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Original Article

Deletion of *Foxa1* in the mouse mammary gland results in abnormal accumulation of luminal progenitor cells: a link between reproductive factors and ER-/TNBC breast cancer?

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Abstract: In humans, parity without breastfeeding increases risk of estrogen receptor-negative (ER-) breast cancer and is associated with hypermethylation of *FOXA1*, a pioneer factor regulating lineage commitment of mammary gland luminal progenitor cells. We postulate that pregnancy-associated repression of *FOXA1* results in the accumulation of aberrant, differentiation-arrested luminal progenitor cells which, following additional genetic and epigenetic insults, may give rise to ER- tumors. Consistent with this hypothesis, we show that deletion of *Foxa1* in the mouse mammary gland results in a two-fold increase in the proportion of luminal progenitor cells and a reduction in mammary gland epithelial cells that stain positive for ER. These results provide compelling support for the notion that reduced *Foxa1* expression is sufficient to alter mammary gland luminal cell fate determination *in vivo*, which could be a mechanism linking parity with ER- breast cancer.

Keywords: Foxa1, mammary gland, pregnancy, breastfeeding, progenitors

Introduction

Although having children reduces risk of ER+ breast cancer, parity has been shown to increase the risk of ER- disease, particularly among women who do not breastfeed. Mechanisms underlying these relationships are unknown, although inflammation during involution of the breast [1] could be exacerbated with abrupt involution when breastfeeding does not occur or is terminated early [2]. This may be especially relevant for U.S. Black women who have a higher incidence of ER- breast cancer, and who are more likely to be parous and not to breastfeed (reviewed in [2]).

Evidence indicates that both ER+ and ER- breast tumors arise from breast luminal progenitors [3]. This includes *BRCA1*-deficient cancers, most of which are ER-, where an expanded population of aberrant luminal progenitor cells has been postulated as the cell-of-origin [4]. Our previous studies of breast cancer showed that the majority of differences in DNA methylation between Black and White women were in ER- breast tumors [5, 6] and that the *FOXA1* gene was one of the most differentially methylated loci between ER- and ER+ cancers. Relationships were most pronounced in tumors from Black women, with *FOXA1* more often hypermethylated and silenced in ER- tumors compared to ER+ [6]. When merging the epigenetic results with epidemiological data, we found that the level of methylation at *FOXA1* in ER- tumors was positively associated with the number of children born to the patient and, importantly, the association was observed only among women who had not breastfed [6]. Consistent with our DNA methylation and RNA-seq studies, we found that FOXA1 protein expression also was higher in ER+ breast tumors compared to ER- tumors, that increased parity was

associated with decreased FOXA1 staining, and that breast feeding partially abrogated this association [7].

Work using breast cancer cell lines has suggested that *FOXA1* drives differentiation of luminal progenitors by inducing "luminal" genes and suppressing "basal" genes [8]. Intriguingly, wildtype BRCA1 protein positively regulates *FOXA1* by preventing its DNA methylation and silencing [9], suggesting that *BRCA1*-mutated breast tumors are usually ER- because mutation/inactivation of *BRCA1* leads to methylation-induced silencing of *FOXA1* in luminal progenitors. We postulate that, analogous to *BRCA1* mutations, pregnancy in the absence of breastfeeding can result in DNA methylation and silencing of *FOXA1* in luminal progenitors leading to the accumulation of aberrant differentiation-arrested cells which, following additional mutations and/or epimutations, could give rise ER- breast tumors. Consistent with this notion, *FOXA1* was shown to become hypermethylated in cancer-free breast tissue obtained from parous women undergoing cosmetic reduction mammoplasty, further suggesting a relationship with reproductive factors [10, 11]. Conditional *Foxa1* knockouts have been previously reported to affect mammary gland morphogenesis and mammary gland tumor incidence [12, 13]. However, there have been no studies describing the effect of *Foxa1* deletion on the relative proportions of the different subtypes of mouse mammary gland epithelial cells. To test the hypothesis that deletion of *Foxa1* in the mouse mammary gland alters its normal proportions of epithelial subtypes, we knocked out *Foxa1* in mouse mammary glands and analyzed the epithelial subtypes by flow cytometry. We observed a 2-fold increase in the proportion of luminal progenitors in *Foxa1*-depleted mammary glands. We suggest that at least a fraction of these cells is differentiation-arrested, and that in the human breast, analogous cells would be predisposed to develop into ER- mammary gland tumors if transformed.

Material and methods

Mice

Mice were handled in accordance with IACUC/AAALAC and ethics guidelines from the Department of Laboratory Animal Resources (DLAR), Roswell Park Comprehensive Cancer Center. Mice carrying a *Foxa1* allele with loxP sites flanking exon 2 (designated as *Foxa1*^{f}) [14] were obtained from Dr. Klaus Kaestner at the University of Pennsylvania and crossed with strains carrying a transgene expressing *Cre*-recombinase under the control of either the mouse mammary tumor virus (MMTV) promoter (MMTV-*Cre*, line D)(Tg[MMTV-cre]4Mam/J, JAX stock no. 003553) or the keratin 14 promoter (*Krt14-Cre*)(Tg[KRT14-cre]1Amc/J, JAX stock no. 004782), to obtain experimental genotypes, *Foxa1*$^{f/f}$; MMTV-*Cre*, Foxa1f/+; MMTV-Cre, and Foxa1f/+; Krt14-Cre as well as controls Foxa1f/f and Foxa1f/+ (Supplementary Figure 1). Exposure to Cre-recombinase results in a 3.5 kb deletion removing approximately 80% of the protein coding sequence [14] (Supplementary Figure 2). Groups of 5-6 virgin females of each genotype produced were sacrificed as 9 weeks old virgins and mammary glands collected.

Mammary gland dissociation and isolation of epithelial cells

Primary mammary epithelial cells (MEC) were isolated from thoracic (the 2nd and 3rd), abdominal (the 4th) and inguinal (the 5th) mammary glands using a protocol provided by Dr. Sung Jin Huh [15]. Chopped tissue was transferred to digestion medium (DMEM/F-12, 10% FBS, 5 mg/ml insulin, pen/strep) containing 2 mg/ml collagenase type 4 (Worthington), incubated at 37°C with shaking for 30 minutes. Following centrifugation, digests were resuspensed in medium and large sized "organoids" collected by filtering through a 40 micron mesh. To obtain single cell suspensions for analysis, organoids were suspended in 0.05% trypsin, incubated at 37°C for 10 minutes, the trypsin inactivated by addition of FBS-containing medium, the cells pelleted and then suspended in medium containing 10 U/ml DNase1 and 5 mM $MgCl_2$.

Flow cytometry analysis

Flow cytometry was carried out using the protocol of Dr. Sung Jin Huh [15]. Freshly prepared single-cell suspensions of MECs were resuspended in 0.5% BSA, 2 mM EDTA in PBS. Approximately 1 × 10^6 cells were incubated for 30 minutes with 5 antibodies (biotin-conjugated rat anti-mouse CD31 [BD Pharmingen, cat no. 558737], biotin-conjugated rat anti-mouse CD45 [BD Pharmingen, cat no. 553077], APC-

labeled anti-mouse CD24 [BioLegend, cat no. 101814], PE-labeled hamster anti-mouse CD61 [BD Pharmingen, cat no. 553347], FITC-labeled rat anti-mouse CD29, [BioLegend, cat no. 102206]) and DAPI. Following washing in PBS, cells were resuspended in PBS containing Alexa750-conjugated streptavidin (Molecular Probes, cat no. S21384). DAPI facilitates removal of dead cells; anti-CD31-biotin and anti-CD45-biotin, together with Alexa750-streptavidin, excludes endothelial cells and leukocytes from the analysis. $CD31^-$ $CD45^-$ cells were further selected with anti-CD24, and these cells analyzed for CD29 and CD61 signals to determine the proportions of the 3 epithelial cell populations (Supplementary Figure 3). Flow cytometry was performed using a BD LSRII Flow Cytometer available in the Roswell Park Flow Cytometry and Imaging Core.

PCR genotyping

Genomic DNA was extracted using the Mouse Tail Direct PCR Kit (Creative Biogene, Shirley, NY) and single-cell suspensions of mammary epithelial cells (described above) using the Wizard Genomic DNA Purification kit (Promega). PCR was carried out using Direct PCR kit and run on a BioRad C1000 Touch Thermal Cycler. For *Foxa1*, the following primers were used: 5'-CTGTGGATTATGTTCCTGAT-3' and 5'-GTGTCAGGATGCCTATCTGGT-3', amplify the wild-type and the floxed *Foxa1* alleles, giving rise to PCR products of 244 and 260 bp, respectively [14]. For the *Cre* gene, 5'-CGACCAGGTTCGTTCACTCA-3' (forward primer), and 5'-CAGCGTTTTCGTTCTGCCAA-3' (reverse primer) resulting in a PCR product of 184 bp. To confirm deletion of *Foxa1*, primers 5'-GATCACTCAAGCAGGCCTGT-3' and 5'-TCCCCCTTTGCCACGTTTTA-3' were used which flank the deleted region, and amplify the deleted *Foxa1* allele, yielding a 530 bp PCR product.

Immunohistochemistry and image analysis

Mammary glands were fixed with 10% buffered formalin overnight, embedded in paraffin, sectioned, and stained with an antibody against estrogen receptor-α (ERα) (Abcam, cat. no. ab3575). Stained slides were digitally imaged at 20x magnification using the Aperio ScanScope XT (Aperio Technologies, Vista, CA). Whole section slides were annotated manually to identify mammary gland epithelial regions for the image analysis. The percent of cells stained was recorded in each intensity category: 0, 1+ (only partial or weak staining), 2+ (moderate and complete staining), and 3+ (intense and complete staining). A histological score (H-score) was calculated by the formula: [1 × (% cells 1+) + 2 × (% cells 2+) + 3 × (% cells 3+)] × 100 [16].

Statistical analysis

The flow cytometry profiles were compared between mouse groups in a pairwise fashion using two-sided Wilcoxon rank sum (WRS) exact tests. Analyses were conducted in SAS v9.4 (Cary, NC) at a nominal significance level of 0.05.

Results

Floxed *Foxa1* alleles were deleted early in mammary gland development using two Cre-driver mouse strains, MMTV-*Cre* (D line) and *Krt14-Cre*, both of which function in the mammary gland [12]. Only virgin females were used in this study to avoid confounding factors associated with changes in mammary gland epithelial subpopulations during pregnancy, lactation, and involution (reviewed in [17]). Mammary glands were collected from 9 week-old virgin mice of 5 different genotypes (Supplementary Figure 1), the cells dissociated, tested for deletion of *Foxa1* (Supplementary Figure 2) and analyzed by flow cytometry (Methods). Following heterozygous deletion of *Foxa1* using *Krt14-Cre*, there was an increase in the proportion of luminal progenitor cells ($CD24^+CD61^+CD29^{lo}$) from approximately 17% in control mice ($Foxa1^{+/f}$) to approximately 42% in $Foxa1^{+/f}$; $Krt14\text{-}Cre^{+/-}$ mice (P = 0.004, Holm-Bonferroni adjusted t-test) (**Figure 1A**, **1B**). A similar trend was observed when *Foxa1* was heterozygously deleted using the MMTV-*Cre* transgenic line, however the increase in luminal progenitors was not statistically significant, possibly due to the mosaic nature of *Cre*-recombinase excision in the mammary gland by MMTV-*Cre* [12]. On the other hand, when *Foxa1* was homozygously deleted in mammary epithelial cells using MMTV-*Cre* ($Foxa1^{f/f}$; MMTV-$Cre^{+/-}$ mice), the fraction of luminal progenitors was also significantly increased compared to its control ($Foxa1^{f/f}$) (P = 0.016, Holm-Bonferroni adjusted t-test) (**Figure 1C**, **1D**). Homozygous deletion of *Foxa1* using *Krt14*-Cre results in the complete

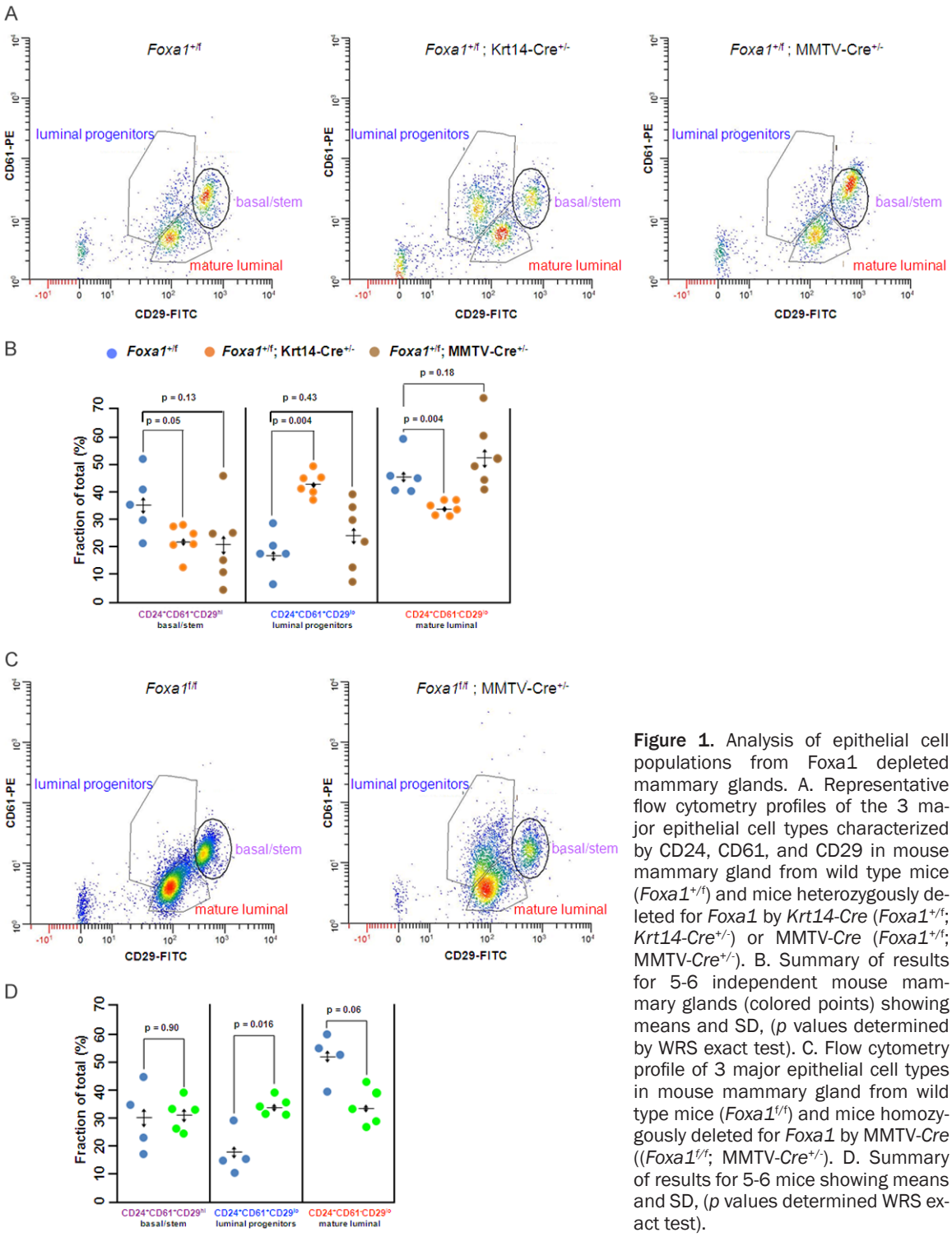

Figure 1. Analysis of epithelial cell populations from Foxa1 depleted mammary glands. A. Representative flow cytometry profiles of the 3 major epithelial cell types characterized by CD24, CD61, and CD29 in mouse mammary gland from wild type mice (*Foxa1*$^{+/f}$) and mice heterozygously deleted for *Foxa1* by *Krt14-Cre* (*Foxa1*$^{+/f}$; *Krt14-Cre*$^{+/-}$) or MMTV-*Cre* (*Foxa1*$^{+/f}$; MMTV-*Cre*$^{+/-}$). B. Summary of results for 5-6 independent mouse mammary glands (colored points) showing means and SD, (*p* values determined by WRS exact test). C. Flow cytometry profile of 3 major epithelial cell types in mouse mammary gland from wild type mice (*Foxa1*$^{f/f}$) and mice homozygously deleted for *Foxa1* by MMTV-*Cre* ((*Foxa1*$^{f/f}$; MMTV-*Cre*$^{+/-}$). D. Summary of results for 5-6 mice showing means and SD, (*p* values determined WRS exact test).

absence of mammary ducts [12, 13] precluding the analysis of mammary epithelial cells. As would be predicted by the postulated blockage of differentiation of luminal progenitors, there was a corresponding decrease in the proportion of mature luminal cells ($CD24^+CD61^-CD29^{lo}$) (P = 0.004, Holm-Bonferroni adjusted t-test) in *Foxa1*$^{+/f}$; *Krt14-Cre* mammary glands, although this was not observed in the *Foxa1*$^{+/f}$; MMTV-*Cre* line. However, we also observed a

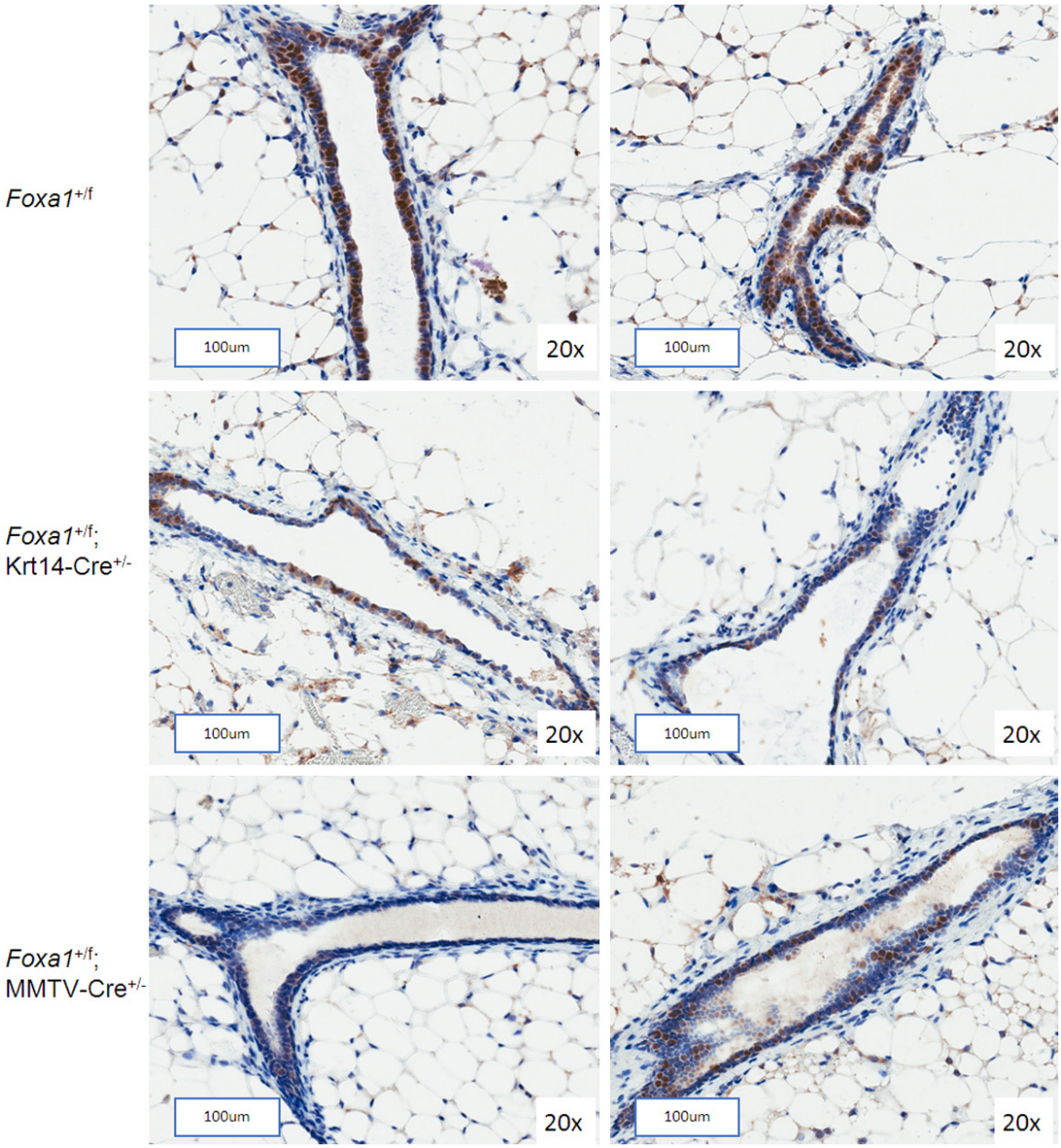

Figure 2. ERα immunostaining of mammary gland sections from conditional Foxa1 knockout mice. Expression of ERα in the ductal epithelial cells of mammary glands from 9-week-old virgin *Foxa1*$^{+/f}$; *Krt14-Cre*$^{+/-}$, *Foxa1*$^{+/f}$; MMTV-*Cre*$^{+/-}$ or control *Foxa1*$^{+/f}$ mice determined by IHC. The scale bars represent 100 μm.

reduction in the proportion of basal/stem cells in both *Foxa1*$^{+/f}$; *Krt14-Cre* and *Foxa1*$^{+/f}$; MMTV-*Cre* lines, although the difference was not statistically significant in the latter mouse strain. Thus, at least for basal/stem cells, the apparent decrease in relative proportion may be indirect due to the expansion of the luminal progenitors in these mice (see discussion).

Since *Foxa1* is indispensable for expression of ERα [18], we assessed the effect of *Foxa1* deletion on the prevalence of ER-positive cells in mammary gland sections by immunohistochemistry. We observed a marked decrease in number of ERα-positive cells in the mammary ducts of *Foxa1*$^{+/f}$; *Krt14-Cre*$^{+/-}$, and to lesser extent in *Foxa1*$^{+/f}$; MMTV-*Cre*$^{+/-}$ mice, compared to the wildtype control *Foxa1*$^{+/f}$ mice (**Figure 2**, Supplementary Figure 4).

Discussion

As a surrogate for DNA methylation-induced silencing, we conditionally deleted *Foxa1* in the

mouse mammary gland using a floxed-*Foxa1* allele in combination with two *Cre*-recombinase drivers. Results demonstrate that depletion of *Foxa1* leads to dramatic changes in the proportions of mammary gland epithelial cell populations, resulting in an abnormal accumulation of luminal progenitors. Similar increases in luminal progenitors occur following deletion of *Gata3*, a gene upstream of *Foxa1* [19]. However, we did not find *GATA3* to be differentially methylated in ER+ versus ER- tumors in our study of human breast cancer [6], supporting instead the importance of *FOXA1* in the etiology of ER- breast tumors. There are at least two different types of luminal progenitor cells in the mouse mammary gland, a large ER- subset that are precursors to the secretory alveolar cells, and a smaller ER+ subset of cells that express high levels of *Foxa1* [17, 20]. These ER+ progenitors probably give rise to ductal luminal cells because they express *Foxa1* and other transcription factors required for ductal development [18, 20], and *Foxa1* ablation leads to complete absence of ductal tree formation [12, 13]. It is therefore likely that ER+ progenitor cells were the cells affected by *Foxa1* depletion in the present study, resulting in blocked differentiation and accumulation in the luminal progenitors in mutant mice. Through its function as a "pioneer factor", *Foxa1* regulates a plethora of genes by binding to transcriptionally repressed chromatin and facilitating subsequent binding by multiple transcription factors including ERα [21]. Thus, its depletion in these cells (or their precursors) may lead to an accumulation of differentiation-arrested luminal progenitors with increased proliferative capacity due to disruption of the FOXA1 and ERα transcriptomes. The overall effect of *Foxa1* deletion on differentiation of luminal epithelial cells remains to be fully elucidated but will become clearer by analysis of mammary glands from conditional *Foxa1* knockouts using single-cell RNA-seq approaches [22-24].

Unexpectantly, we also found a reduction in the proportion of basal/stem cells in some of the *Foxa1* knockout mammary glands. *Foxa1* is not known to be expressed in the basal cell compartment of the mouse mammary gland and therefore its deletion in these cells is not expected to produce a phenotype. Hence, the finding of a reduced proportion of cells in the basal/stem cell compartment may simply reflect a greater absolute number of epithelial cells in the mammary glands in these two mouse lines as a consequence of the increased number of highly proliferative, differentiation-blocked, luminal progenitor cells.

Our results provide support for the notion that reduced *Foxa1* expression alters luminal cell fate determination *in vivo* and proof-of-principle that pregnancy-linked methylation and silencing of *FOXA1* may lead to the accumulation of aberrant differentiation-arrested luminal progenitors in the human breast, which following additional mutations and/or epimutations, could give rise ER- breast tumors. For the most part, a mechanistic link between parity and breastfeeding and increased methylation of *FOXA1* remaine to be elucidated [25]. However, with the convincing evidence in the literature that parity increases risk of ER- breast cancer, particularly among women who do not breastfeed, coupled with our molecular results showing that parity without breastfeeding results in hypermethylation of *FOXA1*, particularly among Black women, these animal experiments provide a reasonable mechanism for these associations; e.g., through abnormal accumulation of luminal progenitor cells. This work is potentially relevant to the important question of why ER- breast cancer is more common in Black women who are more likely to have children and not to breastfeed. Identification of key genes such as *FOXA1* may provide potential targets for cancer prevention, e.g. through the development of targeted epigenetic-modifying agents, to reverse silencing of pro-luminal genes.

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Disclosure of conflict of interest

None.

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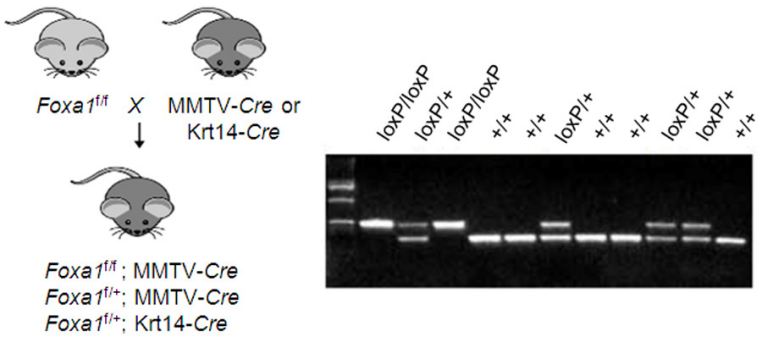
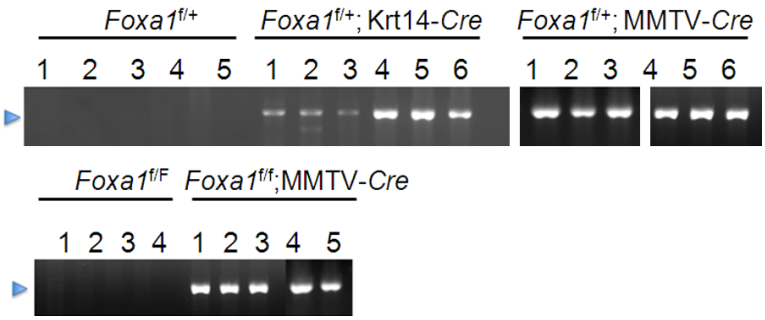
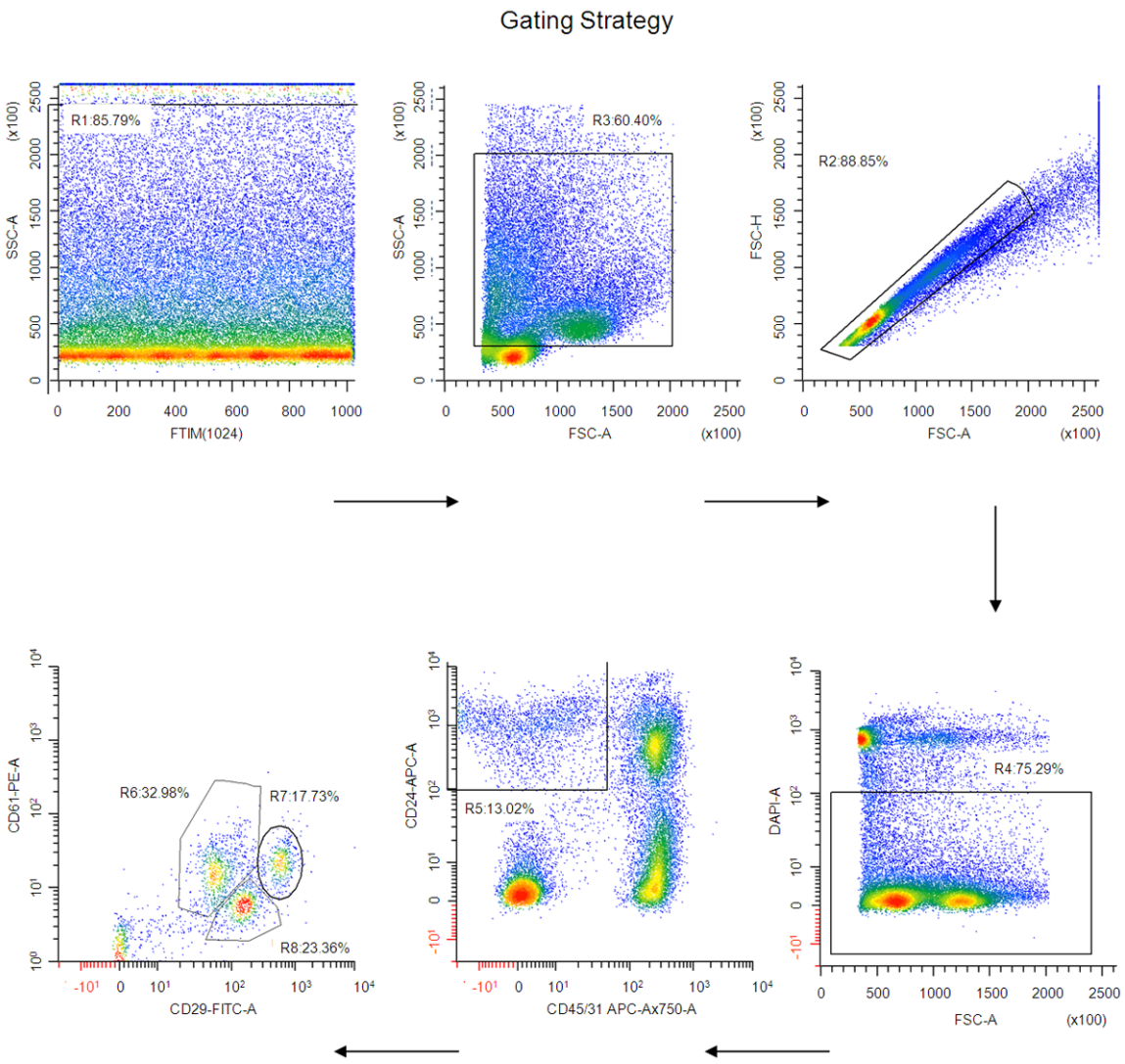


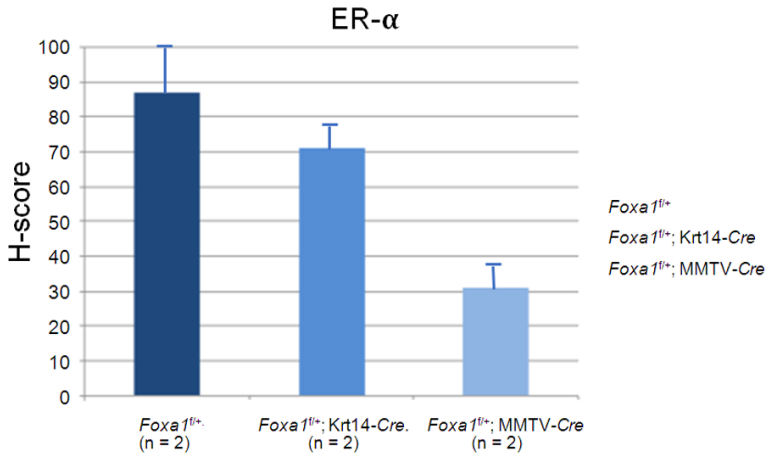
Supplementary Figure 1. Generation of *Foxa1* conditional knockout mice. Schematic representation of the strategy used to target the *Foxa1* gene in the mouse mammary gland. Mice carrying floxed *Foxa1* allele(s) were crossed into mouse strains that express *Cre*-recombinase; MMTV-*Cre* and *Krt14-Cre*. Gels show examples of PCR products representing the floxed and the WT genotypes of knockout mice.



Supplementary Figure 2. Deletion of the floxed *Foxa1* region in the mammary gland of *Foxa1* knockout mice. MMTV- and *Krt14*-driven *Cre*-mediated *Foxa1* deletion as detected by PCR in the mouse mammary gland cells. PCR results showed deletion of *Foxa1* in heterozygous floxed *Foxa1*; *Krt14-Cre*, heterozygous floxed *Foxa1*; MMTV-*Cre* and homozygous floxed *Foxa1*; MMTV-*Cre* positive mice but not the control animals (*Foxa1*$^{f/+}$, *Foxa1*$^{f/f}$).



Supplementary Figure 3. Gating strategy for FACS analysis of three major epithelial cell populations in the murine mammary gland.



Supplementary Figure 4. Quantitation of ERα immunohistochemical staining of mammary glands from conditional *Foxa1* knockout mice. Bars show the mean H-score (Methods) of 2 mice/genotype with errors bars representing the range of H-scores.