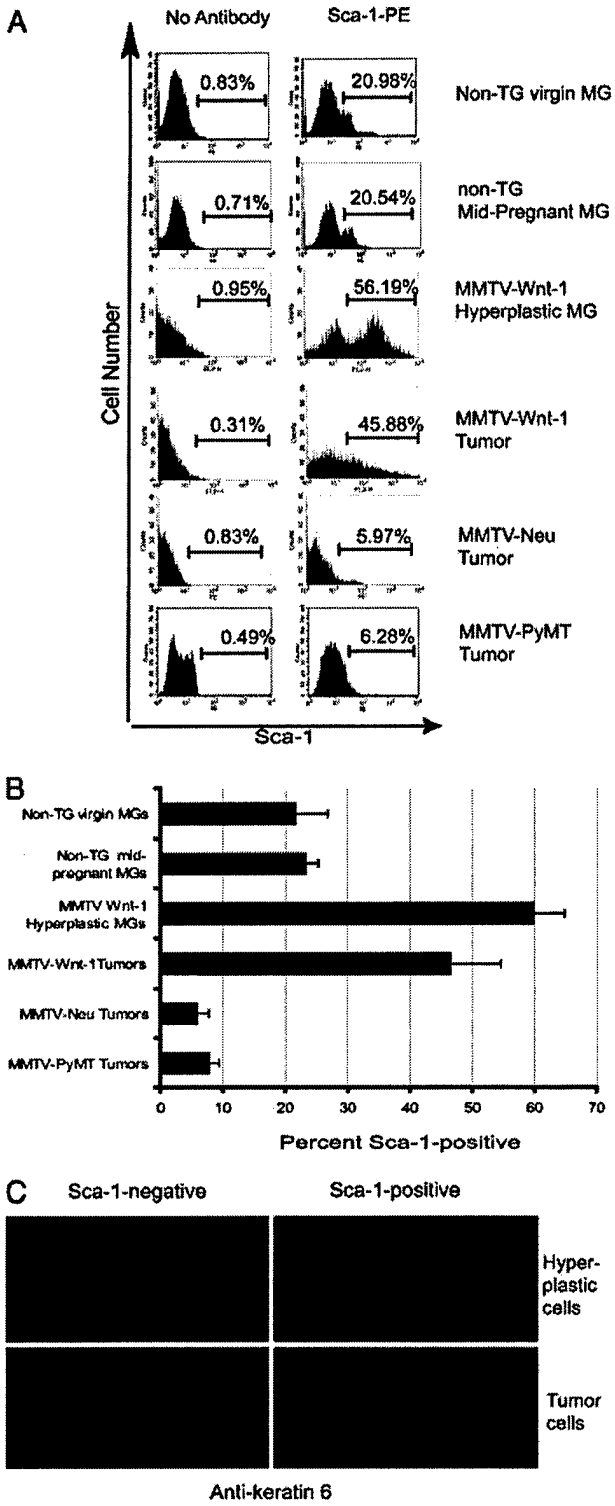

Fig. 3. Increased proportions of Sca-1-positive cells in hyperplastic mammary glands and mammary tumors from MMTV-*Wnt-1* TG mice. Representative FACS histograms (*A*) and a summary bar graph (*B*) representing data from three to five independent FACS experiments are shown for mammary glands from wild-type virgin and mid-pregnancy mice, hyperplastic mammary glands from MMTV-*Wnt-1* TG mice, and mammary tumors from TG mice carrying MMTV-*Wnt-1*, MMTV-*Neu*, or MMTV-*PyMT* transgenes. Hyperplastic cells or tumor cells prepared from MMTV-*Wnt-1* TG mice were stained with anti-Sca-1-FITC, sorted by FACS, and stained for keratin 6 (*C*). Red indicates positive staining for keratin 6. Images were captured by using a ×40 objective.

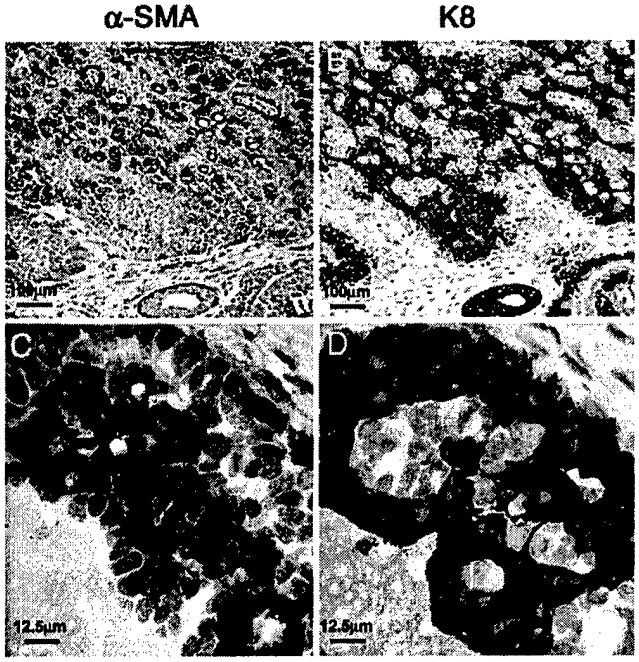

Fig. 4. Abnormal myoepithelial cells in mammary tumors from MMTV-*Wnt-1* TG mice. Consecutive paraffin sections of a mammary tumor from an MMTV-*Wnt-1* TG mouse were stained for α-SMA and keratin 8 as indicated. Note the large nuclei and disorganization of the α-SMA-positive cells in the tumor (*C*). The scales are shown in each panel.

of *Wnt-1*-induced mammary tumors arising in a *Pten* heterozygous background (36); an expanded set of samples is shown in Fig. 5*A*. Because such tumors, like *Wnt-1*-induced tumors in a wild-type *Pten* background, are composed approximately equally of luminal epithelial and myoepithelial cells (Fig. 8), loss of heterozygosity (LOH) must have occurred in both cell types.

We confirmed the absence of Pten protein by both Western blotting and immunohistochemical staining, using a purified rabbit antibody specific for Pten (Fig. 5 *B* and *C*). By Western blotting, Pten was detected in tumors from *Pten*-wild-type, MMTV-*Wnt-1* TG mice and in tumors from MMTV-*Wnt-1* TG/*Pten*$^{+/-}$ mice without LOH; but Pten was almost undetectable in mammary tumors that were MMTV-*Wnt-1* TG/*Pten*$^{+/-}$ with LOH at the *Pten* locus (Fig. 5*B*). In accord with the results of the Western blotting, by immunohistochemical staining, Pten was undetectable in both luminal epithelial and myoepithelial tumor cells in MMTV-*Wnt-1* TG/*Pten*$^{+/-}$ mammary glands that had undergone LOH (Fig. 5*C Lower* and ref. 36). On the other hand, Pten was readily detected in stromal cells within and outside of the tumor and in hyperplastic ducts surrounding the tumor. Pten was also detected in most or all cells in tumors from *Wnt-1* TG/*Pten*$^{+/+}$ mice (Fig. 5*C Upper*). Together, these results strongly suggest that the loss of *Pten* occurs in both luminal epithelial and myoepithelial cells of tumors arising in MMTV-*Wnt-1* TG/*Pten*$^{+/-}$ mice. Because it is unlikely that the two cell types sustained independent mutations, a single event most likely occurred in a common progenitor to these cells.

Discussion

In this study, we present several lines of evidence to support a role for mammary progenitor cells in mammary tumorigenesis induced by Wnt signaling. First, many keratin 6-positve cells are present in both preneoplasias (Fig. 1) and tumors (Fig. 2) from MMTV-*Wnt-1* TG mice. Second, keratin 6-positive hyperplastic and tumor cells in these mice also express Sca-1 (Fig. 3*C*). Third,

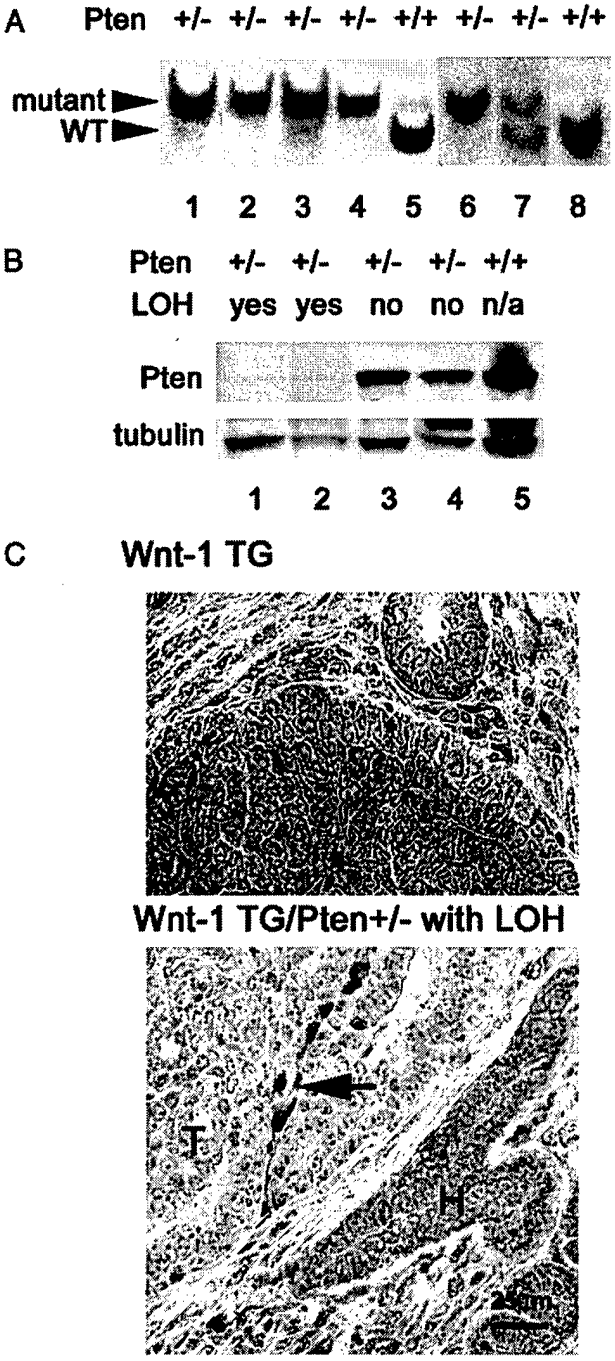

Fig. 5. Loss of *Pten* heterozygosity in both luminal epithelial and myoepithelial cells of mammary tumors from *Pten*$^{+/-}$, MMTV-*Wnt-1* TG mice. (*A*) Southern blotting analysis for LOH at the *Pten* locus in mammary tumors from MMTV-*Wnt-1* TG mice; the *Pten* genotypes are indicated above the panel. Fragments of the wild-type (WT) and target mutant alleles of *Pten* are indicated by arrowheads. The image is a composite of two independent blots (lanes 1–5 and lanes 6–8). (*B*) Western blotting analysis for Pten in MMTV-*Wnt*-1-derived mammary tumors; the *Pten* genotype and LOH status are indicated above the panel. The image of the same blot after a subsequent incubation with antibodies against γ-tubulin is also shown to indicate variations in amounts of protein loaded. The faint signal in the samples with evidence of LOH is most likely due to protein from the stroma. (*C*) Immunohistochemical staining for Pten in mammary tumors; the genotype is indicated above each panel. Areas of tumor (T) and hyperplastic (H) ducts are indicated. The string of positively stained cells (indicated by an arrow) probably represents stroma recruited into the tumor. The scale is shown in *Lower*.

luminal epithelial and myoepithelial cells coexist in these tumors, suggesting transformation of a common precursor. Finally, in some mammary tumors arising in *Pten* heterozygous, MMTV-*Wnt-1* TG mice, the wild-type *Pten* locus is missing in both luminal epithelial and myoepithelial cells (Fig. 5), suggesting that loss of *Pten* occurred in a common precursor to these two populations of tumor cells. Efforts are underway to attempt to define a subset of tumor cells from MMTV-*Wnt-1* TG mice capable of cancer regeneration, in light of a recent report (47) that only a small number of human breast cancer cells have the capacity to regenerate tumors in immunodeficient mice.

Mammary tumors from MMTV-*β-catenin* and MMTV-*c-Myc* mice, which express components of the Wnt signaling pathway, are similar to tumors from MMTV-*Wnt-1* TG mice. Collectively, our data suggest that deregulated Wnt signaling causes excess proliferation of mammary progenitor cells and predisposes them to cancer. This interpretation is consistent with reports that loss of Wnt signaling blocks early mammary development (25, 26), and it is also consistent with the role of Wnt signaling in other tissues. Wnt signaling is now known to play a critical role in the proliferation of hematopoietic stem cells and those in the skin, colon, and other organs (18–28), and deregulated activation of this pathway is a cancer-predisposing factor in several tissues including the colon, skin, and liver (19, 48).

Although progenitor cells are the likely precursors to cancer in mammary glands that have activated the Wnt signaling pathway, keratin 6 and/or Sca-1 are not present in mammary hyperplasias and tumors from several other TG lines, including MMTV-*Neu*, MMTV-*H-Ras*, and MMTV-*PyMT* TG mice (Figs. 2 and 7). In addition, a detectable second tumor cell type, such as myoepithelial tumor cells, is absent in these tumors (Fig. 8). These results are consistent with previous reports that initiating genetic lesions exert a significant influence on both gene expression patterns and histological features of mammary tumors from both humans and TG animals (1–8). However, our findings also suggest that the differentiation status of putative target cells may contribute to the heterogeneity of breast cancer. There are several possible mechanisms by which target cells may influence the histopathology and molecular features of tumors resulting from different oncogenes. Transgenes encoding Neu, H-Ras, and PyMT may transform progenitor cells, but either fail to arrest them at the progenitor stage or actively induce differentiation of the transformed cells. Alternatively, Neu, H-Ras, and PyMT may be able to transform only more differentiated cells that no longer express keratin 6 or Sca-1. In addition, there is a remote possibility that different oncogene RNAs expressed from MMTV are differentially translated in progenitor and differentiated cells. Finally, we cannot rule out the possibility that all of these oncogenes transform differentiated cells, but Wnt-1 signaling leads to dedifferentiation; however, the loss of *Pten* in both myoepithelial and luminal epithelial cells argues strongly against this possibility.

Although it remains to be determined whether the Wnt-1 signaling pathway and the oncogenic pathways mediated by Neu, H-Ras, and PyMT induce tumors in mammary cells at precisely the same stage of differentiation, these transgenes are presumably expressed in similar populations of mammary cells, because they are all under the control of the MMTV LTR. In accord with this assumption, interbreeding of MMTV-*c-Myc* and MMTV-*H-Ras* led to more rapid formation of mammary tumors (33); but differentiation markers have not been studied in these tumors to ask whether the *H-Ras*- or *c-Myc*-induced phenotype predominates. To ask this question in a different context, we have recently bred MMTV-*Wnt-1* with MMTV-*Neu* TG mice. The resulting bi-TG females develop mammary tumors as early as 12 weeks of age, sooner than in mice carrying either transgene alone, implying that both transgenes were expressed in these tumors (K.P. and Y.L., unpublished work). Interestingly, neoplastic cells in these tumors are positive for keratin 6, Sca-1, and myoepithelial markers, and they are histopathologically more similar to *Wnt-1*-induced tumors than to *Neu*-induced tumors (K.P. and Y.L., unpublished work). Therefore, it appears that Neu does not relieve the arrest of differentiation that may be imposed by Wnt-1 signaling in mammary progenitor cells.

In conclusion, we provide several lines of evidence to suggest that components of the Wnt signaling pathway transform mammary progenitors, and that these cells develop into heterogeneous tumors containing different histological cell types expressing markers of both mature and immature epithelial cells. Thus, breast cancer heterogeneity may result from transformation of distinct cell types by different oncogenes.

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