Available online at www.sciencedirect.com
ScienceDirect
Developmental Biology 303 (2007) 29–44
DEVELOPMENTAL BIOLOGY
www.elsevier.com/locate/ydbio

Parity-induced mammary epithelial cells are multipotent and express cell surface markers associated with stem cells

Laurice A. Matulka [a], Aleata A. Triplett [a], Kay-Uwe Wagner [a,b,*]

[a] *Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, 986805 Nebraska Medical Center, Rm. 8009, Omaha, NE 68198-6805, USA*

[b] *Department of Pathology and Microbiology, University of Nebraska Medical Center, 986805 Nebraska Medical Center, Omaha, NE 68198-6805, USA*

Received for publication 5 June 2006; revised 29 November 2006; accepted 7 December 2006
Available online 13 December 2006

Abstract

Parity-induced mammary epithelial cells (PI-MECs) are defined as a pregnancy hormone-responsive cell population that activates the promoter of late milk protein genes during the second half of pregnancy and lactation. However, unlike their terminally differentiated counterparts, these cells do not undergo programmed cell death during post-lactational remodeling of the gland. We previously demonstrated that upon transplantation into an epithelial-free mammary fat pad, PI-MECs exhibited two important features of multipotent mammary epithelial progenitors: a) self-renewal, and b) contribution to ductal and alveolar morphogenesis. In this new report, we introduce a new method to viably label PI-MECs. Using this methodology, we analyzed the requirement of ovarian hormones for the maintenance of this epithelial subtype in the involuted mammary gland. Furthermore, we examined the expression of putative stem cell markers and found that a portion of GFP-labeled PI-MECs were part of the $CD24^{+}/CD49f^{high}$ mammary epithelial subtype, which has recently been suggested to contain multipotent stem cells. Subsequently, we demonstrated that isolated PI-MECs were able to form mammospheres in culture, and upon transplantation, these purified epithelial cells were capable of establishing a fully functional mammary gland. These observations suggest that PI-MECs contain multipotent progenitors that are able to self renew and generate diverse epithelial lineages present in the murine mammary gland.

Keywords: Mammary gland; Differentiation; Cre recombinase; Stem cells; CD24; CD49f; Sca-1; PI-MECs; Side population; Pregnancy; Ovariectomy

Introduction

The mammary gland consists of a network of branching ducts with alveolar units located at their terminal ends. In the murine gland, ducts are embedded within a fibrous and adipocyte-rich stroma, called the mammary gland fat pad. During each pregnancy, precursor cells within alveolar units proliferate and differentiate to form the secretory lobuloalveolar compartment of the mammary gland. After weaning of the offspring and cessation of lactation, these differentiated secretory structures regress swiftly. Although mammary glands of nulliparous (virgin) and nonpregnant, parous females are quite similar in their morphological appearance, significant differences in growth properties, physiological parameters, and gene expression profiles of mammary epithelial cells (MECs) have been reported (for more detailed information on this subject please refer to a recent review by Wagner and Smith, 2005).

One difference between mammary glands of nulliparous and parous females is the existence of a unique epithelial cell population that originates during the first full-term pregnancy cycle. Using a Wap-Cre/Rosa-LacZ double transgenic reporter system, we genetically labeled mammary epithelial cells that are abundant in nonpregnant, parous females and that are virtually absent in virgin animals (Wagner et al., 2002). We therefore named this cell population 'parity-induced mammary epithelial cells' (PI-MECs). In brief, the *WAP* gene promoter-driven Cre recombinase transgene specifically targets hormone

* Corresponding author. Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, 986805 Nebraska Medical Center, Rm. 8009, Omaha, NE 68198-6805, USA. Fax: +1 402 559 4651.
E-mail address: kuwagner@unmc.edu (K.-U. Wagner).

0012-1606/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2006.12.017

responsive alveolar precursors undergoing an advanced differentiation program during the second half of pregnancy and during lactation. Through the Cre-mediated excision of a transcriptional Stop sequence between the *Rosa* promoter and the *LacZ* reporter gene, the transient upregulation of Cre recombinase permanently activates a ubiquitously expressed Rosa-LacZ reporter transgene, whose expression, unlike the Wap-Cre construct, is not dependent on the differentiation status of a given cell. Hence, the constitutive activation of the reporter transgene labels differentiating cells during pregnancy and lactation and their descendents that survive remodeling during involution (i.e. partially committed alveolar cells that are apoptosis-resistant during mammary gland remodeling following a normal pregnancy-lactation cycle).

Using X-Gal-stained histological sections of mammary tissues from parous females, we demonstrated that parity-induced mammary epithelial cells (PI-MECs) are specifically located at terminal ducts within alveolar units (Wagner et al., 2002). In agreement with their location, PI-MECs serve as alveolar progenitors during subsequent pregnancies in multiparous females. These unique cells, however, are not just alveolar precursors. After being transplanted as fragments together with other epithelial subtypes into epithelia-free mammary fat pads, PI-MECs contributed significantly to the reconstitution of branching ducts (Wagner et al., 2002). By performing serial transplantations, Boulanger et al. (2005) recently demonstrated that PI-MECs have the capacity to proliferate and self-renew over several transplant generations. In addition to possessing characteristics of normal mammary epithelial stem cells, we recently found that PI-MECs can serve as cancer-initiating cells (i.e. "cancer stem cells") in Her2/neu-overexpressing transgenic mice that exhibit accelerated tumorigenesis in multiparous females. In agreement with this assumption, the selective growth inhibition of PI-MECs reduced the onset of neoplasia in females expressing the Her2/neu oncogene (Henry et al., 2004).

Various research groups reported that multipotent mammary epithelial subtypes express distinct patterns of putative stem cell markers. The types of cell surface markers and the amount of cells expressing them, however, vary considerably between reports. The existence of a small number of mammary epithelial cells that are able to efflux the dye Hoechst 33342, referred to as the side population (SP), were first described by Welm et al. (2002) and Alvi et al. (2003). Following transplantation, mammary epithelial SP cells were able to generate ducts and alveoli. The selection of mammary epithelial cells expressing the stem cell antigen 1 (Sca-1) also seems to enrich for multipotent stem cells (Welm et al., 2002). Nonetheless, the total amount of cells expressing Sca-1 (approximately 20–30%) greatly exceeds the number of putative stem cells (i.e. about one stem cell in 2500 MECs; Smith, 1996; Kordon and Smith, 1998). Recently, a subpopulation of mammary epithelial cells that express CD24 in combination with CD49f or CD29 has been identified to be highly enriched for multipotent stem cells (Shackleton et al., 2006; Stingl et al., 2006). Functional mammary glands consisting of both lobular and ductal structures were obtained following transplantation of purified MECs that either exhibit a $Lin^{-}/CD29^{hi}/CD24^{+}$ or $CD24^{+}/CD49f^{high}$ marker profile.

As demonstrated in transplantation studies, parity-induced mammary epithelial cells contain multipotent progenitors (Wagner et al., 2002; Boulanger et al., 2005). Since the relative amount of PI-MECs among all mammary epithelial cells in a parous mammary gland exceeds the suggested number of stem cells, we hypothesized that PI-MECs themselves are heterogeneous based on their expression profile of putative stem cell markers. To examine the expression of these markers, we developed a strategy to viably label PI-MECs using the WAP-Cre transgene in combination with a Cre/lox reporter strain expressing the green fluorescent protein (GFP) under a ubiquitous β-actin promoter. Using this approach, we first determined the requirement of ovarian hormones for the maintenance of PI-MECs in the involuted mammary gland. Surprisingly, the results of our experiments demonstrated that the continued presence of PI-MECs in the involuted mammary gland does not depend on ovarian hormones that once were essential to generate this unique epithelial subtype. Using multicolor flow cytometry, we examined the expression of putative stem cell markers and found that GFP-labeled PI-MECs were present in a fraction of $CD24^{+}/CD49f^{high}$ mammary epithelial cells that has been reported recently to contain multipotent stem cells (Stingl et al., 2006). Subsequently, we demonstrated that PI-MECs were able to form mammospheres in culture, and, upon transplantation, purified PI-MECs were able to establish a fully functional mammary gland. Unexpectedly, the transplantation of mammary epithelial cells devoid of PI-MECs did not result in epithelial outgrowths. Based on this finding and other observations in previously published experiments we propose that PI-MECs do not only have stem cell capabilities, but they might also functionally contribute to the maintenance of other pluripotent progenitors as part of the stem cell niche.

Materials and methods

Transgenic mice

Genetically-engineered mice carrying the WAP-Cre transgene (Wagner et al., 1997a) and the Rosa-LacZ Cre/lox reporter construct (Soriano, 1999) have been described previously. Oligonucleotide sequences and PCR conditions utilized to genotype these transgenic strains were published elsewhere (Wagner et al., 2002). Genetically-engineered mice that express the enhanced green fluorescent protein (GFP) under a modified chicken β-actin promoter upon Cre-mediated recombination (CAG-lox-CAT-lox-GFP) were generated by Kawamoto and coworkers (Kawamoto et al., 2000). The presence of the CAG-lox-CAT-lox-GFP (or short CAG-GFP) construct was verified by PCR using the following forward and reverse primers that detect 310 base pairs of the coding sequence of the GFP: 5′-GAC GTA AAC GGC CAC AAG TTC AG-3′ and 5′-GAT GCG GTT CAC CAG GGT GTC G-3′. All animals used in the described studies were treated humanely and in accordance with institutional guidelines and federal regulations.

Mammary epithelial cell cultures

Number four inguinal mammary glands of nonpregnant, parous WAP-Cre/CAG-GFP double transgenic females (30 days of involution) and age-matched WAP-Cre/CAG-GFP virgin controls were dissected and analyzed briefly under a fluorescence stereoscope to verify the presence or absence of GFP-expressing

cells. Primary mouse mammary epithelial cells (MECs) were isolated from these mammary glands by enzymatic digestion (collagenase and hyaluronidase) according to a protocol published by Medina and Kittrell (2000). Cells were cultured for up to 72 h in Dulbecco's modified Eagle's medium (DMEM)/F12 supplemented with 2% fetal bovine serum, 10 μg/ml insulin, 1 μg/ml hydrocortisone, 10 ng/ml epidermal growth factor (EGF), 1 mg/ml bovine serum albumin, 50 μg/ml gentamicin, 20 U/ml fungazone, and 100 U/ml penicillin/streptomycin.

Administration of hormones in vitro and in vivo

Monolayer cultures of MECs or whole mammary gland organ cultures from 6-week-old triple transgenic WAP-Cre/CAG-GFP/Rosa-LacZ nulliparous females were treated with various combinations of steroid and peptide hormones to determine whether the expression of the WAP-Cre transgene could be induced *ex vivo* through hormonal manipulation mimicking pregnancy. The derivation and culture conditions of MECs grown as monolayers are described in the previous paragraph. Mammary gland organ cultures were performed in analogy to a protocol published by Ginsburg and Vonderhaar (2000). In brief, the #4 inguinal glands of virgin females were resected aseptically and cut into ~1 mm^3 fragments. These fragments were randomized and placed into four 10 cm^2 tissue culture dishes. Organ cultures were maintained in Waymouth's media supplemented with 2% fetal bovine serum, gentamicin (50 μg/ml; Invitrogen), fungazone (20 U/ml; Invitrogen), and penicillin/streptomycin (100 U/ml; Invitrogen). The first experimental group of organ cultures and monolayer cultures was treated with estrogen (0.1 μg/ml) and progesterone (0.9 μg/ml). A second experimental group was treated with estrogen, progesterone, and EGF (100 ng/ml). A third experimental group was treated with DIP (i.e. 1 μM dexamethasone, 10 μg/ml insulin, and 1.0 μg/ml prolactin), which is a hormone combination known to induce alveolar differentiation. All experimental groups and untreated controls were maintained for 7 days before analysis of WAP-Cre and CAG-GFP activation. The expression of the GFP reporter construct in monolayer cultures was monitored daily under an inverted fluorescence microscope (Zeiss Axiovert 35) equipped with a digital Nikon Coolpix camera. We observed that the nonspecific autofluorescence of the mammary fat pad of cultured mammary fragments increased significantly during the 7-day treatment period (hormone-treated and untreated controls alike). Therefore, we decided to enzymatically digest the tissue and analyze epithelial cells 72 h later as monolayer cultures. The presence of GFP-expressing MECs was documented, and cells were fixed in glutaraldehyde and stained with X-Gal as described previously (Wagner et al., 2002).

Six-week-old virgin WAP-Cre/CAG-GFP double transgenic females ($n=12$) were randomized into two experimental groups. A mammary gland biopsy of one #4 mammary gland of each animal was performed to assess the absence of GFP expressing cells and the morphology of the gland prior to hormonal stimulation (internal controls). Slow release pellets (Innovative Research of America, Inc.) containing estrogen (1.7 mg) and progesterone (15 mg) or placebo pellets were placed under the skin of both experimental groups. After 21 days of hormonal stimulation, pellets were removed to induce remodeling (i.e. "involution") of alveolar cells during a consecutive period of 28 days. Subsequently, the presence of GFP-expressing epithelial cells was documented by analyzing the remaining #4 mammary glands under a fluorescent stereoscope. To assess the overall morphology of the gland after hormonal stimulation and remodeling, mammary gland whole mounts were fixed in Carnoy's and stained with Carmine Alum as described previously (Wagner et al., 1997b).

Derivation of mammospheres

Mammary epithelial cells from 4-month-old parous, involuted WAP-Cre/CAG-GFP mice were cultured for 72 h followed by FACS (FACSVantage DiVa, Becton Dickinson) to collect the GFP^{pos} and GFP^{neg} populations. Defined numbers of GFP^{pos} and GFP^{neg} mammary epithelial cells were resuspended in DMEM/F12 supplemented with bFGF-2 (40 ng/ml), EGF (20 ng/ml), B27 (20×), and heparin (4 μg/ml) and transferred into a T25 flask. Non-adherent mammospheres began to form within 4 days and were cultured for 7 days prior to analysis. The total number of mammospheres was counted in both cultures.

Ovariectomy

The surgical removal of both ovaries from 4-month-old parous, involuted WAP-Cre/CAG-GFP mice ($n=6$) was carried out according to a standard surgical protocol. Colleagues of the UNMC Mouse Genome Engineering Core Facility assisted during the surgical procedure. Mammary glands were removed 28 days later to analyze their morphological features and to determine the presence and relative amount of parity-induced mammary epithelial cells in the atrophic ductal network. For quantitative analysis of the relative amount of GFP-labeled cells, primary MECs were derived and analyzed by flow cytometry as described above.

Labeling of cell surface markers and flow cytometric analysis

Cell surface markers present on mammary epithelial cells were labeled by incubating cells with primary antibodies against the respective antigen in 1× phosphate buffered saline (PBS) supplemented with 1% bovine serum albumin and 0.1% sodium azide (FACS buffer) for 25 min at 4 °C. Subsequently, cells were washed once in ice-cold FACS buffer to remove excess antibodies. An unconjugated antibody against CD24 (clone M1/69; 1:200 dilution in FACS buffer) and PE-conjugated antibodies against CD49f (clone GoH3; 1:4 dilution) and Sca-1 (clones D7 and E13–161.7; 1:250 dilution) were purchased from BD Pharmingen. A goat-anti-mouse IgG, $F(ab')_2$ fragment conjugated to allophycocyanin (APC, 1:150 dilution; Jackson Immunoresearch) was utilized as a secondary antibody in a three-color flow cytometric analysis to detect CD24-labeled cells. Flow cytometric analyses were performed on a FACSCalibur cytometer at the UNMC Cell Analysis Core Facility. Fluorescence activated sorting of GFP-labeled cells was carried out on a FACSVantage DiVa (Becton Dickinson). The purity of sorted populations was consistently greater than 95%.

Immunofluorescence staining

Primary cells were fractionated into CD24 positive or negative subtypes as well as GFP positive of native populations on a FACSVantage DiVa. After the sorting procedure, cells were plated into chamber slides (50,000 cells/well). Following a culture period of 48 h, the cells were rinsed with 1× PBS, fixed, and permeabilized in cold methanol at −20 °C for 10 min. Subsequently, cells were incubated in bovine serum albumin (BSA) (3% in PBS) for 60 min then treated with primary antibodies (Cytokeratin 14; 1/50; Covance, CA or Cytokeratin 18; 1/20; Progen Biotechnik, Germany) for 24 h at 4 °C. After an additional washing step in 1xPBS, cells were incubated with an isotype-specific, Alexa Fluor® 594-conjugated secondary antibody (Molecular Probes) for 60 min in the dark at room temperature. The slides were washed repeatedly in 1× PBS and mounted with Vectashield containing 1.5 μg of 4′,6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Inc). Digital fluorescence images were taken on a Zeiss Axio Imager microscope equipped with a SPOT FLEX camera (Diagnostic Instruments, Inc.), a Windows/XP-based computer, and image capturing software (SPOTsoftware V.4.6).

Hoechst 33342 staining of epithelial cells

Primary mammary epithelial cells were stained with the Hoechst 33342 dye immediately following the isolation of single cells from enzymatically digested tissues of parous WAP-Cre/CAG-GFP females. MECs were resuspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2% FBS at a final concentration of 1×10^6 cells per ml and incubated at 37 °C for 90 min with 5 μg/ml Hoechst 33342 dye. The sorting of Hoechst-dye effluxing cells was performed on a FACSVantage DiVa (Becton Dickinson) flow cytometer as described above.

Transplantation of GFP-expressing PI-MECs

DeOme and his colleagues originally devised the technique of tissue transplantation into mammary fat pads cleared from endogenous mammary epithelium (DeOme et al., 1959). The surgical procedures for clearing the fat pad of 3-week-old female mice and the method of implanting tissue fragments and

cell suspensions have been described in detail by Lawrence Young (2000). Pure populations of parity-induced mammary epithelial cells (PI-MECs) and their GFP-negative controls were obtained through fluorescence activated cell sorting using a FACSVantage DiVa (Becton Dickinson) as described above. GFP-positive PI-MECs and GFP-negative control MECs were resuspended in DMEM supplemented with 10% FBS. A total number of 1.2×10^4 cells were injected in a volume of 20 μl into each cleared mammary fat pad of 21-day-old recipient females (AthymicNCr-nu/nu females). The recipients were maintained as nulliparous animals for 8 weeks prior to removal and analysis of the transplant. A subset of recipient females was bred 8 weeks after transplantation to induce lobuloalveolar development in the resulting outgrowth. Mammary transplants were resected during mid-pregnancy and analyzed as whole mounts under the stereoscope to visualize GFP-positive ductal structures and alveolar cells. Next, the transplants were treated in Carnoy's fixative for several hours, washed in distilled water, and stained with Carmine Alum (Wagner et al., 1997b) to examine morphological features of the reconstituted mammary ductal network.

Results

Labeling and quantification of viable PI-MECs

In previous studies, we labeled PI-MECs and studied their stem cell properties in fixed mammary tissues from parous, nonpregnant WAP-Cre/Rosa-LacZ double transgenic females. Since β-galactosidase is ubiquitously expressed in these cells, we attempted to utilize the FACS-Gal method (Fiering et al., 1991) to isolate and transplant viable PI-MECs. Initial attempts to use fluorescein di-[beta]-D-galactopyranoside (FDG) failed due to adverse effects of the ice-chilled isotonic incubation medium, which is needed for FDG to diffuse into cells. FGD then has to be hydrolyzed to release the fluorescent dye. After this initial attempt, we reasoned that the use of FDG to label viable stem cells might not be an ideal approach since these unique cells are suggested to express transporters that regulate transmembrane traffic and efficiently efflux drugs. Instead, we developed a strategy to viably label PI-MECs using the WAP-Cre transgene in combination with a Cre/lox reporter strain that upon Cre-mediated recombination expresses the green fluorescent protein (GFP) under the ubiquitously expressed CAG (i.e. CMV enhancer/chicken β-actin) promoter (Fig. 1). We utilized fluorescent stereoscopy on freshly dissected mammary tissues to monitor the expression of GFP in the epithelial compartment of the gland. The fluorescent stereoscope was specifically built in our laboratory by mounting a fluorescence unit with an FITC filter set on a simple Meiji stereoscope (RZ series). The WAP-Cre-mediated excision of the floxed chloramphenicol acetyl transferase (CAT) gene, located between the promoter and the GFP cDNA, led to an efficient activation of GFP in the vast majority of differentiating alveolar cells during pregnancy and lactation (Figs. 2A and B). GFP-labeled PI-MECs were clearly identifiable in mammary tissues from nonpregnant, parous females 4 weeks after weaning of the litter (Figs. 2C and D). Like in the WAP-Cre/Rosa model (Wagner et al., 2002), genetically labeled cells expressing bright GFP fluorescence were confined to terminal ducts and alveolar units, whereas large collecting ducts did not contain a significant amount of GFP positive cells. This observation was confirmed using immunofluorescent staining for GFP in formalin-fixed

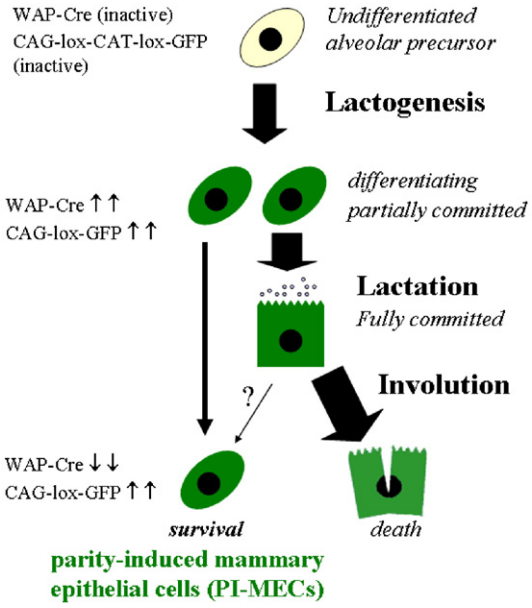

Fig. 1. Methodology to genetically label *viable* parity-induced mammary epithelial cells (PI-MECs) in non-pregnant, parous females. The transient up-regulation of Cre recombinase expressed by the WAP promoter in differentiating epithelial cells during pregnancy permanently activates the expression of the green fluorescence protein (GFP) from a Cre/loxP reporter transgene (CAG-lox-CAT-lox-GFP) due to the Cre-mediated excision of the floxed chloramphenicol acetyltransferase (CAT) gene between the ubiquitously expressed chicken beta-actin (CAG) promoter and the GFP coding sequence. Once activated through Cre-mediated recombination, the cell-type specific expression of the CAG-GFP reporter is not dependent on the differentiation status of a given cell, and the reporter remains active in cells that change their fate during development whether they still express Cre recombinase or not. Hence, the WAP-Cre-mediated, permanent activation of the reporter transgene labels pregnancy hormone-responsive mammary epithelial cells that are apoptosis resistant during involution. Since these genetically-labeled cells are only abundant in the parous mammary gland, they were defined as 'parity-induced mammary epithelial cells'.

sections (data not shown). The majority of age-matched nulliparous (virgin) WAP-Cre/CAG-GFP females did not exhibit GFP-labeled cells throughout their mammary ductal system (data not shown). A few GFP-positive cells could be detected in nulliparous mice at particular stages of the estrus cycle (i.e. during estrus), but these cells are known to disappear during metestrus (Wagner et al., 2002). Nine-month to 1-year-old nulliparous females also did not exhibit significantly higher numbers of GFP-positive cells ($\leq 5\%$, $n=2$), which is another indication that these WAP-Cre-expressing cells are transiently present and do not accumulate in aging mice. Mammary glands of CAG-GFP single transgenic mice did not exhibit GFP positive cells (data not shown), suggesting that spontaneous activation of this reporter transgene does not occur.

Next, we wanted to determine whether the activated CAG-GFP transgene is also expressed in explanted mammary epithelial cells. We dissected both inguinal #4 mammary glands from primiparous WAP-Cre/CAG-GFP females (>30 days involution) and their age-matched nulliparous controls. Before enzymatically dissociating the tissues, we verified the presence of PI-MECs in primiparous glands under the fluorescent stereoscope. Differential centrifugation was performed to re-

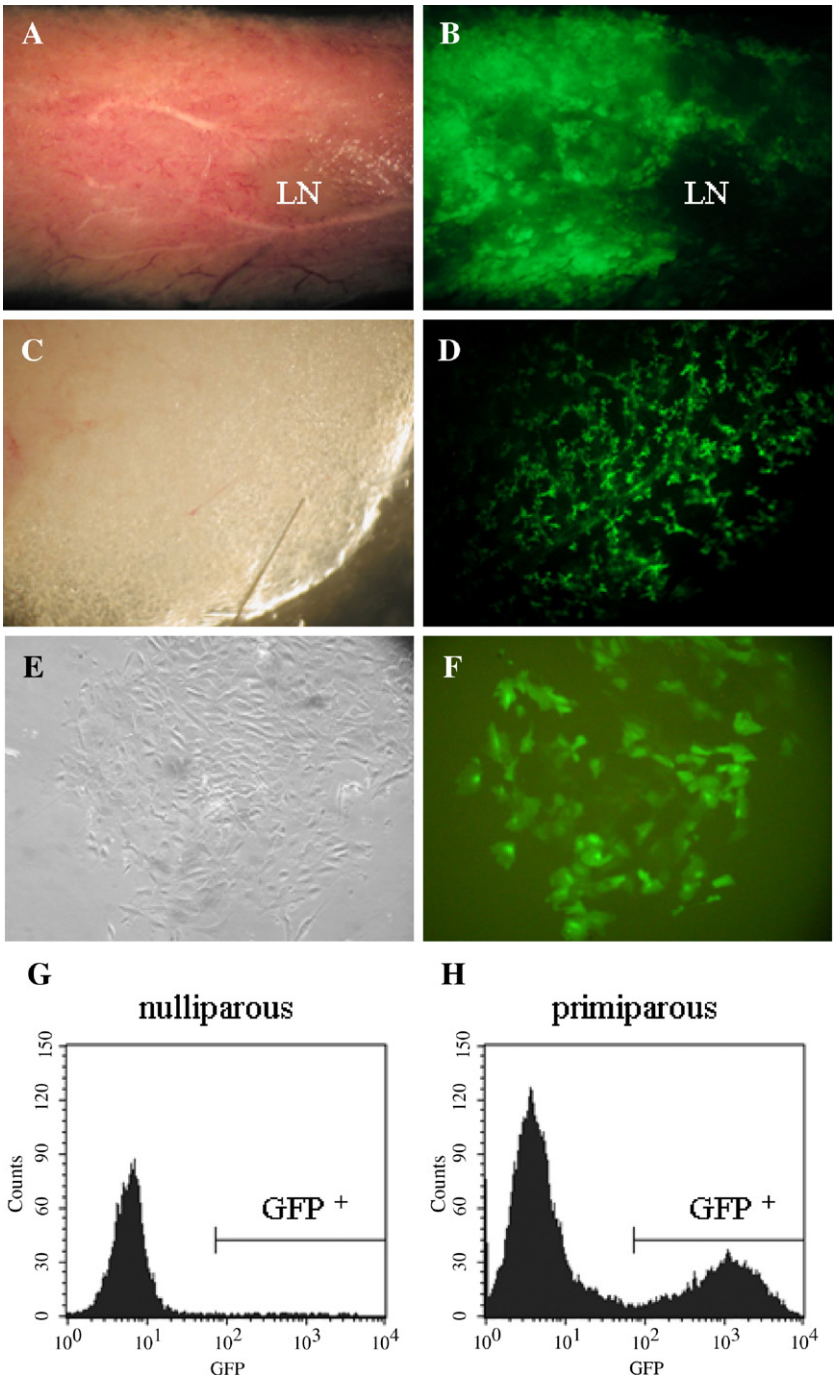

Fig. 2. Analysis of GFP expression in mammary epithelial cells of WAP-Cre/CAG-GFP double transgenic females using fluorescence microscopy (A–F) and flow cytometry (G, H). Brightfield pictures (A, C) and corresponding GFP fluorescence images (B, D) of freshly isolated mammary glands at day 10 of lactation (A, B) and after 30 days of postlactational involution (C, D) using a fluorescence stereoscope (10× magnification). Note that the lymph node (LN) and blood vessels do not express GFP. Phase contrast (E) and GFP fluorescence image (F) of primary mammary epithelial cells derived from parous double transgenic females (200× magnification). Comparison of GFP expression in mammary epithelial cells of nulliparous (G) and primiparous (H) females using flow cytometry.

move a fraction of stromal cells, and mammary organiods and single cells were plated into plastic dishes without using adhesion substrates. Notably, the expression of GFP was temporarily reduced in enzymatically dissociated cells. PI-MECs regained strong GFP expression within 48 h after attaching to the dish (Figs. 2E and F). To verify that the collagenase/hyaluronidase treatment is responsible for this temporarily weakened GFP expression, we dissociated mammary glands by mechanical means using a Tekmar Stomacher (data not shown). Unlike enzymatic treatment, the mechanical dissociation of the tissue did not affect the expression of GFP, but this method yielded fewer single cells. Therefore, we preferred enzymatic dissociation and short-term culturing of primary cells for up to 72 h as the primary technique to isolate viable PI-MECs. Subsequently, we used flow cytometry to determine the relative amount of GFP-labeled cells in primary cell cultures

from primiparous mammary glands and age-matched nulliparous controls (Figs. 2G and H). Thus far, we analyzed 50 cultures of primiparous WAP-Cre/CAG-GFP females, and on average 16% (±9.05%) of the primary cells expressed GFP. As expected, only 2.36% (±2.34%, $n=10$ mice) of cultured cells were GFP-positive in nulliparous control glands. GFP-labeled PI-MECs were identifiable in involuted mammary glands of primiparous and multiparous mice ranging in age from 9 months to 1 year. This suggests that unlike transiently WAP-Cre-expressing cells in nulliparous females (see previous paragraph), PI-MECs are an epithelial subtype that permanently populates the involuted mammary gland after a single full-term pregnancy.

The origination of PI-MECs requires the hormonal milieu of a full-term pregnancy, the presence of the stroma, and a correct 3D architecture of the mammary gland

To address whether the presence of pregnancy hormones is sufficient to activate the WAP-Cre transgene in cultured primary cells, we treated epithelial monolayer cultures derived from nulliparous WAP-Cre/CAG-GFP females for 7 days with three hormonal combinations: a) estrogen and progesterone, b) estrogen, progesterone, and EGF, and c) dexamethasone, insulin, and prolactin. In a nutshell, none of these hormonal cocktails were able to induce the activation of the WAP-Cre transgene and the GFP reporter construct in monolayer cultures, suggesting that the presence of steroid and peptide hormones is not sufficient to trigger advanced differentiation and activation of *Wap* regulatory elements (data not shown). Next, we asked whether the efficacy of these hormones could be intensified by preserving the correct 3D architecture of the mammary gland when treated *ex vivo*. For this purpose, we maintained mammary gland fragments from nulliparous WAP-Cre/CAG-GFP females for 7 days in Waymouth's media containing 2% fetal bovine serum supplemented with one of the three hormonal cocktails. Due to a bright yellow autofluorescence of the mammary gland fat pad that emerged on the second day of culturing fragments in all experimental groups including untreated controls, we were unable to directly assay the activation of the GFP reporter by examining the fragments under the stereoscope. Therefore, we enzymatically dissociated the

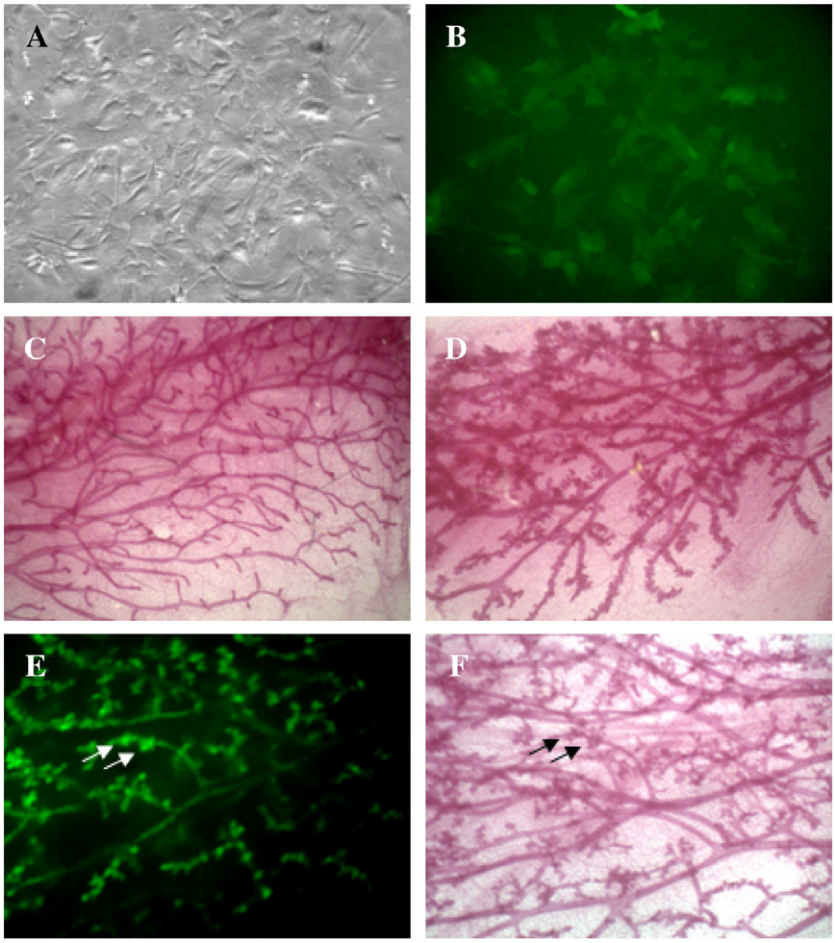

Fig. 3. WAP-Cre-mediated activation of the CAG-GFP reporter in mammary epithelial cells of nulliparous, WAP-Cre/CAG-GFP double transgenic females after treatment with steroid and peptide hormones. A and B: Brightfield image and corresponding GFP fluorescence of isolated epithelial cells from mammary gland fragments that were treated for 7 days in culture with dexamethasone, insulin, and prolactin. C and D: Carmine Alum-stained mammary glands of a nulliparous female prior to hormonal stimulation (C) and after 21 days of continuous administration of estrogen and progesterone (D). E and F: GFP fluorescence image and corresponding Carmine Alum-stained whole mount of a mammary gland 28 days after withdrawal of estrogen and progesterone. Note that cells with strong GFP expression were mainly localized within alveolar units at terminal ends of selected ducts (arrows).

fragments after a week of hormonal treatment to remove the fat cells and to examine epithelial cells as monolayers 96 h later. GFP-labeled cells were only visible in significant numbers in the experimental group treated with dexamethasone, insulin, and prolactin (Figs. 3A and B). Very few GFP-expressing cells were observed in fragments treated with estrogen, progesterone, and EGF (not shown). Estrogen and progesterone alone, however, were insufficient to induce alveolar differentiation and activation of the WAP-Cre transgene in this 3D culture model. In summary, our observations suggest that the presence of the stroma and a correct 3D architecture of the mammary gland are necessary to facilitate the function of pregnancy hormones to induce functional differentiation and activation of the WAP-Cre transgene.

Administration of extraphysiological levels of estrogen and progesterone are often used to mimic early pregnancy and to induce alveolar proliferation *in vivo*. Since these hormones alone had little effect on alveolar specification and WAP-Cre activation *in vitro*, we asked whether the effects of estrogen and progesterone can be enhanced by other growth factors such as prolactin, insulin, and glucocorticosteroids that are present at physiological levels in nulliparous females. A mammary gland biopsy of one #4 mammary gland was performed on 12 nulliparous WAP-Cre/CAG-GFP double transgenic females 6 weeks of age to verify the absence of GFP expressing cells (data not shown) and to assess the typical morphology of a virgin gland prior to hormonal stimulation (Fig. 3C). Next, these mice were randomized into two experimental groups. Slow release pellets containing estrogen and progesterone were placed subcutaneously into animals of the experimental group. Females of the control group received placebo pellets. Alveolar bud formation, which is an indicator for the correct delivery of estrogen and progesterone, was determined by Carmine Alum staining of one mammary gland of each experimental group at day 21 of hormonal treatment (Fig. 3D). After 21 days of hormonal stimulation, pellets were removed to induce remodeling (i.e. "involution") of alveolar cells that transiently express WAP-Cre (i.e. similar to partially differentiated cells that normally disappear during metestrus). After a remodeling period of 28 days, the presence of GFP-expressing epithelial cells was documented by analyzing #4 mammary glands under a fluorescent stereoscope (Fig. 3E). Clusters of GFP-positive cells were clearly visible at the terminal ends of some ducts. To assess the overall morphology of ductal structures containing GFP-expressing cells, mammary gland whole mounts were subsequently fixed and stained with Carmine Alum (Fig. 3F). This experiment shows that prolonged exposure to estrogen and progesterone can partially induce parity-induced epithelia-like structures in some regions of the mammary gland, but the number and distribution of GFP-positive cells are much lower and quite irregular compared to a mammary gland after a single full-term pregnancy. Hence, the majority of PI-MECs do not appear during early pregnancy in response to elevated levels of estrogen and progesterone. They seem to preferentially originate during the second half of pregnancy when specification and functional differentiation in response to prolactin signaling occurs.

PI-MECs do not require ovarian hormones for their persistent presence in the involuted mammary gland

The hormonal milieu of pregnancy is important for the appearance of PI-MECs during the first full-term gestation period. After analyzing the genesis of PI-MECs in response to

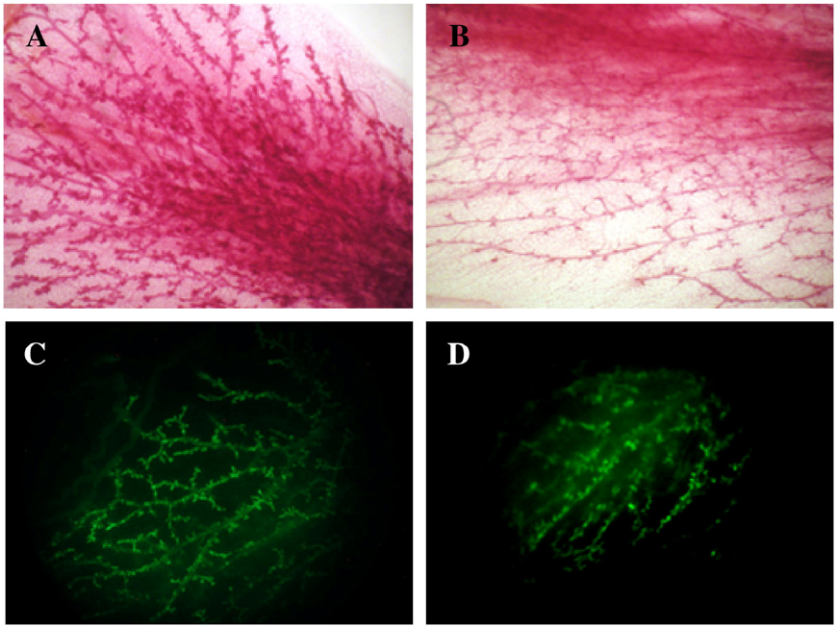

Fig. 4. Presence of PI-MECs in the mammary gland of parous females that were ovariectomized after a full-term pregnancy and lactation–involution cycle. A: Carmine Alum-stained typical mammary gland of a primiparous female after 30 days of involution. B: Carmine Alum-stained mammary gland of a primiparous female 28 days following bilateral ovariectomy. C and D: GFP fluorescence analysis of PI-MECs in unfixed and unstained mammary tissue prior to ovariectomy (C) and 28 days following bilateral ovariectomy (D).

hormonal stimulation, we asked whether ovarian hormones are also necessary for the maintenance of this unique epithelial subtype in the remodeled gland following postlactational involution. To address this question, we assessed the appearance of GFP-labeled PI-MECs in primiparous WAP-Cre/CAG-GFP double transgenic females before and after bilateral ovariectomy. The typical morphology of a primiparous mammary gland after 30 days of involution is shown in Fig. 4A. We removed one inguinal #4 mammary gland prior to ovariectomy to verify the presence of PI-MECs in these glands (Fig. 4C) and to determine the percentage of PI-MECs in primiparous females with intact ovarian function (internal control). In this particular experimental group, PI-MECs comprised 9.25% (±1.8%, $n=8$ glands) of the total number of primary cells. Within 28 days following bilateral ovariectomy, mammary glands underwent epithelia atrophy due to the loss of ovarian hormones (Fig. 4B). The entire ductal system, in particular collecting ducts, appeared much thinner, but tertiary side branches and alveolar units at duct termini were still present. Surprisingly, the loss of ovarian hormones had virtually no effect on the existence of PI-MECs (Fig. 4D). The relative amount of GFP-positive cells was slightly higher in ovariectomized females (13.63±8.44%, $n=5$), but this difference was statistically insignificant due to variability between ovariectomized animals. We demonstrate in the next section that virtually all GFP-labeled PI-MECs were positive for the extra-cellular marker CD24. About one-third of CD24-positive mammary epithelial cells (35.4±4.9%) had detectable levels of GFP prior to ovariectomy. The relative amount of GFP-positive cells varied to a greater extent in ovariectomized females. Although the total number of mammary epithelial cells might decline following ovariectomy, the mean relative number of labeled PI-MECs among all CD24-positive cells in mammary glands of ovariectomized females (41.9±11.6%) was not different from those prior to ovariectomy. In conclusion, a very significant portion of PI-MECs that are present in the involuted mammary gland of nonpregnant females do not depend on ovarian hormones that once were essential to generate this unique epithelial subtype during a full-term pregnancy.

A subset of PI-MECs express cell surface markers associated with multipotent mammary epithelial stem cells

Recent studies by Stingl et al. (2006) and Shackleton et al. (2006) suggest that multipotent mammary epithelial stem cells reside within a population of cells that express the heat-stable antigen (CD24) in combination with α6-integrin (CD49f) or β1-integrin (CD29). These studies were performed on nulliparous females, and the effect of pregnancy on the expression of these stem cell markers had not been determined. Since PI-MECs exhibit characteristics of multipotent stem cells when transplanted into an epithelia-free mammary stroma, we assumed that this epithelial subtype might contain cells that display a similar combination of these putative stem cell markers. We used triple-color flow cytometry to determine the co-expression of CD24 and CD49f in all epithelial cells and in GFP-labeled PI-MECs that were cultured for 72 h. Along with these cells from primiparous WAP-Cre/CAG-GFP females, we also examined age-matched, nulliparous WAP-Cre/CAG-GFP controls to study the effects of a single pregnancy on possible changes in the relative number of cells expressing the two putative stem cell markers. All flow cytometric analyses were performed using appropriate negative controls for each marker (not shown). The initial comparison of the scatter plots (Fig. 5) show that under our experimental conditions, the majority of

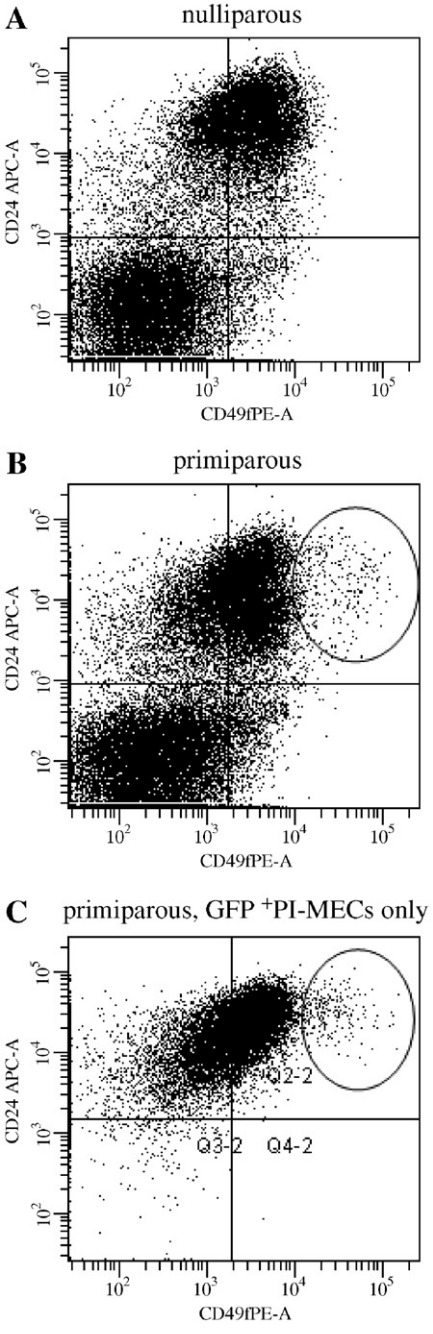

Fig. 5. Co-expression of CD24 and CD49f on the surface of mammary epithelial cells in nulliparous (A) and primiparous (B) females. Using triple-color flow cytometry, both cell surface markers shown in panel B were gated for GFP-positive PI-MECs (C). Note that virtually all PI-MECs express CD24, and the majority of this epithelial subtype co-expresses CD49f. PI-MECs were also present in a CD24/CD49f double positive population with exceptionally high expression of CD49f (B and C, circle).

cultured primary cells can be classified into CD24 positive and negative subtypes. In contrast, CD49f is expressed on a sliding scale, i.e. cells expressing CD49f display more variant levels of this marker on their surface. In general, medium and high levels of CD49f appeared to be more prominent in cells that are also positive for CD24 (Figs. 5A and B, upper right quadrant). Sleeman et al. (2006) recently suggested that the various mammary epithelial subtypes express distinctive levels of CD24, whereas the CD24 negative population represents stromal cells. We verified these observations under our experimental conditions using immunofluorescence staining for cytokeratin 14 and 18 on primary cells that were sorted into CD24 positive and negative populations (Fig. 6A). In this experiment, the CD24 negative population was not exclusively comprised of stromal cells but also contained a fraction of keratin 14 expressing mammary epithelial cells. Nonetheless, CD24 negative cells did not express keratin 18, suggesting that the vast majority of mammary epithelial cells (luminal and basal) were present in the CD24 positive population. Accordingly, GFP-labeled PI-MECs were virtually all expressing CD24 (98±1%), and the majority of them co-expressed CD49f (Fig. 5C). Fig. 6B illustrates the average percentages of GFP-labeled PI-MECs from eight primiparous females stratified according to their expression levels of CD24 and CD49f. PI-MECs constituted approximately 12% (11.6±3.1%) of all primary cells in this particular experimental group. About 30% (29.1±9.3%) of all primary mammary cells that express medium to high levels of CD24 were GFP positive.

Next, we analyzed the ratio of dual stained cells among all CD24 positive cells depending on the reproductive status of a female. We found that a single full-term pregnancy did not significantly alter the percentage of dual labeled cells within the $CD24^+$ population (73.4±11.8% in virgins, $n=10$; 71.4±11.8% in primiparous females, $n=8$). However, while analyzing the

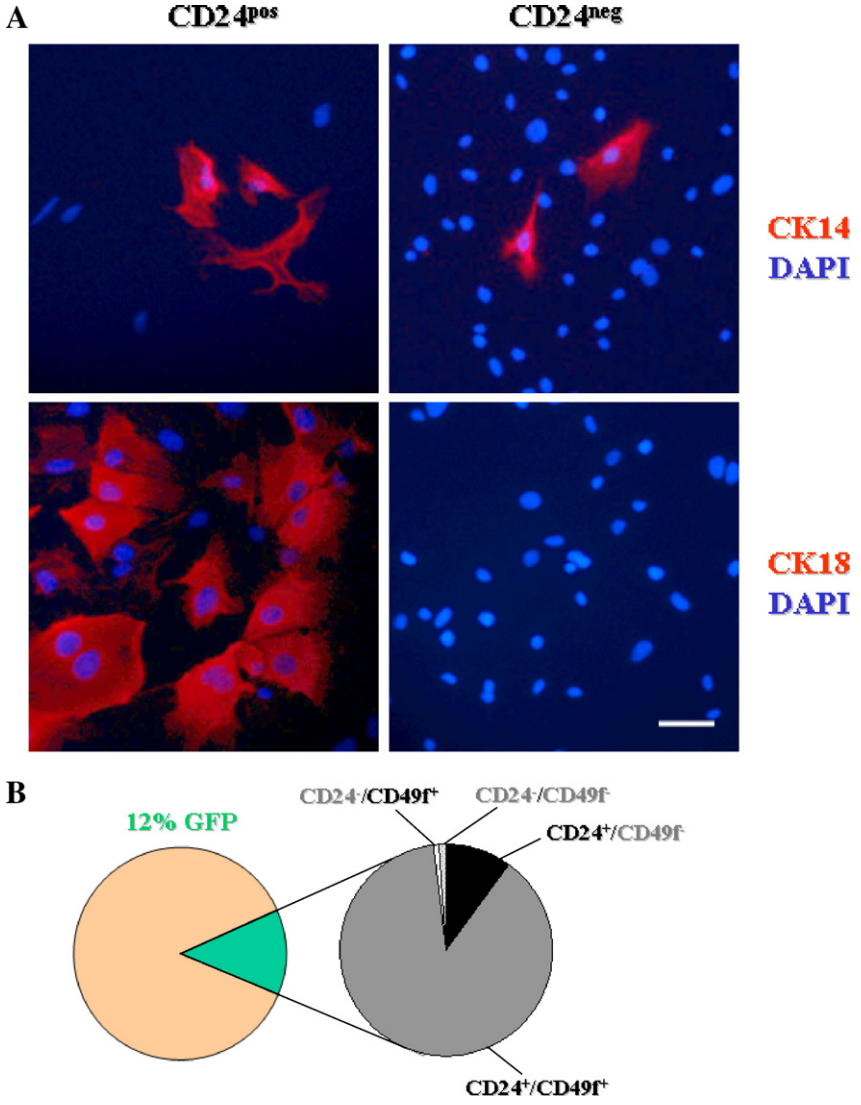

Fig. 6. A: Immunofluorescent staining of cytokeratin 14 and 18 in CD24 positive and negative primary cells of the mammary gland that were separated using fluorescent activated cell sorting (FACS). The bar represents 50 μm. B: Average percentage of GFP-labeled PI-MECs expressing CD24 alone or in combination with CD49f in mammary glands of primiparous WAP-Cre/CAG-GFP females. PI-MECs represented approximately 12% (11.6±3.1%) of all primary cells isolated from individual mammary glands. Note that virtually all PI-MECs express CD24 and the vast majority co-expresses CD49f.

Table 1
Percentage of $CD24^+/CD49f^+$ dual positive mammary epithelial cells among all CD24-expressing cells or only GFP-labeled PI-MECs in eight different primiparous females

Sample	All $CD24^+$ (%)	PI-MECs (%)
1	58.8	93.9
2	57.5	89.8
3	61.9	72.4
4	78.0	90.2
5	66.5	73.0
6	75.7	98.0
7	86.0	96.6
8	87.2	96.0
Mean	71.4±11.8	88.7±10.3

Virtually all (>98%) PI-MECs expressed CD24. Note that the percentage of dual stained cells is higher among PI-MECs in comparison to all CD24 positive cells in each sample.

scatter plots of parous and nulliparous females, we noticed that parous females had a more pronounced occurrence of cells that were positive for CD24 and that expressed maximum levels of CD49f (Fig. 5B, circle). More interestingly, a significant fraction of these cells were GFP-labeled, and therefore represent a subpopulation of PI-MECs (Fig. 5C, circle). The high representation of PI-MECs within the CD24/CD49f double-positive population may also be apparent from the analysis of individual animals (Table 1). In each parous female, the percentage of dual labeled cells within the CD24 positive epithelial cell population was higher among the GFP-labeled PI-MECs compared to the entire $CD24^+$ population of primary cells.

To assure the accuracy of our results, we also addressed whether culturing the cells for 72 h had an effect on the co-expression of these putative stem cell markers. In this experiment, we were able to identify approximately 17% GFP-positive cells immediately after enzymatic dissociation of mammary tissues from parous WAP-Cre/CAG-GFP females. All GFP-labeled cells expressed CD24, and again, the vast majority of them were positive for CD49f (data not shown). This suggests that the co-expression of CD24 and CD49f on the cell surface of PI-MECs was not induced by the culture conditions.

In addition to the markers described above, we also examined the expression of Sca-1 in parous females. Approximately 30% of freshly isolated mammary epithelial cells expressed Sca-1 (data not shown). When placed into culture for 48 to 72 h, virtually all epithelial cells displayed Sca-1 on

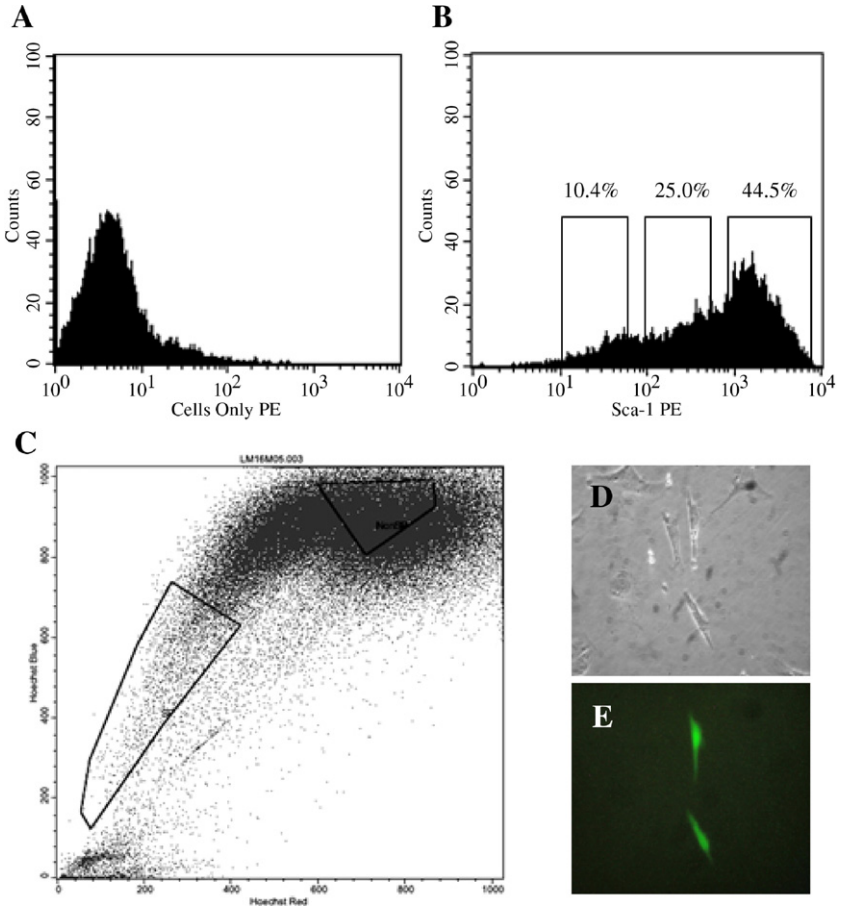

Fig. 7. Contribution of PI-MECs to Sca-1-positive mammary epithelial cells (A, B) and the mammary epithelial "side population", i.e. SP cells (C–E). A and B: No primary antibody control (A) and staining for Sca-1 (B) of mammary epithelial cells cultured for 48 h. Note that in comparison to the negative control, all MECs express Sca-1 at various levels when cultured *ex vivo*. The relative amount of GFP-labeled PI-MECs within the total number of mammary epithelial cells expressing low, medium, or high levels of Sca-1 is indicated in panel B. C: Flow cytometric analysis of Hoechst 33342 dye-effluxing cells (SP cells) in the mammary gland of parous mice. D and E: Analysis of GFP expression in sorted SP cells. Although a small number of SP cells were GFP positive (E), the majority of PI-MECs are not represented by the side population.

their cell surface (Figs. 7A and B). This result is surprising since we used similar culture conditions and Sca-1 labeling procedures that were described by Welm et al. (2002). To ensure the integrity of this observation, we repeated the study with a different Sca-1 antibody, and we used dilution series of both antibodies. The results were identical, suggesting that primary MECs upregulate Sca-1 when placed into culture. Despite its presence on all cultured epithelial cells, Sca-1 expression seemed to vary between individual cells. We therefore examined the percentage of PI-MECs that correspond to cellular populations expressing low, medium, or high levels of Sca-1 (Fig. 7B). For this experiment, we analyzed three parous females (28 days of involution), and PI-MECs constituted 21–23% of the primary cells in this experimental group. PI-MECs were present in all three fractions of Sca-1-expressing cells, but the majority (i.e. 44%) of PI-MECs resided within cells that expressed high levels of Sca-1.

Next, we examined whether a subset of PI-MECs belongs to the 'side population (SP)', i.e. cells that are able to efflux the Hoechst 33342 dye due to the presence of the ATP-binding cassette transporters Abcg2 and Mdr1a/1b (Jonker et al., 2005a). In an initial experiment, we investigated possible effects of the culture conditions on the relative number of Hoechst 33342 dye-effluxing cells. We observed that culturing MECs for up to 72 h significantly increased the percentage of SP cells. An overnight culture contained 7% Hoechst 33342 dye-effluxing cells, and this relative number increased to 26% after 72 h. Hence, we were faced with the dilemma that SP cells needed to be analyzed from freshly dissociated mammary glands. However, as mentioned earlier, PI-MECs often transiently exhibited lower levels of GFP expression during the enzymatic dissociation procedure. To circumvent this problem, we used a reciprocal experimental design. First, we isolated SP cells from freshly prepared single cell suspensions (Fig. 7C). Subsequently, we cultured isolated SP cells for 72 h to resume maximal expression of GFP. In this experiment, SP cells represented less than 2% (1.4%) of all MECs. Next, we examined the expression of GFP directly under a fluorescent microscope (Figs. 7D and E) and subsequently counted GFP-positive cells using flow cytometry. This analysis of adherent cells after the sorting procedure also facilitates the exclusion of hematopoietic SP cells. Under our experimental conditions, 8.6% of mammary epithelial cells capable of effluxing the Hoechst 33342 dye were GFP positive (Fig. 7E). The majority of SP cells, however, were GFP negative. PI-MECs were present in a higher percentage (16.8%) in MECs that did not belong to the 'side population'. These observations suggest that PI-MECs do not represent a significant subset of SP cells in the parous mammary gland.

PI-MECs are able to form mammospheres in culture

It has been proposed recently that human multipotent mammary epithelial cells can be propagated in suspension cultures as 'nonadherent mammospheres' (Dontu et al., 2003). According to this report, these progenitors can be selectively maintained in an undifferentiated state using this methodology. We asked whether PI-MECs are capable of forming spheres in nonadherent cultures since these cells cannot be simply referred to as 'undifferentiated progenitors" due to their hormone responsiveness during pregnancy and the subsequent activation of the *Wap* gene promoter, which is commonly regarded as an indication for differentiation. Mammary epithelial cells from parous WAP-Cre/CAG-GFP females were separated into GFP-positive (PI-MECs) and GFP-negative fractions using fluorescence activated cell sorting (FACS). In this experiment, GFP-labeled PI-MECs represented only 7.6% of all primary cells. We plated 4×10^4 PI-MECs and 6×10^4 GFP-negative MECs into ultralow attachment plates and maintained both cultures for 7 days as described in the Materials and methods section. The formation of mammospheres was examined under an inverted microscope equipped with a fluorescence unit. PI-MECs as well as their GFP-negative controls were able to form spheres with a typical round-shaped morphology. Although PI-MECs represent a pure GFP-positive fraction, a number of resulting spheres did not express GFP (Figs. 8A–C). This might be the result of silencing the CAG-GFP reporter gene in a subset of

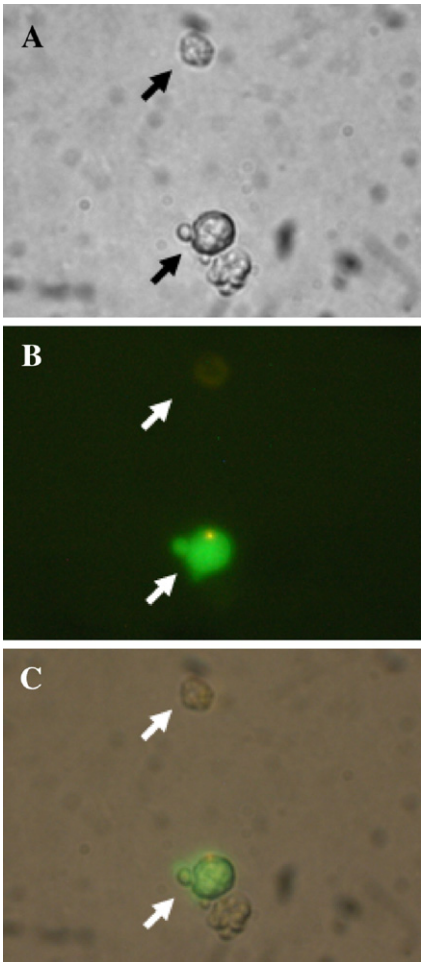

Fig. 8. Mammosphere formation in nonadherent cultures of purified PI-MECs. A: Phase contrast image of mammospheres in a 7-day culture. B: GFP expression in nonadherent mammospheres. C: Composite image of A and B. Arrows indicate the location of mammospheres containing cells with differences in the level of GFP expression.

nonadherent cells, since spherical cells are able to regain GFP expression when subsequently grown in adherent culture conditions on plastic (data not shown). Following the 7-day culture period, we harvested and counted the spheres and calculated the relative number of sphere-forming cells within both cellular populations. We counted a total number of 375 spheres derived from GFP-labeled cells, suggesting that 0.94% of PI-MECs were able to form mammospheres. GFP-negative mammary epithelial cells gave rise to 500 spheres. Due to the higher numeric input of cells, we calculated that approximately 0.83% of GFP-negative MECs were capable of forming mammospheres. Subsequently, all mammospheres were

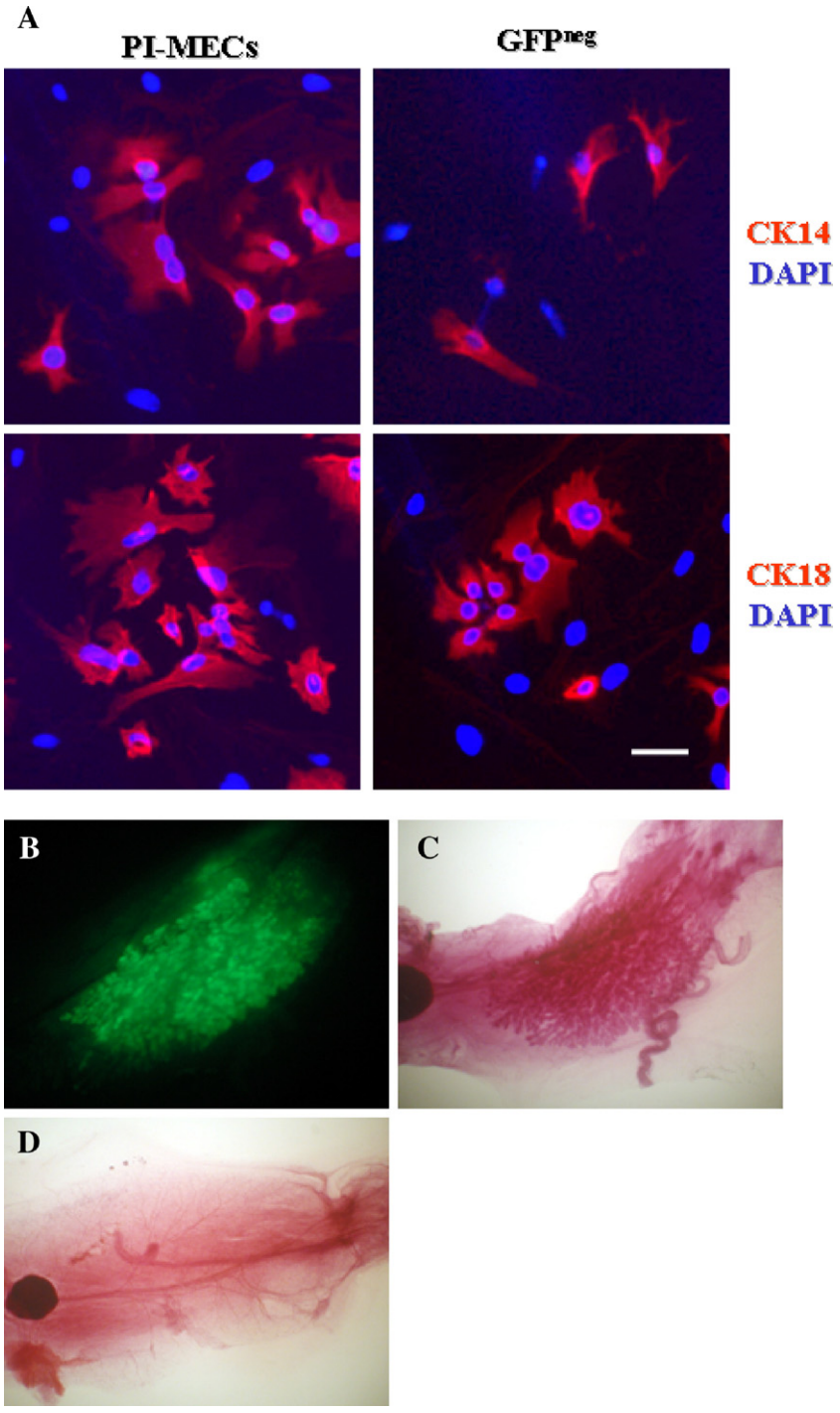

Fig. 9. A: Immunofluorescent staining of cytokeratin 14 and 18 in primary cells of the mammary gland to assess the presence of luminal and basal epithelial subtypes in PI-MECs and GFP-negative cells prior to transplantation into recipient females. The bar represents 50 μm. Note that PI-MECs as well as the GFP-negative fraction contain luminal cells (CK18) and basal cells (CK14). B–D: Transplantation of GFP-positive PI-MECs and GFP-negative mammary cells into the cleared fat pad of recipient females. B: Complete GFP-positive outgrowth arising from purified PI-MECs in a mid-pregnant host. C: Carmine Alum-stained mammary transplant shown in panel B. Note that GFP-positive PI-MECs contributed to the formation of ducts and alveoli. D: Empty fat pad resulting from a transplant of GFP-negative MECs deprived of PI-MECs (10× magnification).

enzymatically dissociated to calculate the relative number of cells per sphere. We determined that approximately 100–200 cells were present per mammosphere in our cultures. In summary, the results of this study suggest that sphere formation is not a sole property of cells that are commonly referred to as 'undifferentiated progenitors". In addition to the expression of stem cell markers, the ability to form mammospheres is another indication for the multipotency of a subset of PI-MECs. Nevertheless, enriching for PI-MECs only resulted in a marginal increase in mammosphere formation.

Purified PI-MECs are multipotent and capable of reconstituting the entire mammary ductal tree

While the capability to form mammospheres might be a first indication about the multipotency of PI-MECs, only the transplantation experiment of *purified* PI-MECs is the ultimate proof to determine whether these cells are actually able to give rise to all epithelial lineages. Primary mammary epithelial cells of primiparous WAP-Cre/CAG-GFP double transgenic females were cultured for 72 h and subsequently separated into GFP-positive (PI-MECs) and GFP-negative populations using FACS. In this experiment, PI-MECs constituted approximately 15% of all primary cells. A small fraction of the sorted GFP positive and GFP negative cells were cultured to verify that both populations contain viable cells. Subsequently, these cells were used for immunofluorescence staining against cytokeratins 14 and 18 (Fig. 9A). Interestingly, PI-MECs do not exclusively represent keratin 18 expressing luminal mammary epithelial cells as a significant number of GFP-labeled cells also expressed the basal marker keratin 14. As expected, both K14 and K18 positive cells were present in the GFP negative fraction.

Immediately following the cell sorting procedure, 1.2×10^4 cells of each population were transplanted into #4 epithelia-free mammary fat pads of 11 wildtype recipients (i.e. 22 transplants). Smith and coworkers suggested that one multipotent stem cell is present in every 2500 MECs (Smith, 1996; Kordon and Smith, 1998). If this calculation is correct, we transplanted sufficient stem cells into each fat pad to ensure engraftment. The actual number of transplanted stem cells might be skewed depending on whether one of the fractions was enriched or depleted for stem cells. Each recipient received GFP-positive and GFP-negative cells injected separately into the bilateral #4 mammary gland fat pads. The recipients were maintained as nulliparous females for 8 weeks to allow the transplanted cells from the transgenic donors to penetrate the wild-type fat pad and form a ductal tree. Subsequently, recipient mice were impregnated to induce lobuloalveolar development. We chose late pregnancy as the study endpoint to be able to identify lobule-limited and ductal-limited structures. Both bilateral fat pads were examined under the fluorescent stereoscope and subsequently processed for whole mount staining. We observed complete outgrowths, (i.e. ductal and alveolar structures) in 4 out of 11 fat pads injected only with GFP-positive PI-MECs (Figs. 9B and C). Surprisingly, we did not obtain any type of outgrowth (lobule-limited, ductal-limited, or complete) in the 11 fat pads injected only with GFP-negative cells (Fig. 9C). In summary, these observations suggest that PI-MECs contain multipotent stem cells that are able to reconstitute the entire ductal tree. The absence of positive takes after transplantation of a similar number of primary cells that were depleted of PI-MECs might provide additional experimental evidence that PI-MECs are an essential part of the stem cell niche in the mammary gland of parous females as discussed in the next section.

Discussion

Using a GFP-based Cre/lox reporter strain, we developed a strategy to viably label parity-induced mammary epithelial cells (PI-MECs). This strategy allowed us to determine the relative number of PI-MECs in mammary glands of primiparous and multiparous females using flow cytometry. Next, we were able to examine the role of steroid and peptide hormones on the genesis of this unique epithelial subtype. In addition, this labeling method enabled us to perform a time course experiment to monitor the continued presence of PI-MECs following ovariectomy. More importantly, marking viable PI-MECs was crucial for the flow cytometric analysis of stem cell markers as well as the sorting and transplantation of purified cell populations to study stem cell characteristics of isolated PI-MECs *in vivo*.

The role of pregnancy hormones for the genesis and maintenance of PI-MECs

The results of our studies demonstrate that an optimal activation of the WAP-Cre transgene requires the hormonal milieu of pregnancy and the correct 3D architecture of the mammary gland. Using cultured fragments of the mammary gland, Yale Topper demonstrated many years ago that milk protein synthesis (i.e. casein and WAP) occurred only in the combined presence of insulin, hydrocortisone and prolactin (Topper and Freeman, 1980; Pittius et al., 1988). These three hormones, however, were unable to induce the expression of WAP-Cre in epithelial monolayer cultures. In analogy to Topper's observations, dexamethasone, insulin, and prolactin (DIP) were sufficient to upregulate the WAP-Cre transgene when fragments of nulliparous mammary glands were cultured *ex vivo*. It is known that proper expression of the endogenous *WAP* gene requires cell-to-cell contact and the formation of a closed lumen (i.e. a correct 3D structure of an alveolus) (Chen and Bissell, 1989). In this regard, the randomly inserted WAP-Cre transgene accurately follows the regulation of the endogenous *WAP* locus, and, therefore, a short-term culture of epithelial cells as monolayers has no effect on the WAP-Cre-mediated genetic labeling of PI-MECs. The administration of estrogen and progesterone (E+P) *in vivo* was able to induce alveolar proliferation and partial activation of the WAP-Cre transgene. Although these ovarian hormones are essential for alveolar genesis during pregnancy (and therefore also for the origin of PI-MECs), the number of GFP-labeled cells after prolonged E+P treatment was significantly lower compared to a full-term pregnancy. This observation confirms that a) other pregnancy-induced hormones and local growth factors (such as DIP; see above) control optimal *WAP* expression, and b) like the

endogenous *WAP* locus, the WAP-Cre transgene is highly expressed during the last phase of pregnancy, i.e. a developmental stage that is not accurately mimicked by E+P administration alone. Regardless of an optimal activation of WAP-Cre, elevated levels of E+P during pregnancy are essential for alveologenesis and the origin of PI-MECs during the first gestation cycle. Since PI-MECs in nonpregnant, primiparous females serve as alveolar progenitors during subsequent gestation cycles (Wagner et al., 2002), we assumed that these cells are not only responsive to E+P, but the majority of PI-MECs might actually be dependent on the presence of ovarian hormones. It has been suggested recently that subtypes of multipotent mammary epithelial stem cells are positive for ER and PR (Clarke et al., 2005), and, therefore, both stem cell models are not mutually exclusive. Unexpectedly, our experiments clearly demonstrated that the continued presence of a significant portion of PI-MECs in the involuted mammary gland does not depend on ovarian hormones that once were essential to generate this unique epithelial subtype. This result also explains why PI-MECs were present in old female animals past their reproductive capabilities. In conclusion, a single full-term pregnancy produces parity-induced mammary epithelial cells that are present in a female beyond menopause, and, consequently, this might have implications for the prevention of chemically-induced mammary tumorigenesis, which, in mice and rats, is significantly reduced in parous females in comparison to virgin controls (Russo and Russo, 1996; Medina and Smith, 1999). In support of this conclusion, we never detected GFP-labeled PI-MECs in the few mammary tumors that arose in parous WAP-Cre/CAG-GFP females treated with the chemical carcinogen 7,12-dimethylbenz[*a*]anthracene (DMBA) (Matulka, Triplett, and Wagner, unpublished).

PI-MECs express putative stem cell markers

A number of putative markers for multipotent mammary epithelial stem cells have been reported in the past. For example, it has been suggested that stem cells reside within the 'side population' (SP) and Sca-1 positive epithelial subtypes in the murine mammary gland (Welm et al., 2002). However, in a follow-up study, Alvi et al. (2003) were unable to verify these observations. These authors suggested that the 'side population' contains lobuloalveolar-restricted progenitors instead of multipotent stem cells. Recent work by Sleeman et al. (2006) suggested that epithelial cells expressing low levels of CD24 have the ability to repopulate a cleared fat pad. In their recent landmark paper, Shackleton et al. (2006) proposed that an entire mammary ductal tree can be reestablished by transplanting a single stem cell expressing CD24 and CD29^{high}. Neither Sca-1^{high} nor SP cells appeared to be enriched in the Lin^{-}/CD24^{+}/CD29f^{high} epithelial subtype. In an accompanying article by Stingl et al. (2006), multipotent mammary progenitors were defined as cells expressing CD24 and high levels of CD49f. No mammary stem cells were detected in the Sca-1^{high} subset of CD49f^{+} cells. Based on inconsistent observations made by different research groups over the last 5 years, the discussion about the legitimacy of particular markers to identify epithelial stem cell lineages is still ongoing.

Using transplantation of mammary fragments or isolated, unfractionated epithelial cells, we demonstrated that parity-induced mammary epithelial cells contain stem cells. In multiple experiments, the vast majority (60–90%) of resulting outgrowths contained genetically labeled PI-MECs and their descendants (Wagner et al., 2002; Boulanger et al., 2005). In this study, we wanted to know which combination of putative stem cell markers best describes this unique epithelial subtype. The Abcg2 transporter, which mediates part of the SP phenotype (Jonker et al., 2005a), is upregulated in differentiating alveolar cells during pregnancy and lactation (Jonker et al., 2005b), and it was therefore reasonable to assume that PI-MECs display a relatively high number of SP cells. Surprisingly, we found that PI-MECs did not represent a significant subset of SP cells in the parous mammary gland. Also, the expression level of Sca-1 did not correlate with the genetic labeling of PI-MECs. More than half of all PI-MECs did not exhibit high expression of this marker. In addition, we observed that culturing mammary epithelial cells appeared to upregulate the expression of Sca-1. A similar observation was recently reported by Stingl et al. (2006). Unlike Sca-1, the expression of CD24 and CD49f was not significantly altered by culture conditions. Using these two markers, we found that a single pregnancy did not significantly alter the overall proportion of cells expressing CD24 alone or in combination with CD49f. Virtually all GFP-labeled PI-MECs expressed moderate to high levels of CD24. However, we also observed that parity seemed to augment the occurrence of CD24^{+} cells that display a very high expression of CD49f. More importantly, GFP-labeled PI-MECs were represented in this specific fraction of double positive epithelial cells. According to Stingl et al. (2005, 2006), CD24^{+}/CD49f^{high} mammary epithelial cells represent the multipotent stem cell population within the mammary epithelium. If this model is correct, then a fraction of PI-MECs with a CD24^{+}/CD49f^{high} marker expression profile belongs to the multipotent stem cell population.

Multipotency of a subset of PI-MECs and a potential role for PI-MECs in the stem cell niche

Through the use of the CAG-GFP reporter construct, we were able to transplant purified PI-MECs, thus allowing us to assess a) whether PI-MECs are sufficient to contribute to ductal and alveolar morphogenesis, and b) whether the depletion of PI-MECs has an effect on the growth properties of other stem cell populations or restricted progenitors. The transplantation of 12,000 GFP-positive PI-MECs immediately after the sorting procedure resulted in complete outgrowths in more than 30% of the transplants, suggesting that purified PI-MECs contain stem cells that are able to reconstitute the entire ductal tree. Previous studies have shown that the transplantation of limiting dilutions of unsorted PI-MECs together with other epithelial subtypes resulted in both lobule-limited and ductal-limited outgrowths that were entirely comprised from PI-MECs and their LacZ labeled descendents (Boulanger et al., 2005). These results

indicated that all luminal, myoepithelial, and cap cells of terminal buds may be derived from PI-MECs and their progeny. PI-MECs are therefore not only self-renewing, but they are multipotent as well. The transplantation of purified PI-MECs provided experimental evidence that this assumption might be correct and that the stem cell properties of PI-MECs are not restricted to lineage-specific progenitors of ducts and alveoli.

Although this epithelial subtype, which is unique for parous females, contains stem cells, the total number of PI-MECs greatly exceeds the suggested quantity of putative stem cells in the mammary gland (1 in 2500). Therefore, it cannot be assumed that *all* PI-MECs are stem cells, and due to the restricted location of PI-MECs at terminal ducts, it is also unlikely that these cells represent all multipotent progenitors in a parous mammary gland. Surprisingly, the depletion of PI-MECs appeared to have a profound impact on the regenerative capacity of non-labeled mammary epithelial cells. We did not observe any type of outgrowth (lobule-limited, ductal-limited, or complete) in fat pads injected only with GFP-negative MECs. Based on this observation, it might be possible that many PI-MECs, that are not genuine multipotent stem cells, play an important role in stem cell maintenance as part of the stem cell niche. This hypothesis might also explain why the absence of PI-MECs in serial transplants over several transplant generations was associated with growth senescence (Boulanger et al., 2005). In addition, Boulanger and colleagues reported that a successful transplant of limiting dilutions of epithelial cells from parous females always contained PI-MECs, and no outgrowth was entirely comprised of unlabeled epithelial cells. Finally, our hypothesis about a functional role of PI-MECs as part of the stem cell niche might be supported by the fact that the targeted expression of the negative growth regulator TGF-β1 selectively inhibited the capacity of PI-MECs to self-renew. WAP-TGF-β1-expressing mammary epithelial cells exhibit an early growth senescence (Boulanger and Smith, 2001), and second-generation transplants undergoing senescence from parous WAP-Cre/Rosa26-LacZ/WAP-TGF-β1 triple transgenic females were devoid of PI-MECs and their progeny. In summary, we have evidence from limiting dilution studies, serial transplantations, and TGF-β1-induced growth senescence to suggest that PI-MECs do not only have stem cell capabilities, but they might also fundamentally contribute to the maintenance of stem cells. The depletion of PI-MECs, which greatly affected the self-renewal capacity of all types of progenitors (i.e. stem cells, as well as duct and alveolar-limited progenitors), might provide additional experimental evidence that PI-MECs are an essential part of the stem cell niche in the mammary gland of parous females.

Acknowledgments

This work was supported, in part, by the Susan G. Komen Breast Cancer Foundation (BCTR0402956). Additional financial support was provided to K.U.W. by the Nebraska Cancer and Smoking Disease Research Program (NE DHHS LB506 2007-43). Funding from the Nebraska Research Initiative (NRI) was imperative to finance, in part, the maintenance of genetically-engineered mouse models. L.A.M. currently receives a post-doctoral fellowship from the Susan G. Komen Breast Cancer Foundation (PDF83506). The authors are grateful to Jeffrey Segall (A. Einstein College of Medicine) for providing the GFP reporter strain. We thank Charles Kuszynski and Linda Wilkie of the UNMC Cell Analysis Facility for performing flow cytometric analyses and cell sorting procedures. Judy Stribley and Michael Salbaum of the UNMC Mouse Genome Engineering Core Facility provided expertise and practical training during ovariectomy. The authors acknowledge Dan Medina (Baylor College of Medicine) for advice regarding the hormonal treatment of mice. We thank Gilbert Smith and Brian Booth (NIH) for providing protocols about the derivation and growth conditions of mammospheres as well as the hormonal treatment of whole organ cultures. The authors are very grateful to John Stingl (British Columbia Cancer Agency), who shared his protocols and observations about the expression of putative stem cell markers on primary mammary epithelial cells prior to their publication.

References

Alvi, A.J., Clayton, H., Joshi, C., Enver, T., Ashworth, A., Vivanco, M.M., Dale, T.C., Smalley, M.J., 2003. Functional and molecular characterisation of mammary side population cells. Breast Cancer Res. 5, R1–R8.

Boulanger, C.A., Smith, G.H., 2001. Reducing mammary cancer risk through premature stem cell senescence. Oncogene 20, 2264–2272.

Boulanger, C.A., Wagner, K.U., Smith, G.H., 2005. Parity-induced mouse mammary epithelial cells are pluripotent, self-renewing and sensitive to TGF-beta1 expression. Oncogene 24, 552–560.

Chen, L.H., Bissell, M.J., 1989. A novel regulatory mechanism for whey acidic protein gene expression. Cell Regul. 1, 45–54.

Clarke, R.B., Spence, K., Anderson, E., Howell, A., Okano, H., Potten, C.S., 2005. A putative human breast stem cell population is enriched for steroid receptor-positive cells. Dev. Biol. 277, 443–456.

DeOme, K.B., Faulkin, L.J., Bern, H.A., Blair, P.E., 1959. Development of mammary tumors from hyperplastic alveolar nodules transplanted into gland-free mammary fat pads of female C3H mice. Cancer Res. 19, 515–520.

Dontu, G., Abdallah, W.M., Foley, J.M., Jackson, K.W., Clarke, M.F., Kawamura, M.J., Wicha, M.S., 2003. In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. Genes Dev. 17, 1253–1270.

Fiering, S.N., Roederer, M., Nolan, G.P., Micklem, D.R., Parks, D.R., Herzenberg, L.A., 1991. Improved FACS-Gal: flow cytometric analysis and sorting of viable eukaryotic cells expressing reporter gene constructs. Cytometry 12, 291–301.

Ginsburg, E., Vonderhaar, B.K., 2000. Whole organ culture of the mouse mammary gland. In: Ip, M.M., Ash, B.B. (Eds.), Methods in Mammary Gland Biology and Breast Cancer. Kluwer Academic/Plenum Publishers, New York, pp. 147–154 (Chapter 14, 11-1).

Henry, M.D., Triplett, A.A., Oh, K.B., Smith, G.H., Wagner, K.U., 2004. Parity-induced mammary epithelial cells facilitate tumorigenesis in MMTV-neu transgenic mice. Oncogene 23, 6980–6985.

Jonker, J.W., Freeman, J., Bolscher, E., Musters, S., Alvi, A.J., Titley, I., Schinkel, A.H., Dale, T.C., 2005a. Contribution of the ABC transporters Bcrp1 and Mdr1a/1b to the side population phenotype in mammary gland and bone marrow of mice. Stem Cells 23, 1059–1065.

Jonker, J.W., Merino, G., Musters, S., van Herwaarden, A.E., Bolscher, E., Wagenaar, E., Mesman, E., Dale, T.C., Schinkel, A.H., 2005b. The breast cancer resistance protein BCRP (ABCG2) concentrates drugs and carcinogenic xenotoxins into milk. Nat. Med. 11, 127–129.

Kawamoto, S., Niwa, H., Tashiro, F., Sano, S., Kondoh, G., Takeda, J., Tabayashi, K., Miyazaki, J., 2000. A novel reporter mouse strain that

expresses enhanced green fluorescent protein upon Cre-mediated recombination. FEBS Lett. 470, 263–268.

Kordon, E.C., Smith, G.H., 1998. An entire functional mammary gland may comprise the progeny from a single cell. Development 125, 1921–1930.

Medina, D., Kittrell, F.S., 2000. Establishment of mouse mammary cell lines. In: Ip, M.M., Ash, B.B. (Eds.), Methods in Mammary Gland Biology and Breast Cancer. Kluwer Academic/Plenum Publishers, New York, pp. 137–145 (Chapter 13, 11-1).

Medina, D., Smith, G.H., 1999. Chemical carcinogen-induced tumorigenesis in parous, involuted mouse mammary glands. J. Natl. Cancer Inst. 91, 967–969.

Pittius, C.W., Sankaran, L., Topper, Y.J., Hennighausen, L., 1988. Comparison of the regulation of the whey acidic protein gene with that of a hybrid gene containing the whey acidic protein gene promoter in transgenic mice. Mol. Endocrinol. 2, 1027–1032.

Russo, I.H., Russo, J., 1996. Mammary gland neoplasia in long-term rodent studies. Environ. Health Perspect. 104, 938–967.

Shackleton, M., Vaillant, F., Simpson, K.J., Stingl, J., Smyth, G.K., Asselin-Labat, M.L., Wu, L., Lindeman, G.J., Visvader, J.E., 2006. Generation of a functional mammary gland from a single stem cell. Nature 439, 84–88.

Sleeman, K.E., Kendrick, H., Ashworth, A., Isacke, C.M., Smalley, M.J., 2006. CD24 staining of mouse mammary gland cells defines luminal epithelial, myoepithelial/basal and non-epithelial cells. Breast Cancer Res. 8, R7.

Smith, G.H., 1996. Experimental mammary epithelial morphogenesis in an in vivo model: evidence for distinct cellular progenitors of the ductal and lobular phenotype. Breast Cancer Res. Treat. 39, 21–31.

Soriano, P., 1999. Generalized lacZ expression with the ROSA26 Cre reporter strain. Nat. Genet. 21, 70–71.

Stingl, J., Raouf, A., Emerman, J.T., Eaves, C.J., 2005. Epithelial progenitors in the normal human mammary gland. J. Mammary Gland. Biol. Neoplasia 10, 49–59.

Stingl, J., Eirew, P., Ricketson, I., Shackleton, M., Vaillant, F., Choi, D., Li, H.I., Eaves, C.J., 2006. Purification and unique properties of mammary epithelial stem cells. Nature 439, 993–997.

Topper, Y.J., Freeman, C.S., 1980. Multiple hormone interactions in the developmental biology of the mammary gland. Physiol. Rev. 60, 1049–1106.

Wagner, K.U., Smith, G.H., 2005. Pregnancy and stem cell behavior. J. Mammary Gland. Biol. Neoplasia 10, 25–36.

Wagner, K.U., Wall, R.J., St-Onge, L., Gruss, P., Wynshaw-Boris, A., Garrett, L., Li, M., Furth, P.A., Hennighausen, L., 1997a. Cre-mediated gene deletion in the mammary gland. Nucleic Acids Res. 25, 4323–4330.

Wagner, K.U., Young, W.S., Liu, X., Ginns, E.I., Li, M., Furth, P.A., Hennighausen, L., 1997b. Oxytocin and milk removal are required for post-partum mammary-gland development. Genes Funct. 1, 233–244.

Wagner, K.U., Boulanger, C.A., Henry, M.D., Sgagias, M., Hennighausen, L., Smith, G.H., 2002. An adjunct mammary epithelial cell population in parous females: its role in functional adaptation and tissue renewal. Development 129, 1377–1386.

Welm, B.E., Tepera, S.B., Venezia, T., Graubert, T.A., Rosen, J.M., Goodell, M.A., 2002. Sca-1(pos) cells in the mouse mammary gland represent an enriched progenitor cell population. Dev. Biol. 245, 42–56.

Young, L.J.T., 2000. The cleared mammary fat pad and the transplantation of mammary gland morphological structures and cells. In: Ip, M.M., Ash, B.B. (Eds.), Methods in Mammary Gland Biology and Breast Cancer. Kluwer Academic/Plenum Publishers, New York, pp. 67–74 (Chapter 6, 11-1).