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Chapter 6

Bmi1 regulates stem cells but also proliferation and differentiation of committed cells in mammary gland epithelium

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Chapter 6: Role of Bmi1 in mammary gland development

Bmi1 regulates stem cells but also proliferation and differentiation of committed cells in mammary gland epithelium

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PolycombGroup (PcG) proteins are epigenetic silencers involved in maintaining cellular identity, and their deregulation can result in cancer1. Mice without the PcG gene Bmi1 are runted and suffer from progressive loss of hematopoietic and neural stem cells2-4. Here, we assess the effects of Bmi1 on stem cells and differentiation of an epithelial tissue in vivo. We chose the mammary gland because it allows limiting dilution transplantations5,6 and because Bmi1 is overexpressed in breast cancer7,8.

Our analyses show that Bmi1 is expressed in all cells of the mouse mammary gland, and is especially high in luminal cells. Loss of Bmi1 results in a severe mammary epithelium growth defect, which can be rescued by codeletion of the Ink4a/Arf locus or pregnancy. Even though mammary stem cells are present in the absence of Bmi1, their activity is reduced and this is only partially due to Ink4a/Arf expression. Interestingly, loss of Bmi1 causes premature lobuloalveolar differentiation, whereas overexpression of Bmi1 inhibits lobuloalveolar differentiation induced by pregnancy hormones.

Because Bmi1 affects not only mammary stem cells but also more committed cells, our data warrant a more detailed analysis of the different roles of Bmi1 in breast-cancer etiology.

Results & Discussion
Impaired mammary gland development in Bmi1-knockout mice
To evaluate the role of Bmi1 in mammary epithelium, we compared mammary glands from Bmi1+/− mice with those of their wild-type littermates by wholemount analysis. At 5 weeks postpartum, mammary ducts in wildtype mice have started to colonize the fat pad under the influence of pubertal hormones. By this age the mammary glands exhibit Terminal End Buds (TEB), containing stem cells and their highly proliferative progeny9, that have extended beyond the lymph nodes (Figure 1A, left panel). In contrast, the development of the mammary gland in Bmi1−/− littermates was severely inhibited (Figure 1A, right panel). Only occasionally did we observe an isolated TEB in a Bmi1−/− mammary gland. In general, the Bmi1-deficient ductal epithelium failed to invade the fat pad and mostly resembled the rudimentary mammary tree that is laid down during embryogenesis, indicating that Bmi1 plays an essential role in postnatal mammary development. Histological analysis of the Bmi1−/− mammary glands demonstrated that the mammary architecture remains intact, i.e. the mammary ducts consist of a single layer of luminal cells surrounded by a layer of myoepithelial cells (Figure 1B). This suggests that Bmi1 is not required to establish the fate of these cells. Immunostaining of wildtype mammary
glands showed that Bmi1 expression is not restricted to a minority of the epithelial cells, as might be expected from a protein best known for its role in stem cells, but instead Bmi1 expression can be detected in all cells. Overall, Bmi1 expression is variable in TEBs, low in the myoepithelium and highest in the luminal compartment (Figure 1B and S1).

To determine whether the growth defect of Bmi1-/- mammary glands is due to a defect in the epithelial cells or caused by a deficiency in the environment of the cells, we isolated Bmi1-/- mammary epithelial cells (MECs), briefly expanded them in culture and transplanted them into wildtype mice. When 100,000 MECs lacking Bmi1 were injected into cleared fat pads of wildtype mice, they still displayed a severe growth defect ten weeks after transplantation, in contrast to wildtype cells that were injected contra-laterally. To exclude possible artifacts from the in vitro culture, we repeated this experiment by transplanting a small piece of mammary tissue from the Bmi1-/- mice from the nipple area directly into the cleared fatpad of a wildtype mouse. Even though mammary epithelium was detectable upon transplantation, there was virtually no growth of the Bmi1-deficient tissue in contrast to wildtype tissue, demonstrating that Bmi1 expression in mammary epithelial cells is required for their proliferation (Figure 1C).

Several, but not all, defects of Bmi1-/- mice are caused by inappropriate Ink4a/Arf expression. p16Ink4a and p19Arf, which induce cell cycle arrest and apoptosis through activation of Rb and p53 respectively, are not expressed in normal tissue, but are induced upon oncogenic signaling. In mammary epithelium, repression of the Ink4a/Arf locus is also dependent on Bmi1 because we found that the transcripts of both Ink4a and Arf are increased in Bmi1-/- MECs compared to wildtype MECs (Figure S2, bars on the left, labeled “virgin”). When we codeleted the Ink4a/Arf locus together with Bmi1 and transplanted this Bmi1;Ink4a/Arf-deficient mammary tissue and cells, we found an almost complete rescue of mammary growth (Figure 1D). This demonstrates that the lack of repression of the Ink4a/Arf locus is mostly responsible for the growth defect of Bmi1-/- tissue.

Because growth defects in mammary gland development can often be rescued by pregnancy, we tested this for Bmi1-/- breast tissue as well. Because of the ill health of the Bmi1-knockout mice, we used transplanted Bmi1-/- tissue pieces in wildtype mice, which we mated with males four weeks after transplantation. Interestingly, we observed a near-complete rescue of Bmi1-deficient mammary outgrowth during pregnancy (Figure 2A). We hypothesized that the strong growth stimulation during pregnancy could activate multiple pathways that result in inhibition of the cell-growth inhibitors p16Ink4a and p19Arf. To test this, we isolated mammary epithelial cells from pregnant recipients that had been previously transplanted with wildtype and Bmi1-/- tissue and determined the expression of Ink4a and Arf. Surprisingly, Ink4a and Arf RNA and protein levels were still high in Bmi1-deficient tissue (Figure S2 and Figure 2B), even though there is extensive proliferation in these transplants (Figure S2C). Apparently, pregnancy hormones overcome the effects of these cell cycle inhibitors to allow cell cycle progression, possibly through induction of cyclin D1.

During pregnancy, lobules of alveolar cells are formed and differentiate to become sites of milk production, a process termed lobuloalveolar differentiation. In pregnant recipients, this process occurs in the Bmi1-deficient outgrowths that are indistin-
Figure 1 - Bmi1-Knockout Mice Show a Severe Defect in Mammary Development.
A. Carmine-stained inguinal mammary glands from FVB littermates, 38 days old, wild-type (WT) or knockout (BmiKO) for Bmi1. "LN" stands for lymph node. Data are represented as mean ± SD, n = 4 glands per condition (30 to 40 days old). B. Immunohistochemistry on mammary glands from 5-week-old wild-type and Bmi1-knockout mice (top and bottom panel, respectively). There is no Bmi1 staining in the Bmi1-knockout mouse, as expected. Myoepithelial (smooth muscle actin [SMA]) and luminal (E-cadherin) cells are present and properly organized (H&E) in Bmi1-knockout mammary glands. C. Five Bmi1−/− tissue pieces and two cell isolations were injected into cleared fat pads of 21-day-old wild-type recipients, and the outgrowth capacity was analyzed 10 weeks later. The site of injection of the wild-type tissue is indicated by an arrowhead. Seven glands per genotype were analyzed from four independent donor pairs (littermates). D. Five tissue transplants and three cell transplants of Bmi1/Ink4a/Arf-deficient mammary glands were performed. As a control, Ink4a/Arf−/− mammary tissue or cells were each time transplanted on the contralateral side. The sites of injection are indicated by arrowheads. Eight glands per genotype were analyzed (donor material from six independent littermate pairs). The scale bars represent 1 mm in (A, C, and D) and 50 μm in (B).
guishable from the contralateral wild-type control transplants, as judged by morphology and histology. In addition, one of the constituents of milk, β-casein, is induced to comparable levels in transplants of Bmi1-deficient breast tissue and wildtype transplants (Figure 3C), indicating that the Bmi1-deficient mammary tissue is undergoing functional differentiation as well. This suggests that pregnancy can override the block in proliferation due to the absence of Bmi1, and Bmi1 is not required for lobuloalveolar differentiation.

Figure 2 - Pregnancy Rescues Bmi1-Deficient Mammary Outgrowth.
A. Four tissue pieces and three cell isolates of wild-type and Bmi1⁺⁺ mammary glands were transplanted, and the capacity to reconstitute a cleared fat pad was analyzed after 15–18 days pregnancy of the wild-type recipients. The sites of injection are indicated by arrowheads. Seven glands per genotype were quantified (donor material from seven littermate pairs). B. MECs were isolated from two late-pregnant hosts (M1 and M2) that had been grafted with Bmi1⁻⁻ (KO) and wild-type (WT) mammary tissue. Protein lysates were probed for Ink4a and subsequently for Arf expression (asterisk indicates Ink4a signal from first incubation; Arf signal is marked by the arrowhead). C. Carmine staining of mammary glands from a 15-day pregnant wild-type recipient that was transplanted with wild-type and Bmi1⁻⁻ mammary tissue reveals equal lobuloalveolar morphology. mRNA levels of β-casein are measured by Q-PCR and normalized against beta-actin. Values are the mean of three independent MEC isolations per genotype. The scale bars represent 1 mm.
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Loss of Bmi1 affects both mammary stem cells and progenitors

The complete reconstitution of a mammary tree in pregnant recipients illustrates that mammary stem cells are present in the rudimentary mammary structures observed in the Bmi1−/− mouse. To obtain a quantitative measurement of the effect of Bmi1 loss in mammary stem cells in vivo, we performed limiting dilution transplantations. First, we transplanted Bmi1−/− tissue and wildtype tissue as a control into wildtype recipients. After rescuing the Bmi1−/− growth by pregnancy, we harvested these cells for use in a limiting dilution series in new recipients to analyze the minimum number of cells required for growth of mammary epithelial tissue; this numbers serves as an indication of the amount of stem cells that were present in the original cell isolate6, see Figure S3 for experimental set-up). When half a million cells are injected and the recipient is made pregnant, both wildtype and Bmi1-deficient cells are capable of reconstituting a mammary gland (Figure S4A). Upon reduction of the amount of injected cells however, it becomes apparent that there is a 14-fold reduction in mammary repopulating units (MRUs) derived from the Bmi1−/− mammary gland (Table 1). Apparently, mammary stem cells are less dependent on Bmi1 than for instance hematopoietic stem cells2,3,19.

To determine whether loss of the Ink4a/Arf locus can rescue stem cell activity in Bmi1-deficient mammary cells, we compared MECs with or without Bmi1 in the absence of the Ink4a/Arf locus. Also in this case, we first transplanted Bmi1/Ink4a/Arf−/− tissue and Ink4a/Arf−/− tissue as a control into wild-type recipients to obtain sufficient cells for the limiting dilution (Figure 1D and Figure S3). Loss of Ink4a/Arf by itself does not affect the repopulation activity (Table 1), suggesting that Ink4a/Arf levels in wildtype stem cells are negligible. However, in Bmi1−/− absence, which results in derepression of the Ink4a/Arf locus, deletion of Ink4a/Arf does have an effect on stem cell activity, albeit partial (3-fold reduced compared to wildtype or Ink4a/Arf−/− cell isolates, Table 1). Importantly, the frequency of MRU remains similar in pregnant and virgin recipients (compare wildtype vs Ink4a/Arf−/−, Table 1), consistent with previous reports demonstrating an increase in stem cells accompanying the increase in overall cell number during pregnancy20. Because we measure functional stem cell activity we can not gauge the relative contribution of pregnancy-induced stem cells21, but our data demonstrate that there is an overall reduction in stem cell frequency in the absence of Bmi1. Because the rescue upon codelletion of the Ink4a/Arf locus was incomplete,

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Table 1 - Mammary Stem Cell Activity Is Reduced in the Absence of Bmi1.

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The frequency of mammary-gland repopulating units (MRUs) was determined by limiting dilution transplantations of Bmi1+/+ and wild-type MECs after rescue by pregnancy and for Bmi1/Ink4a/Arf and Ink4a/Arf deficient cells in virgin recipients. See Figures S3 and S4 for experimental details. The fold reduction is relative to wild-type cells, and goodness of fit to a single-hit Poisson model was tested with standard Chi-square statistics.
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**Figure 3 - Loss of Bmi1 Facilitates Lobuloalveolar Differentiation.**

**A.** Carmine-stained wholemount analysis. During pregnancy, the ducts that have a smooth appearance in the wild-type virgin mouse become lined with budding alveoli (left and right panels). The limited outgrowths of Bmi1−/− transplants in virgin recipients form structures that morphologically resemble alveoli (middle). **B.** Immunofluorescent staining on paraffin sections from mammary glands as indicated. Luminal cells of virgin ducts show clear membrane staining of the NKCC1 transporter (left panel), whereas this expression is largely lost during pregnancy (right panel). Several Bmi1−/− ducts transplanted in virgin recipients have also lost NKCC1 staining (Tp BmiKO). Conversely, the Npt2b transporter is not expressed in virgin tissue (left panel) but becomes expressed at the lumen of alveoli during pregnancy (right panel). Almost half of Bmi1-deficient mammary structures in virgin recipients exhibit properly localized Npt2b staining (Tp BmiKO and **C**). **C.** The percentage of Npt2b-positive ducts in tissue sections of at least three independent transplantations (left side) or three independent mammary glands (right side, “straight”) was determined per genotype. The scale bars represent 500 μm in (A) and 50 μm in (B).
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it is likely that Bmi1 is required to repress additional target genes for the maintenance of mammary stem cells. Future studies should elucidate whether these target genes are additional cell cycle regulators such as p21, or whether they belong to the class of developmental regulators that are bound by PcG in ES cells.

The fact that injections of high numbers of Bmi1-deficient MECs, containing multiple mammary stem cells, did not show any growth upon transplantation (Figure 1C), suggests that proliferation of mammary progenitor cells is also hampered by the absence of Bmi1. In contrast, loss of hematopoietic stem cells in the Bmi1-deficient mouse is initially compensated by increased proliferation of their immediate descendants. Furthermore, Molofsky et al. showed that neural progenitors from the forebrain proliferate normally in the absence of Bmi1, although granule cell precursor cells require Bmi1 for their proliferation. These data indicate that not all progenitors are equally dependent on Bmi1 expression for their proliferation. There may be a tissue-specific dependency on silencing of PcG targets such as the Ink4a/Arf locus, which in certain circumstances might not be activated or the function of Ink4a and Arf may be overruled downstream, thereby alleviating dependence on Bmi1 for proliferation. This is illustrated by our observation that pregnancy induces proliferation in the mammary gland, regardless of high Ink4a and Arf expression.

In vitro studies have suggested that in breast epithelium, like in neural cells, Bmi1 is a downstream effector of sonic hedgehog (Shh) signaling, and is required for mammary stem cells. In those studies, nonadherent cultures of human MECs, termed mammospheres, are used as a measurement for stem cell and progenitor activity. However, a recent publication showed that even though Shh activation in mouse MECs likewise increases the number of mammospheres, there is an actual reduction in the number of cells that are capable of reconstituting the mammary gland. Therefore, the authors suggest that the mammosphere assay measures proliferation and anchorage-independent growth rather than stem cell activity. Our in vivo data demonstrate that loss of Bmi1 has an effect on mammary stem cell activity but is also required for proliferation of all progeny. Thus, the effect of Bmi1 loss in mammospheres probably reflects both functions of Bmi1: a partial loss of stem cell activity as well as a block in proliferation.

Bmi1 prevents premature initiation of lobuloalveolar differentiation

Upon close examination, Bmi1-deficient mammary tissue showed morphological signs of lobuloalveolar differentiation when it was transplanted into the fat pad of a virgin recipient. This suggests that without Bmi1, mammary cells may enter premature differentiation (Figure 3A). To further define this morphological phenotype in the Bmi1-deficient transplants, we used two well-established markers to discriminate between virgin and pregnant tissue, NKCC1 and Npt2b, respectively. In the transplants of Bmi1-deficient breast tissue, some ducts showed a virgin-like expression of NKCC1 (data not shown) but other structures have lost such NKCC1 staining, similar to pregnant tissue (Figure 3B). The Na-Pi type IIb cotransporter (Npt2b) is not detectable in virgin tissue, but becomes expressed at the apical membrane from day 15 of pregnancy and remains present during lactation. Strikingly, in the Bmi1-deficient transplants more than half of the mammary structures expressed Npt2b, indicating that this process is normally re-
pressed by Bmi1 in wildtype cells (Figures 3B and 3C). Interestingly, when transplants from Bmi1/Ink4a/Arf-deficient tissue were stained for Npt2b, the number of positive ducts was still increased compared to wildtype or Ink4a/Arf−/− transplants (Figure 3C). Since these cells are proliferating and reconstituting a cleared fat pad, this indicates that the precocious differentiation in the absence of Bmi1 alone is not initiated by expression of Ink4a and Arf or a default response to a block in proliferation. This suggests that Bmi1 generally prevents premature differentiation in the virgin mammary gland through repression of genes other than Ink4a and Arf.

To further substantiate the role of Bmi1 in lobuloalveolar differentiation, we manipulated Bmi1 levels in HC11 cells. This mouse mammary cell line can be induced to differentiate when the cells have established cell-cell contacts upon confluency combined with the addition of lactogenic hormones. We induced differentiation by adding lactogenic hormones (DIP) to HC11 cells after knockdown of Bmi1 by two independent shRNAs (63% and 85% reduction in mRNA levels for shRNA#1 and #2, respectively) or after overexpression of Bmi1 in HC11 cells. Knockdown of Bmi1 increases β-casein production in HC11 cells, while overexpression of Bmi1 represses β-casein production. Western-blot analysis of Bmi1 protein levels at various stages of wild-type mammary-gland development, as follows: virgin (V), 7, 15, and 19 days pregnant, 11 days lactating (L), and 3 days after involution (I). Samples are representative of two independent sets of MECs. Tubulin levels are shown as a loading control.
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hormones\textsuperscript{28}. Functional differentiation can be subsequently quantified by determining the expression of milk proteins, such as β-casein\textsuperscript{29}. Strikingly, knockdown of Bmi1 in HC11 cells greatly increased the β-casein production upon differentiation (Figure 4A). Conversely, overexpression of Bmi1 prevented β-casein expression completely, comparable to HC11 cells that have not been induced to differentiate (Figure 4B). This observation predicts that Bmi1 should be downregulated during pregnancy to allow efficient alveolar differentiation and milk production. We isolated MECs from two independent sets of mice at different stages of development and pregnancy. During the initial stages of pregnancy, Bmi1 levels are upregulated, possibly related to the increased mammary cells proliferation that occurs at this time. However, at later time points we observed a significant decrease in Bmi1 levels, especially during lactation and involution (Figure 4C).

In summary, a picture emerges whereby wildtype levels of Bmi1 are required to prevent spurious activation of terminal differentiation, whereas overexpression of Bmi1 inhibits activation of this program, even in the presence of the appropriate signals\textsuperscript{30}. For instance, wildtype Bmi1 levels prevent Ink4a and Arf expression and thereby premature senescence. Upon oncogenic signaling such as constitutive activation of c-Myc or Ras, the Ink4a/Arf locus is expressed, which functions as a failsafe mechanism against tumorigenesis\textsuperscript{31}. Bmi1 overexpression however overrules this tumor-suppressive response by hyper-repressing the Ink4a/Arf locus\textsuperscript{32}. Overall, Bmi1 levels seem to determine the threshold for signals to activate its target genes. In the mammary gland, Bmi1 fulfills this function not only on the Ink4a/Arf locus, but also on (as yet unidentified) inducers of alveolar differentiation. Whereas genome-wide mapping of PcG targets in embryonic stem cells have already suggested a role for PcG proteins in preventing differentiation\textsuperscript{23,33}, we now show for the first time that Bmi1 is functionally required for the prevention of premature differentiation in more committed cells as well.

Implications for breast cancer
Several correlative studies implicate Bmi1 in breast cancer\textsuperscript{7,8}, but it is unknown whether Bmi1 has a causal role in this malignancy and if so, what is the relative importance of Bmi1’s roles in the mammary gland. As our data show, Bmi1 affects mammary stem cells’ proliferation and differentiation. Liu et al. observed that Bmi1 levels are increased in a subpopulation of breast cancer cells, and this led to the speculation that Bmi1 is involved in the self-renewal of cancer stem cells\textsuperscript{25}. In addition, continuous overexpression of Bmi1 would be expected to prevent the tumor-suppressive activity of both Ink4a and Arf. A study by Yi et al.\textsuperscript{34} suggested that loss of Arf endows mammary cells with indefinite reconstitution capacity. Overexpression of Bmi1 could have an analogous effect, namely prevention of Arf activation, thereby creating immortal cells. Moreover, Bmi1 overexpression may contribute to the immortality of mammary cells via induction of telomerase\textsuperscript{7}. In that respect, it would be interesting to assess the serial transplantation capacity of Bmi1 overexpressing MECs. Our current study provides yet another possible contribution of Bmi1 overexpression to breast cancer: Bmi1 overexpression might contribute to an increase in the population of cells that could be susceptible to oncogenic transformation by blocking terminal differentiation.

Together, our data demonstrate that Bmi1 is required for mammary gland development during puberty, but not for alveolar differentiation during pregnancy. We show
that mammary stem cells are partially dependent on Bmi1 and that Bmi1 is required to allow proliferation of mammary epithelial cells and to prevent premature differentiation. The relative contributions of these functions of Bmi1 to its role in breast cancer will be the focus of our future studies. In general, our data illustrate the flexibility of epigenetic silencing by Pcg proteins that cater to the plasticity that is required for cell fate regulation during development and adult life.

Acknowledgements
We wish to thank Nancy Hynes for the HC11 cells, Jim Turner and Jürg Biber for their generous gifts of antibodies against NKCC-1 and Npt-IIb, respectively, John Hassell and members from his laboratory for technical demonstrations, Marleen Blom and John Zevenhoven for excellent technical assistance, Michael Hauptman for advice on statistical analyses; and Mathijs Voorhoeve and Anke Sparmann for critically reading the manuscript. This work was supported by a grant from the Dutch Cancer Society (NKI 2007-3803).

References


Supplementary information

Experimental Procedures

Mouse crosses
Bmi1-/- mice1 and Ink4a/Arf-/- mice2, were both maintained on a FVB background. Animals were kept according to the “Guide for the Care and Use of Laboratory Animals” and all animal studies were approved by an independent Animal Ethical Committee (Dutch equivalent of the IACUC).

Whole gland morphological analysis
Inguinal mammary glands were dissected, fixed overnight in methacarn (60% methanol, 30% chloroform and 10% glacial acetic acid), and subsequently stained in 0.2% carmine alum. After dehydration in 70%, 90% and 100% ethanol series, the glands were submerged in methyl salicylate (Sigma) and photographed under an Olympus SZX12 microscope. The percentage of mammary epithelial tissue that filled the fat pad is measured as maximal length x maximal width of the ductal structure relative to the maximal width x maximal length of the fat pad.

Immunohistochemistry and immunofluorescence analysis
For histological analysis, thoracic and inguinal mammary glands were fixed in formalin, embedded in paraffin, sectioned, and either stained with Hematoxylin-Eosin or used for immunolocalization studies. Immunostaining was performed after microwave antigen retrieval (20 minutes) in 10 mM sodium citrate and blocking in 5% normal goat serum in phosphate-buffered saline. The following primary antibodies were used: mouse monoclonal against Bmi1 (1:50, Upstate), E-cadherin (1:200, BD Biosciences), Smooth Muscle Actin (1:4000, Immunotech), Ki-67 (1:40, Dako), NKCC1 (1:1000, kindly provided by dr. Jim Turner) and Npt-IIb (1:200, a kind gift from dr. Jürg Biber).

Transplantation assays
Primary mammary epithelial cells (MECs) were isolated essentially as described3 and resuspended at the desired concentration in L15 medium with 6% fetal calf serum in a total volume of 10 ul. MECs were subsequently injected into cleared fat pads of inguinal mammary glands of 21-day old female wildtype FVB mice4. In all cases, control cells were injected contra-laterally from Bmi1-/- cells. At the appropriate time after transplantation (see text), inguinal glands were excised and stained as wholemounts. Glands showing at least 5% fat pad filling were scored as positive 'take'. Among the positive takes, there was no significant difference between the extent of the outgrowth of Bmi1-deficient and controls transplantations. For tissue transplantations, a small piece of ~1 mm³ was cut from the nipple area of mice with the desired genotype and these were inserted into the cleared fat pads of 3-week-old FVB recipients with a Dumont#5 surgical forceps. The frequency of MRUs in the cell suspension being transplanted was calculated using Poisson statistics and the method of maximum likelihood using L-Calc software (StemCell Technologies)5.

HC11 differentiation assays
HC11 cells (a kind gift from dr. Nancy Hynes) were grown in RPMI 1640 medium containing 10% fetal calf serum, 10 ug/ml insulin and 10 ng/ml epidermal growth factor. Subconfluent cell layers were infected with retroviruses expressing Bmi1 cDNA, pRetroSuper viruses expressing two independent short hairpins that target mouse Bmi1 mRNA (siBmi#1: TGGCCCACTACCTTTGAAA;
siBmi#2: GATTGATCGGAAAATGAAA), or an empty control virus. For efficient knockdown of Bmi1, cells were subjected to two rounds of infection. Twenty-four hours after the last infection, cells were selected by the addition of 2 ug/ml puromycin to the medium. When cells reached confluency (usually after about two days), cells were distributed in 6-well plates and allowed to reach confluency. Differentiation was induced one day after reaching confluency with medium containing 5 ug/ml insulin, 10⁻⁶ M dexamethasone and 5 ug/ml prolactin for 3 days. Differentiation was monitored by quantification of β-casein expression with Q-PCR. In different experiments, Bmi1 levels were reduced to varying degrees. In all cases, lower levels of Bmi1 resulted in higher β-casein expression (10 to 40-fold induction).

Quantitative RT-PCR
Total RNA was extracted using Trizol Reagent (Invitrogen) and 1 ug per sample was first treated with DNase and then reverse-transcribed following the manufacturer’s protocol (Invitrogen). The resulting cDNA was analyzed using SYBR Green (PCR master mix, Roche) on an ABI 7000 SDS (Applied Biosystems). Product accumulation was evaluated using the comparative Ct method (deltadelta Ct method), with β-actin and HPRT as endogenous controls for normalization. The following primers (all for mouse transcripts) were used:

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Western blot analysis
MEC pellets were lysed in RIPA buffer, equal amounts of protein were separated on 4-12% precast gels (Invitrogen) and blotted onto 0.2 um Protran nitrocellulose membranes (Whatman). Bands were visualized using enhanced chemiluminescence (Amersham). Primary antibodies were used against Bmi1 (F6, Upstate), Ink4a (M156, Santa Cruz), Arf (ab80, Abcam) and tubulin (Sigma). Secondary antibodies were goat-anti-mouse (ZyMed) or goat-anti-rabbit (BioSource), both HRP-conjugated.

References
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Figure S1 - Expression of Bmi1 in mouse mammary gland.
Immunohistochemistry on mammary gland from 8-week old wildtype virgins. Bmi1 is widely expressed in the mammary gland, including TEBs (left) and with the strongest expression in the luminal cells (middle and right).

Figure S2 - Bmi1 loss results in the upregulation of Ink4a and Arf mRNA.
A. and B. mRNA levels of Ink4a and Arf are measured by Q-PCR and normalized against β-actin. Triplicate measurements of independent isolates are shown, derived from virgin mice (wildtype (WT) or Bmi1-knockout (BmiKO)) or from pregnant wildtype recipients (mouse (M) 1-3). The RNA from M1 and M2 were derived from the same cell isolates that were used for the protein analysis shown in Figure 3B. C. Proliferation is marked by Ki-67 staining on paraffin sections of wildtype or Bmi1−/− transplants in a 15-days pregnant wildtype recipient. Scale bars represent 25 μm.
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Figure S3 - Experimental design of the limiting dilution assays.

A. Equally-sized mammary tissue pieces from the nipple region of Bmi1−/− and wildtype mice were transplanted into the cleared fat pads of wildtype recipients. Pregnancy is required to rescue the growth of the Bmi1−/− transplants (see Figure 2). Upon pregnancy, cells were isolated, dissociated and injected in limiting numbers into new wildtype recipients. Reconstitution capacity was analyzed upon pregnancy of these secondary recipients.

B. Equally-sized mammary tissue pieces from the nipple region of Bmi1/Ink4a/Arf− and Ink4a/Arf-deficient mice were transplanted into the cleared fat pads of wildtype recipients. In 10 weeks, these grafts reconstitute the fat pads of virgin mice (see Figure 1D). Next, cells were isolated and injected in decreasing concentrations into new wildtype recipients. Reconstitution capacity in these secondary recipients was analyzed after another 10 weeks.
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A

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<th>Number of outgrowths</th>
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B

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Figure S4 - Loss of Bmi1 affects mammary stem cells.  
A. Limiting dilutions of Bmi1-deficient MECs. Bmi1-deficient tissue from three independent donor mice was first transplanted into six wildtype recipients that were then made pregnant, to rescue growth. With these cells, and wildtype cells from littermates that had been transplanted contra laterally, a limiting dilution series was injected into new wildtype recipients (4 to 8 per dilution, see table). After these recipients were pregnant, the reconstitution capacity was analysed as a measurement of Mammary gland Repopulating Units (MRUs). A positive take constitutes more than 5% filling of the fat pad. B. Loss of stem cell activity in the absence of Bmi1 is partially rescued by codeletion of the Ink4a/Arf locus. Mammary tissue from Bmi1/Ink4a/Arf and Ink4a/Arf-deficient mice (three donors each) was transplanted into six wildtype recipients to rescue growth. After 10 weeks, cells were isolated and injected in decreasing concentrations into wildtype recipients (2 to 8 recipients per dilution, see table). After another 10 weeks, the percentage reconstitution of the mammary gland was determined.