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FOXA1 is an essential determinant of ERα expression and mammary ductal morphogenesis

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SUMMARY

FOXA1, estrogen receptor α (ERα) and GATA3 independently predict favorable outcome in breast cancer patients, and their expression correlates with a differentiated, luminal tumor subtype. As transcription factors, each functions in the morphogenesis of various organs, with ERα and GATA3 being established regulators of mammary gland development. Interdependency between these three factors in breast cancer and normal mammary development has been suggested, but the specific role for FOXA1 is not known. Herein, we report that *Foxa1* deficiency causes a defect in hormone-induced mammary ductal invasion associated with a loss of terminal end bud formation and ERα expression. By contrast, *Foxa1* null glands maintain GATA3 expression. Unlike ERα and GATA3 deficiency, *Foxa1* null glands form milk-producing alveoli, indicating that the defect is restricted to expansion of the ductal epithelium, further emphasizing the novel role for FOXA1 in mammary morphogenesis. Using breast cancer cell lines, we also demonstrate that FOXA1 regulates ERα expression, but not GATA3. These data reveal that FOXA1 is necessary for hormonal responsiveness in the developing mammary gland and ERα-positive breast cancers, at least in part, through its control of ERα expression.

KEY WORDS: FOXA1, ERα, GATA3, Mammary gland, Breast cancer, Mouse

INTRODUCTION

The epithelium of the mammary gland is composed of luminal and basal/myoepithelial cell lineages (Richert et al., 2000). Luminal cells line the ductal lumen and secrete milk upon terminal differentiation into lobulo-alveolar cells. Basal/myoepithelial cells reside between the luminal cells and the basement membrane and are necessary for ductal contractility. Breast cancer subtypes (luminal versus basal) have been defined by patterns of gene expression that reflect these lineages (Sorlie et al., 2003). Luminal subtype tumors maintain a more differentiated state and are less aggressive than basal subtype cancers. Processes of normal postnatal mammary gland development directly mirror those of tumorigenesis (e.g. invasion, proliferation, angiogenic remodeling and apoptotic resistance) (Wiseman and Werb, 2002). Hence, determining how cell fate is regulated during normal mammary gland development should facilitate identifying the mechanistic basis for phenotypic differences between luminal and basal breast cancers, and should advance the development of subtype-specific therapeutics.

Expression of the transcription factors ERα (ESR1 – Mouse Genome Informatics), GATA3 and FOXA1 strongly correlates with the luminal subtype of breast cancer and favorable patient prognosis (Badve et al., 2007; Habashy et al., 2008; Mehra et al., 2005; Sorlie et al., 2003). Estrogenic signaling through ERα, a member of the nuclear receptor superfamily, is a primary determinant of luminal tumor biology, and patients with luminal tumors have a better prognosis, owing partly to estrogen-targeted therapies (EBCTCG, 2005). The consistent concomitant expression of FOXA1, ERα and GATA3 in this subtype is suggestive of a co-modulatory loop that may be responsible for maintaining the luminal phenotype. In breast cancer cells, FOXA1 facilitates estrogen responsiveness by modulating ERα binding to a subset of target gene promoters (Carroll et al., 2005; Laganiere et al., 2005). For example, FOXA1 is specifically required for ERα-induced transcription of *CCND1* (cyclin D1) (Eeckhoute et al., 2006), an established oncogene in breast cancer (Buckley et al., 1993; Eeckhoute et al., 2006). In contrast to the role of FOXA1 in ERα activity, ERα and GATA3 have been suggested to function in a positive feedback loop, in which expression of ERα is required for the transcription of *GATA3*, and vice versa (Eeckhoute et al., 2007). These data imply an interdependence of FOXA1, ERα and GATA3 in the maintenance of luminal breast cancer. Further defining this collaboration should provide insight into how ERα-positive tumors become resistant to anti-hormone therapies as well as reveal the function of FOXA1 that occurs in tumors in the absence of ERα (Habashy et al., 2008) (R.A.K., unpublished).

The ability of FOXA1, ERα and GATA3 to form a regulatory network in luminal breast cancer cells suggests that they may also co-modulate normal mammary gland morphogenesis, a process

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that requires ERα and GATA3. ERα is expressed within a subset of the normal luminal epithelial and stromal populations of the mammary gland (Haslam and Nummy, 1992). Disruption of *Esr1* blocks development at a rudimentary ductal structure, and signaling from estradiol through ERα during puberty is required for mammary epithelial proliferation, ductal elongation, bifurcation and invasion throughout the mammary fat pad (Feng et al., 2007; Mallepell et al., 2006; Mueller et al., 2002). GATA3 is also necessary for mammary gland development (Asselin-Labat et al., 2007; Kouros-Mehr et al., 2006). Specifically, *Gata3* deficiency leads to expansion of the luminal progenitor population positive for CD61 (ITGB3 – Mouse Genome Informatics), indicating that GATA3 is necessary for terminal differentiation of the luminal lineage (Asselin-Labat et al., 2007). Further investigation revealed that forced expression of GATA3 induces tumor differentiation and inhibits metastatic progression (Kouros-Mehr et al., 2008). In support of a transcriptional interdependence between ERα, GATA3 and FOXA1, loss of *Gata3* in the normal mammary gland decreases the ERα-expressing luminal population (Asselin-Labat et al., 2007; Kouros-Mehr et al., 2006), and overexpression of GATA3 in murine mammary tumors (Kouros-Mehr et al., 2008) and a human embryonal kidney epithelial cell line increases *FOXA1* mRNA (Usary et al., 2004). Moreover, chromatin immunoprecipitation (ChIP) in primary mammary cells revealed that GATA3 binds, and can potentially regulate, transcription of *Foxa1* (Kouros-Mehr et al., 2006), although this has not been directly demonstrated.

Although FOXA1, GATA3 and ERα are positively correlated in breast tumors and form a co-regulatory network in breast cancer cell lines, the functional relationship between these three factors during development and tumor initiation has not been fully explored. In particular, the role of FOXA1 in mammary morphogenesis remains unknown. Herein, we report that *Foxa1* deficiency in the mammary gland results in loss of ERα, a block in terminal end bud formation and an inability of the ducts to properly invade the mammary fat pad in response to pubertal or pregnancy hormones. By contrast, *Gata3* expression and formation of lobulo-alveoli are independent of FOXA1. These data provide the first direct evidence that FOXA1 is crucial for mammary gland morphogenesis and maintenance of ERα expression.

MATERIALS AND METHODS

Immunohistochemistry and immunofluorescence

For analysis of proliferation, mice were injected i.p. with 10 mg/g BrdU (Sigma) 2 hours before sacrifice. Glands were fixed in 4% paraformaldehyde for 4 hours, transferred to 1×PBS, paraffin embedded and sectioned (5 μm). Sections were re-hydrated, and antigen retrieval performed using 10 mM sodium citrate (pH 6) in a pressure cooker (125°C for 10 minutes; 90°C for 2 minutes) or for milk antibody, incubated in 10 μg/ml pepsin in 0.01 N HCl for 15 minutes at room temperature. Sections were blocked with peroxidase blocking reagent (DAKO) and incubated with primary antibody overnight at 4°C [FOXA1, Santa Cruz; ERα, Santa Cruz; E-cadherin, Cell Signaling; CK8 (TROMA-1), Developmental Studies Hybridoma Bank, University of Iowa; α-SMA, Sigma; BrdU, Becton-Dickinson) or at room temperature for 1 hour (milk, Nordic Immunology; PR, DAKO)]. Secondary detection of FOXA1, ERα, E-cadherin, milk and PR was performed using the appropriate Vectastain Elite ABC Kit (Vector Laboratories) as per the manufacturer's recommendations. CK8, α-SMA and BrdU were detected using the EnVision+System-HRP for mouse antibodies (DAKO) as recommended. Secondary conjugates were detected using 3,3′-diaminobenzidine (DAKO). Sections were counterstained with Gill's #3 Hematoxylin (Fisher), dehydrated and mounted. TUNEL was performed as per the manufacturer's recommendations except using Gill's #3 Hematoxylin as a counterstain (ApopTag Peroxidase In Situ Apoptosis Detection Kit, Chemicon). Alexa fluor 596 (anti-goat) and Alexa fluor 488 (anti-rabbit) secondary antibodies (Invitrogen) were used for detection of FOXA1 and ERα by immunofluorescence (IF). IF and immunohistochemistry (IHC) were quantified by counting the percentage of positive epithelium in at least two to five fields per section per mouse. Hematoxylin and Eosin staining was performed by the Case Western Reserve University Tissue Procurement and Histology Core Facility.

Animal breeding

All animal procedures, except production of Ex3αERKO and *MMTV-cre;Gata3$^{f/f}$* mice, were approved by the Case Western Reserve University IACUC. Ex3αERKO mice were generated under an approved protocol at the National Institute of Environmental Health Sciences/NIH, and a contract to Xenogen (Caliper Life Sciences) using a strategy similar to that described previously (Dupont et al., 2000). This resulted in an *Esr1* gene with exon 3 flanked by *loxP* sites. Exon 3 was deleted by crossing mice carrying the floxed exon 3 *Esr1* to a global *Sox2-cre* mouse line [Tg(Sox2-cre)1Amc/J; Jackson Labs]. DNA was evaluated by PCR using P1 and P3 primers as described (Dupont et al., 2000). The *MMTV-cre;Gata3$^{f/f}$* mice were generated at the Walter and Eliza Hall Institute of Medical Research as described (Asselin-Labat et al., 2007). *Foxa1$^{+/-}$* males (Kaestner et al., 1999) were bred with wild-type C57BL/6 females generating *Foxa1$^{+/-}$* progeny that were intercrossed to generate *Foxa1$^{-/-}$* progeny. Transgenic mice were identified by PCR using primers as described (Kaestner et al., 1999).

Mammary anlagen transplantation

Transplantation of mammary anlagen into recipient mice has been described (Robinson et al., 2000). Briefly, the mammary anlagen of embryonic day 14 (E14) female mouse embryos were dissected and cultured at 37°C/5% CO_2 in DMEM/F12 (supplemented with 10% FBS, 1% Pen-Strep, 2 mM L-Glutamine and 0.75 μg/ml Fungizone) until the genotypes were determined. Three-week-old recipient C57BL/6 females were anesthetized with 2.5% avertin, and inguinal fat pads were cleared of endogenous epithelium. The cleared fat pad was examined by whole mount to verify successful clearing. A *Foxa1$^{+/+}$* anlage was inserted into the cleared fat pad, and a *Foxa1$^{-/-}$* anlage was inserted into the contralateral cleared fat pad of the same mouse. The incision was sutured and infiltrated with marcaine (0.25%). Recipient mice were aged 5 or 8 weeks, and the transplanted glands were collected and whole mounts examined. Alternatively, recipient mice were aged 8 weeks, mated with C57BL/6 males, and the transplanted glands collected and whole mounts assessed at 18.5 days postcoitum (dpc).

Renal capsule grafting

Tissue grafting into the renal capsule has been described (Cunha et al., 2000). Briefly, inguinal fat pads of postnatal day 1 female pups were removed, and incubated at 4°C in DMEM/F12 culture media as described above until the genotypes were determined. Recipient C57BL/6 females were anesthetized with 2.5% avertin, and a kidney exteriorized. A small incision was made to separate the kidney capsule from the parenchyma. A polished glass pipette was used to create a pocket between the kidney capsule and parenchyma. The inguinal fat pad from a *Foxa1$^{+/+}$* pup was grafted into the pocket, and the kidney placed back into the body cavity. The same procedure was used to graft the inguinal fat pad from a *Foxa1$^{-/-}$* pup on to the contralateral kidney. The incisions were closed using wound clips, and the wound infiltrated with marcaine (0.25%). Recipient mice were aged either 2 or 4-5 weeks, and the glands harvested for further analysis. Alternatively, recipients were aged 4-5 weeks, mated, and the glands were harvested at 18.5 dpc.

Mammary gland whole mounts

Glands were fixed in Kahle's fixative for at least 4 hours, washed in 70% ethanol, gradually rehydrated to 100% water, stained with carmine alum, dehydrated, cleared in xylenes and mounted as previously described (Rasmussen et al., 2000). Ductal area was obtained by taking the area of a box drawn around the gland, including the nipple. Ductal length was obtained by measuring from the farthest edge of the lymph node from the

nipple to the end of the longest duct. When the duct did not reach the lymph node, the distance from the end of the duct to the farthest edge of the lymph node from the nipple was measured and given a negative value.

Mammary cell preparation and FACS

Inguinal mammary glands from ten (per experiment) wild-type FVB/N virgin females (~8 weeks of age) were isolated and prepared as described (Shackleton et al., 2006). Experiments #1 and #2 were sorted by fluorescence-activated cell sorting (FACS) using antibodies against CD24, CD29 (ITGB1 – Mouse Genome Informatics) and CD61 as described (Asselin-Labat et al., 2007). Experiment #2 was sorted twice to enhance purity.

Quantitative real-time PCR

Total RNA was isolated using TRIzol Reagent (Invitrogen), treated with DNAse I (DNA-*free*, Ambion), and cDNA produced using SuperScript II Reverse Transcriptase (Invitrogen). Real-time PCR was performed using Applied Biosystems TaqMan Gene Expression Assays (Assay IDs=*Foxa1*, Mm00484713_m1; *Pgr*, Mm00435625_m1; *Gata3* Mm00484683_m1; *Krt8*, Mm00835759_m1; *ESR1* Hs01046817_m1; *GAPDH*, Hs99999905_m1) or SYBR-green primers (*Foxa1*-Foward: GGATCCC-CGCTACTCCTTTA; *Foxa1*-Reverse: AGCACGGGTCTGGAATA-CAC).

Cell culture and RNA interference

All cell lines were obtained from ATCC. MCF7 cells were grown in DMEM (Mediatech); T47D cells in RPMI 1640 (Gibco). Media was supplemented with 10% FBS and 1% Penicillin-Streptamycin (Invitrogen). Cells were seeded in 100 mm dishes to be 30-50% confluent upon transfection. siRNA targeting firefly luciferase mRNA (siCONTROL Non-targeting siRNA #2, Dharmacon) or human *FOXA1* mRNA (siGENOME M-010319-01 and -04, Dharmacon) were transfected in OPTI-MEM media (Invitrogen) using Lipfectamine 2000 (Invitrogen) to a final concentration of 100 nM. Culture media was changed to complete growth medium after 16-24 (MCF7) or 24 (T47D) hours. Cells were harvested 36 (MCF7) or 72 (T47D) hours post-transfection.

Immunoblots

Cells were lysed [50 mM Tris-HCl, pH 7.4; 100 mM NaCl; 1 mM EDTA; 1 mM EGTA; 1 mM NaF; 0.1% SDS; 0.5% Sodium Deoxycholate; 1% Triton-X-100; 10% Glycerol; 2 mM Sodium Orthovanadate; Protease Inhibitor Cocktail (Sigma)], and protein levels quantified (Bradford Assay, Biorad). Protein lysate was resolved using SDS-PAGE, and transferred to PVDF membrane (BioRad). Blots were blocked (5%-milk-1XPBST) and incubated overnight at 4°C with primary antibody (FOXA1, Santa Cruz; ERα, Santa Cruz; GATA3, Santa Cruz; β-actin, Sigma) diluted in 5%-BSA-1XPBST. Blots were incubated with an HRP-conjugated secondary antibody (Santa Cruz) diluted in 5%-milk-1XPBST and developed using ECL reagent (Amersham). Quantification of protein levels was determined using Image J (Abramoff et al., 2004).

Chromatin immunoprecipitation

ChIP was performed as previously described (Wittmann et al., 2005). For the FOXA1 ChIP analysis, MCF7 cells were treated with vehicle or 17β-estradiol (10^{-7} M) for 45 minutes. Cleared lysate was incubated with either normal goat IgG or FOXA1 antibody (Abcam, Ab5089). For RNA polymerase II ChIP analysis, MCF7 cells were transfected with a non-targeting siRNA or *FOXA1* siRNA as described above. Cells were harvested 36 hours post-transfection. Cleared lysate was incubated with either normal mouse IgG or RNA polymerase II antibody (Covance, 8WG16). Binding of FOXA1 and RNA polymerase II to the *ESR1* proximal promoter was detected using the following primers: 5′-AG-GAGGGGGAATCAAACAGA-3′ and 5′-TTTACTTGTCGTCGCT-GCTG-3′. Quantification of precipitated DNA relative to input was accomplished using Image J (Abramoff et al., 2004).

Statistical methods

Significance was determined by Student's *t*-test assuming a two-tailed distribution and equal variance among sample populations.

RESULTS

FOXA1 is expressed in the developing mammary gland in conjunction with ERα

The consistent expression of FOXA1 in luminal breast cancers led us to postulate that FOXA1 may also regulate luminal epithelial cells in the normal breast. To begin to address this possibility, we examined the pattern of FOXA1 expression throughout various stages of murine mammary gland development. Given the ability of FOXA1 to regulate ERα activity at numerous target genes in breast cancer, we also assessed the pattern of ERα expression. FOXA1 is expressed in the majority of body cells (i.e. luminal progenitors), but is absent from cap cells (i.e. myoepithelial progenitors) within the terminal end bud (TEB) (Fig. 1A). TEBs appear at the leading edge of the duct during puberty, and are the highly proliferative structures required for ductal elongation and branching of the mammary epithelium throughout its associated fat pad (Richert et al., 2000). The expression pattern for FOXA1 was similar to that observed for ERα in the pubertal gland (Haslam and

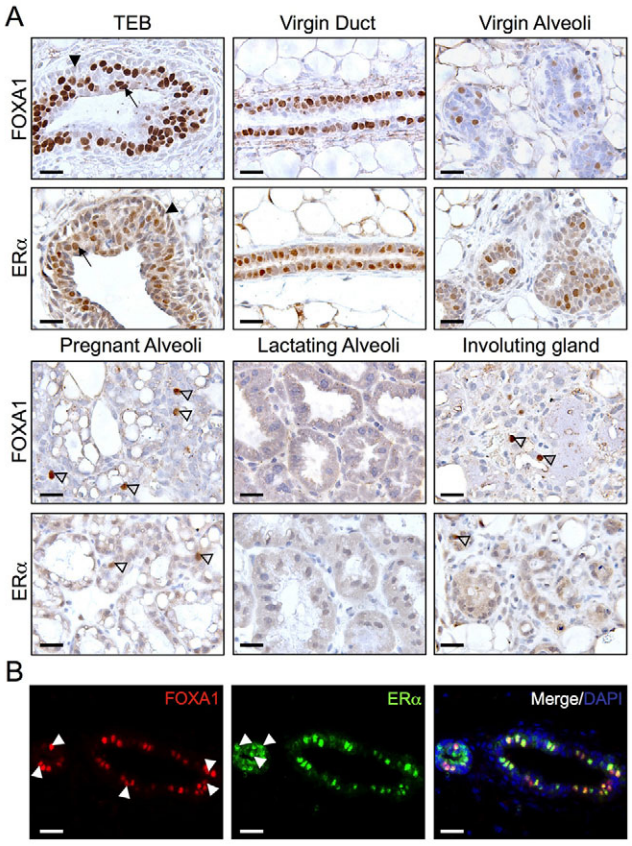

Fig. 1. FOXA1 is expressed in the developing mammary gland in conjunction with ERα. (**A**) Representative images of FOXA1 and ERα IHC in virgin terminal end buds (TEBs) (5 weeks), virgin ductal epithelium (8 weeks), virgin alveoli (20 weeks), pregnant alveoli (day 18), lactating alveoli (day 2) and an involuting gland (day 5). Within the TEB, arrows mark the luminal progenitor cells and arrowheads mark the basal/myoepithelial progenitors. Unfilled arrowheads indicate expressing cells in the pregnant alveoli and involuting gland. FOXA1 and ERα expression (brown nuclei) is counterstained with Hematoxylin. (**B**) Representative image of dual IF for FOXA1 and ERα in virgin ductal epithelium (n=4). The luminal epithelium consists of four populations: cells co-expressing FOXA1 and ERα (31.8±4.4%) (yellow cells in 'Merge'), expressing FOXA1 (12.1±5.0%) or ERα (3.8±0.6%) alone (arrowheads), or expressing neither (52.3±6.8%). Scale bars: 20 μm.

Nummy, 1992). Both FOXA1 and ERα are maintained in the ductal epithelium of post-pubertal virgin mammary glands, but the subset of cells that are positive for either protein decreases within the virgin alveolar population and is further reduced during pregnancy, when only a few positive cells are present per field. Importantly, lobulo-alveoli do not express either FOXA1 or ERα. Detection of the cell population that expresses FOXA1 and ERα is restored as the mammary gland undergoes involution. These data indicate that FOXA1 is present within the structures that are necessary for puberty-associated mammary morphogenesis (i.e. TEBs) and in the same developmental stages as ERα. To define whether FOXA1 and ERα are co-expressed within the same cells, we performed dual IF within the adult virgin gland (Fig. 1B). At this stage, approximately 30% of luminal epithelial cells express both FOXA1 and ERα, whereas a subset of cells express FOXA1 alone, or to a lesser degree, ERα alone. In addition, whereas ERα is present within the stroma, FOXA1 expression is undetectable (data not shown).

FOXA1 is essential for mammary ductal invasion

The pattern of FOXA1 expression in the TEB (Fig. 1A) suggests that it may contribute to mammary morphogenesis. To determine whether loss of *Foxa1* disrupts embryonic development of the mammary primordium, we analyzed mammary rudiments from *Foxa1*$^{+/+}$ (wild-type) and *Foxa1*$^{-/-}$ (null) mice (Kaestner et al., 1999) on postnatal day 1 (see Fig. S1A in the supplementary material). Using whole mount analysis, we observed no difference between *Foxa1*$^{+/+}$ and *Foxa1*$^{-/-}$ rudiments in the number of terminal ducts or area occupied by ducts (see Fig. S1B in the supplementary material). These results indicate that FOXA1 expression is not required for embryonic development of the mammary ductal rudiment.

Other than early rudiment formation, most mammary gland development occurs postnatally with the onset of puberty. Thus, we next examined the impact of FOXA1 on postnatal mammary morphogenesis in *Foxa1*$^{-/-}$ mice. These mice exhibit postnatal lethality as a result of severe hypoglycemia and dehydration (Kaestner et al., 1999). Thus, we implemented two well-established rescue strategies to investigate postnatal mammary gland development: orthotopic transplantation and renal capsule grafting (Cunha et al., 2000; Robinson et al., 2000). The orthotopic transplantation paradigm examines whether epithelia are capable of growing and invading the wild-type stroma in response to the pubertal and post-pubertal hormonal milieu of the recipient. Mammary anlagen were collected from E14 *Foxa1*$^{+/+}$ or *Foxa1*$^{-/-}$ embryos and inserted into the cleared fat pads of wild-type syngeneic recipient females (Fig. 2). Transplanted glands were retrieved from recipient mice 5 and 8 weeks later (Fig. 2A,B). In addition, a subset of the 8-week recipients was mated, and transplanted glands were collected from mice with a verified pregnancy (Fig. 2C). At 5 weeks post-transplant, 50% (3/6) of the wild-type anlagen formed mammary ductal outgrowths. This take-rate is consistent with previous reports using this approach (Chakravarty et al., 2003; Robinson et al., 2001). In contrast to the wild-type donor glands, no detectable outgrowths occurred from *Foxa1*$^{-/-}$ anlagen (0/5). At 8 weeks post-transplant, 59% (10/17) of the wild-type vs 0% (0/9) of the *Foxa1*$^{-/-}$ anlagen formed mammary ductal outgrowths. Even when exposed to pregnancy-associated hormones, all of the *Foxa1*$^{-/-}$ mammary anlagen failed (0/9) to develop, whereas 71% (5/7) of the wild-type anlagen formed outgrowths with extensive alveoli. Although FOXA1 is not necessary for formation of the primordial ductal tree at postnatal day 1 (see Fig. S1 in the supplementary material), these results reveal that the presence of FOXA1 in the mammary epithelium is essential for the ductal outgrowth that occurs with puberty.

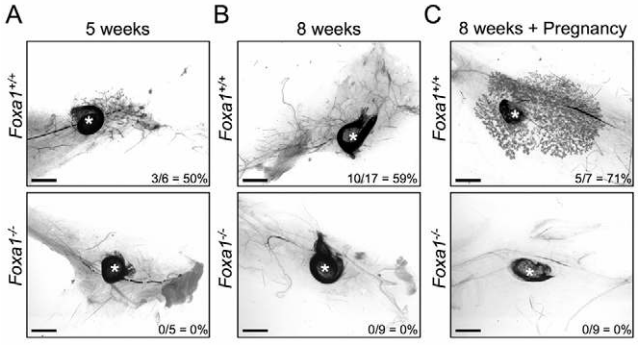

Fig. 2. FOXA1 is required for mammary ductal outgrowth in an orthotopic transplantation model. (**A-C**) Representative whole mounts of ductal outgrowths arising from mammary anlagen collected from E14 *Foxa1*$^{+/+}$ and *Foxa1*$^{-/-}$ mice and transplanted into cleared fat pads of 3- to 4-week-old syngeneic C57BL/6 recipients. (**A**) Recipients aged 5 weeks post-transplant. (**B**) Recipients aged 8 weeks post-transplant. (**C**) Recipients aged 8 weeks post-transplant with subsequent pregnancy (18.5 dpc). Epidermal cysts form as a result of co-transplantation of hair follicles along with the mammary gland. The number and percentage of mammary outgrowths for each donor genotype is indicated. Scale bars: 2 mm. *, epidermal cysts.

The complete absence of ductal outgrowth in transplanted glands from *Foxa1*$^{-/-}$ mice could be a result of either an invasion defect of the developing gland, or a loss of epithelial cells from the transplanted embryonic anlagen. To test this directly, we utilized renal capsule grafting of postnatal day 1 glands (Cunha et al., 2000). This approach maintains the endogenous epithelial-stromal architecture, rather than requiring the formation of a tissue recombinant. In the case of *Foxa1*$^{-/-}$ glands, the intact mammary rudiment/fat pad serves as an ideal source of donor tissue because the glands have a normal rudimentary ductal structure postnatally (see Fig. S1 in the supplementary material). The grafts were harvested 2 weeks after transplantation, and whole-mount analysis revealed numerous TEBs actively invading the fat pad within wild-type glands (Fig. 3A). By contrast, TEBs are not present in *Foxa1* null glands. Rather, the rudimentary ducts have not extended into the fat pad. Hence, loss of *Foxa1* leads to failed development of TEBs and a subsequent inability to invade the mammary fat pad in response to an adult milieu of mammogenic hormones. Whole mount analysis at 4-5 weeks post-transplant demonstrates that even after wild-type glands have completed invasion of the fat pad, *Foxa1*$^{-/-}$ glands remain severely dysmorphic (Fig. 3B).

The TEB contains a high frequency of luminal progenitors (Smalley and Ashworth, 2003), and FOXA1 is expressed in a subset of the body cells in this structure (Fig. 1A). To determine whether *Foxa1* is expressed specifically within luminal progenitors, we compared expression levels between sorted epithelial cell populations enriched for normal mammary stem cell (MaSC), CD61-positive luminal progenitor and mature luminal epithelial populations (Fig. 3C) (Asselin-Labat et al., 2007). When compared with the MaSC-enriched population, *Foxa1* mRNA expression is increased in the luminal progenitor population, and is further

increased in mature luminal cells. These results, in combination with the pattern of expression of FOXA1 in the TEB, suggest that FOXA1 may contribute to specification of the luminal lineage. To test this directly, we analyzed the expression of proteins that distinguish luminal [E-cadherin (CDH1 – Mouse Genome Informatics) and cytokeratin-8 (CK8; KRT8 – Mouse Genome Informatics)] and basal/myoepithelial [α-smooth muscle actin (α-SMA; ACTA2 – Mouse Genome Informatics)] lineages (see Fig. S2 in the supplementary material). Expression and localization of CK8, E-cadherin and α-SMA are unaltered in renal grafts of *Foxa1*$^{-/-}$ glands harvested at 4-5 weeks post-transplantation. Both lineages were also observed in *Foxa1*$^{-/-}$ renal transplanted glands harvested at 2 weeks post-transplant and during pregnancy (data not shown). Hence, FOXA1 is not necessary for lineage specification, but is essential for expansion and invasion of ductal cells.

The blockade of ductal invasion observed in the complete absence of *Foxa1* led us to investigate whether *Foxa1* displays haploinsufficiency. Unlike *Foxa1*$^{-/-}$ mice, *Foxa1*$^{+/-}$ mice are viable, precluding the need for transplantation. Mammary gland development was analyzed at both mid-puberty (5 weeks) and late-puberty (7 weeks), and a significant decrease in ductal invasion was observed at both time points (see Fig. S3A-D in the supplementary material). Ovariectomy and estradiol plus progesterone (E+P) replacement did not rescue this defect (see Fig. S3E,F in the supplementary material), indicating that it is not the result of an ovarian steroid deficiency. *Foxa1*$^{+/-}$ mice are capable of lactating (data not shown); thus loss of a single allele delays, but does not prevent, mammary gland development. Growth inhibition was associated with an increase in epithelial apoptosis without a change in proliferation (see Fig. S3G-J in the supplementary material). These data suggest that the expression level of FOXA1 may be crucial for ductal expansion and invasion as a consequence of its regulation of ductal cell survival.

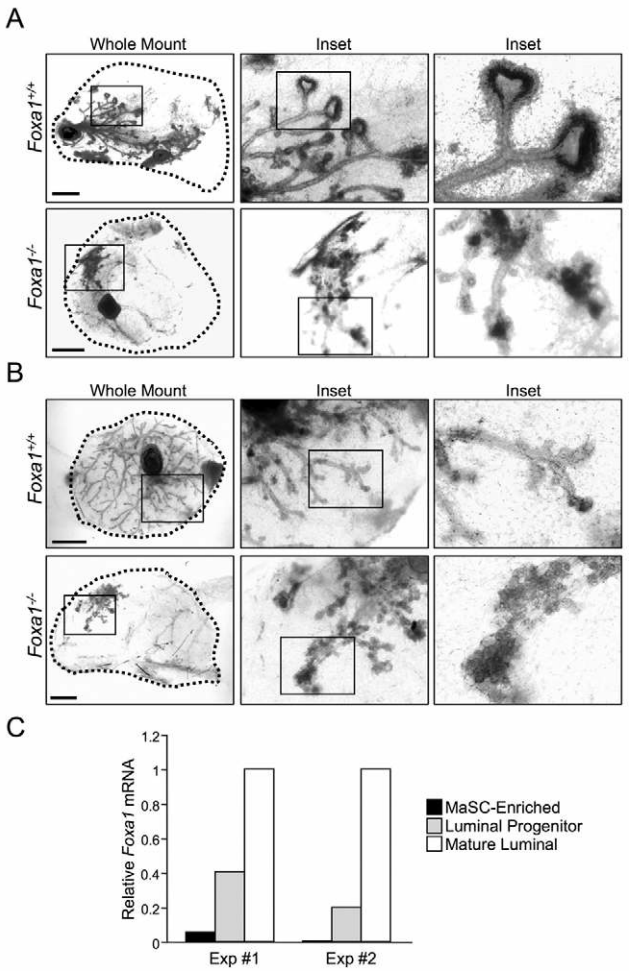

Fig. 3. FOXA1 is required for TEB formation and ductal invasion. (**A**,**B**) Representative whole mounts of renal grafts of *Foxa1*$^{+/+}$ and *Foxa1*$^{-/-}$ mammary glands (into wild-type C57BL/6 recipients) harvested at (A) 2 weeks ($^{+/+}$, *n*=4; $^{-/-}$, *n*=3) and (B) 4-5 weeks post-transplantation ($^{+/+}$, *n*=4; $^{-/-}$, *n*=4). Broken lines outline the mammary fat pad. (**C**) Quantitative real-time PCR of *Foxa1* mRNA levels in the MaSC-enriched population (CD24+/CD29hi), the luminal progenitor population (CD24+/CD29lo/CD61+), and the mature luminal population (CD24+/CD29lo/CD61-) isolated from wild-type FVB/N inguinal mammary glands (*n*=10 per independent experiment). The results of two independent cell-sorting experiments are shown. Values were normalized to *18S* rRNA (Exp#1) or *Gapdh* mRNA (Exp#2) and then expressed relative to the values obtained with the mature luminal population. Scale bars: 1 mm.

Alveologenesis is independent of FOXA1

The mammary luminal lineage terminally differentiates into secretory lobulo-alveolar cells during pregnancy and lactation. To determine whether FOXA1 is necessary for alveolar differentiation, recipients of renal capsule grafts were mated at 4-5 weeks post-transplantation, and transplanted glands were harvested at 18.5 dpc. Alveoli fill the fat pads of wild-type glands, whereas *Foxa1*$^{-/-}$ glands remain severely hampered from invading the surrounding stroma (Fig. 4A). However, histological evaluation of epithelium in *Foxa1*$^{-/-}$ glands revealed the presence of alveoli immediately surrounding the truncated ducts that contained lipid droplets indistinguishable from wild-type controls (Fig. 4A). Expression of milk protein was confirmed in both wild-type and *Foxa1*$^{-/-}$ glands, indicating that the stunted, non-invaded *Foxa1*$^{-/-}$ glands can undergo terminal differentiation (Fig. 4B). Consistent with these data, the E+P-treated *Foxa1*$^{+/-}$ mammary glands have increased alveoli compared with *Foxa1*$^{+/+}$ controls (see Fig. S3E,F in the supplementary material), suggesting that suppression of FOXA1 may promote alveologenesis.

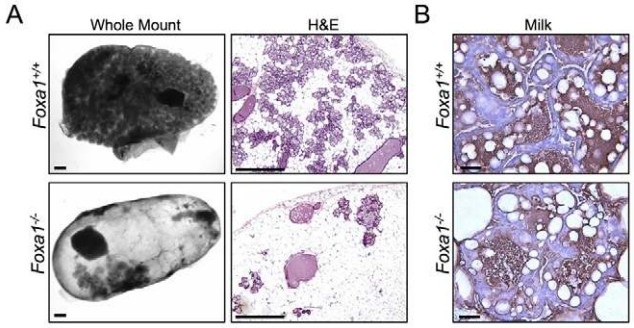

Fig. 4. FOXA1 is not required for alveolar differentiation during pregnancy. (**A**) Representative whole mounts and Hematoxylin and Eosin-stained sections ($^{+/+}$, *n*=5; $^{-/-}$, *n*=3) and (**B**) images of milk protein IHC (brown) ($^{+/+}$, *n*=3; $^{-/-}$, *n*=3) in renal grafts from *Foxa1*$^{+/+}$ and *Foxa1*$^{-/-}$ mammary glands harvested 4-5 weeks after transplantation and during late pregnancy (18.5 dpc). Sections were counterstained with Hematoxylin. Scale bars: 0.5 mm in A; 20 μm in B.

FOXA1 is required for ERα expression in the mammary epithelium

The inability of the *Foxa1*$^{-/-}$ glands to properly invade the mammary fat pad is a phenocopy of the ERα-knockout (αERKO) mouse (Feng et al., 2007; Mallepell et al., 2006; Mueller et al., 2002), and FOXA1 expression colocalizes with ERα in ~30% of luminal cells, suggesting that *Foxa1* may be epistatic with *Esr1* within luminal cells. Analysis of ERα expression in renal transplanted mammary glands revealed that ERα is undetectable within the epithelium of *Foxa1*$^{-/-}$ glands (Fig. 5A). By contrast, ERα is readily detected in the epithelium of wild-type transplanted controls and in the stromal population of both wild-type and *Foxa1*$^{-/-}$ glands. To confirm that ERα activity is lost in *Foxa1*$^{-/-}$ glands, we assessed expression of the progesterone receptor (PR), an established transcriptional target of ERα (Clarke et al., 1997). Similar to ERα, PR expression is undetectable in *Foxa1*$^{-/-}$ epithelium, although maintained in wild-type glands and *Foxa1*$^{-/-}$ stroma (Fig. 5A). PR mRNA (*Pgr*) is similarly decreased (Fig. 5B). These data indicate that FOXA1 is necessary for ERα and PR expression and that this requirement is epithelium-specific. The percentage of epithelial cells expressing ERα and PR was unchanged in pubertal *Foxa1*$^{+/-}$ versus *Foxa1*$^{+/+}$ control glands, (51±6% vs 51±2%, 48±3% vs 47±2%, respectively; *n*=3-4 per group), indicating that retention of one FOXA1 allele is sufficient to maintain the percentage of cells expressing these receptors.

To verify that FOXA1 is upstream of ERα during normal mammary gland development, we analyzed FOXA1 expression in ERα knockout (Ex3αERKO) mice. These mice are devoid of all ERα transcriptional activity as a result of genomic deletion of exon 3, the coding region for the DNA binding domain, in *Esr1*. *Foxa1* mRNA and FOXA1 protein levels are maintained in Ex3αERKO mammary glands compared with wild-type controls (Fig. 5C,D). Combined, these results indicate that FOXA1 functions upstream of, and is necessary for, ERα expression in the normal mammary gland.

It has been proposed that FOXA1, ERα and GATA3 collaborate during mammary morphogenesis (Kouros-Mehr et al., 2006); thus we also evaluated *Gata3* expression in the absence of FOXA1. We found no significant change in *Gata3* mRNA in *Foxa1*$^{-/-}$ glands (Fig. 5B), indicating that FOXA1 is not required for *Gata3* transcription in the mammary gland. In addition, the presence of *Gata3*, but absence of ERα, in *Foxa1*$^{-/-}$ epithelium suggests that, in contrast to breast cancer cells (Eeckhoute et al., 2007), transcription of *Gata3* in normal mammary epithelium may be independent of ERα. This dichotomy was further confirmed by the sustained expression of *Gata3* mRNA in Ex3αERKO mammary glands (Fig. 5E). We also evaluated whether GATA3 regulates expression of FOXA1 using mammary glands deficient for *Gata3* (*MMTV-cre;Gata3*$^{f/f}$) (Asselin-Labat et al., 2007). FOXA1 expressing cells in *MMTV-cre;Gata3*$^{f/f}$ (null) versus *Gata3*$^{+/f}$ (intact) controls were indistinguishable (Fig. 5F).

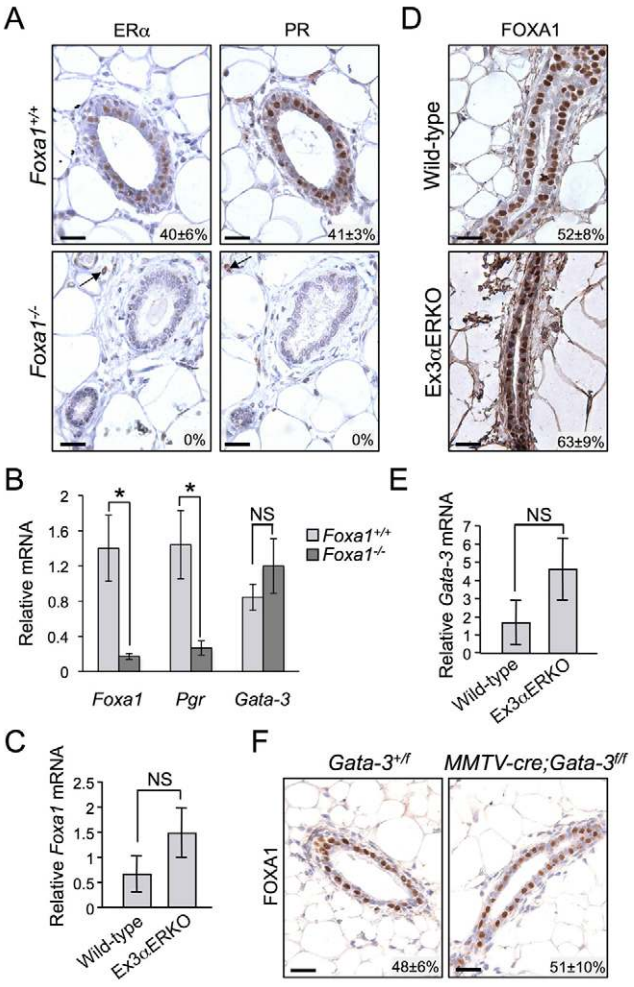

Fig. 5. FOXA1 is required for expression of ERα in the normal mammary gland. (**A**) Representative images of ERα and PR IHC (brown nuclei) in renal grafts from *Foxa1*$^{+/+}$ and *Foxa1*$^{-/-}$ mammary glands harvested 4-5 weeks post-transplantation ($^{+/+}$, *n*=3; $^{-/-}$, *n*=3). ERα and PR are maintained in the stroma of *Foxa1*$^{-/-}$ glands (arrows). (**B**) *Foxa1*, *Pgr* and *Gata3* mRNA levels in renal transplanted *Foxa1*$^{+/+}$ and *Foxa1*$^{-/-}$ mammary glands. Values represent the average ± s.d. and are relative to *Krt8* mRNA ($^{+/+}$, *n*=3; $^{-/-}$, *n*=3; **P*<0.01). (**C**) Quantitation of *Foxa1* mRNA and (**D**) representative images of FOXA1 IHC (brown) in wild-type and Ex3αERKO mammary glands. Values represent the average ± s.d. and are relative to *Krt8* mRNA (wild-type, *n*=3; Ex3αERKO, *n*=3). (**E**) *Gata3* mRNA levels in wild-type and Ex3αERKO mammary glands. Values represent the average ± s.d. and are relative to *Krt8* mRNA (wild-type, *n*=3; Ex3αERKO, *n*=3). (**F**) Representative images of FOXA1 IHC in *Gata3*$^{+/f}$ and *MMTV-cre;Gata3*$^{f/f}$ mammary glands ($^{+/f}$, *n*=3; $^{f/f}$, *n*=3). IHC quantification is depicted in the bottom right corner of each image. All sections were counterstained with Hematoxylin. Scale bars: 20 μm. NS, not significant.

FOXA1 regulates transcription of *ESR1*

Loss of ERα in *Foxa1* null mammary glands could be a result of either a loss of FOXA1/ERα-expressing cells or a specific requirement for FOXA1 to induce expression of ERα. To determine whether FOXA1 regulates expression of ERα, we silenced *FOXA1* expression and assessed the impact on *ESR1* mRNA and ERα protein expression in MCF7 (Fig. 6A-C) and T47D (see Fig. S4 in the supplementary material) breast cancer cell lines, both of which endogenously express FOXA1 and ERα (Williamson et al., 2006). Transient knockdown of *FOXA1* resulted in a significant reduction in ERα protein levels in both cell lines (Fig. 6A,B; see Fig. S4A,B in the supplementary material), recapitulating the loss of ERα in *Foxa1* null mammary glands. *ESR1* mRNA levels were also significantly decreased, suggesting that FOXA1 may regulate its transcription (Fig. 6C; see Fig. S4C in the supplementary material). Importantly, knockdown of *FOXA1* in MCF7 cells did not affect *GATA3* mRNA or GATA3 protein levels (data not shown and Fig. 6A), providing additional evidence that FOXA1 is not required for GATA3 expression.

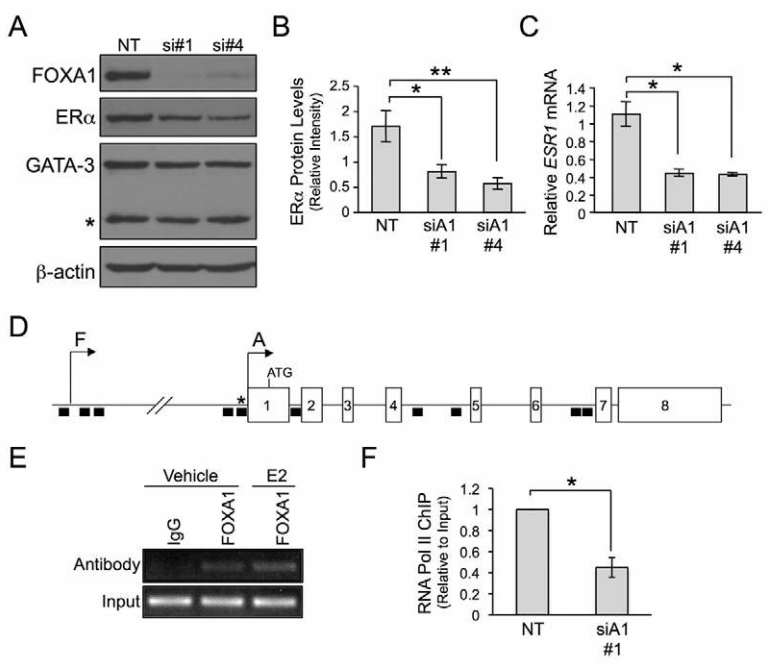

Fig. 6. FOXA1 regulates transcription of *ESR1*. (**A-C**) MCF7 cells were transiently transfected with non-targeting or two different siRNAs targeting *FOXA1* (si#1 and si#4). (A) Representative immunoblots of FOXA1, ERα and GATA3 (l=3) [*, mutant form of GATA3 (Usary et al., 2004)]. (B) Quantitation of ERα protein levels relative to β-actin. Bars represent the mean of three experiments ± s.d. (*P<0.01; **P<0.005). (C) Quantitation of *ESR1* mRNA levels. Bars represent the mean of three experiments ± s.d. relative to *GAPDH* mRNA (*P<0.005). (**D**) *ESR1* is comprised of eight exons and at least seven promoters (only A and F are shown) (Reid et al., 2002). Regions previously identified to bind FOXA1 by ChIP-chip are indicated by black boxes (Lupien et al., 2008). (**E**) Representative (n=3) FOXA1 ChIP of the *ESR1* promoter using primers amplifying a predicted binding site (* in D). MCF7 cells were treated with and without 17β-estradiol (E2). (**F**) MCF7 cells were transiently transfected with NT or *FOXA1* si#1. Quantification of RNA polymerase II ChIP of the *ESR1* promoter (n=3). Bars represent the average fold change relative to input and normalized relative to NT ± s.d. (*P<0.0005). NT, non-targeting siRNA.

To investigate the mechanism underlying regulation of ERα by FOXA1, we queried a publicly available dataset of genome-wide FOXA1 binding sites in MCF7 cells (Lupien et al., 2008). This dataset indicates that FOXA1 binds to ten distinct regions of the *ESR1* gene, with five sites in the promoter and five in intragenic regions (Fig. 6D). We then confirmed FOXA1 binding to one of these predicted regions within the *ESR1* proximal promoter through ChIP followed by site-directed PCR. FOXA1 binds to this region independently of estradiol treatment (Fig. 6E). To ascertain whether FOXA1 regulates transcription of *ESR1*, we examined binding of RNA polymerase II to the *ESR1* proximal promoter following transient knockdown of *FOXA1* (Fig. 6F). Silencing FOXA1 reduces RNA polymerase II binding by ~50%, which is comparable to the reduction in *ESR1* mRNA levels after *FOXA1* knockdown (Fig. 6C). Combined, these data reveal a previously unrecognized requirement for FOXA1 in regulating ERα expression, suggesting that FOXA1 may directly regulate *ESR1*, although these experiments do not rule out an indirect effect of FOXA1 on *ESR1* transcription.

DISCUSSION

FOXA1 is necessary for both ERα expression and functional activity

Previous studies using breast cancer cells revealed that FOXA1 is required for ERα binding to target gene promoters, and subsequent estrogen responsiveness (Carroll et al., 2005; Laganiere et al., 2005). We predicted that FOXA1 might function similarly during mammary morphogenesis. We found that FOXA1 and ERα follow identical expression patterns throughout normal development, and are co-expressed in luminal epithelial cells. Our studies also revealed that FOXA1 is unnecessary for embryonic development of the mammary rudiment, but is required for mammary ductal invasion in three different models: orthotopic and renal capsule transplantation, and *Foxa1* heterozygous null mice. The absence of TEBs in renal transplanted *Foxa1* null glands, along with the presence of *Foxa1* in the luminal progenitor population, indicates that FOXA1 is essential for ductal lineage expansion and morphogenesis (see model, Fig. 7). The loss of epithelial ERα in *Foxa1* null glands provides a specific mechanism for this phenotype because ERα is also essential for TEB development and ductal morphogenesis (Feng et al., 2007; Mallepell et al., 2006; Mueller et al., 2002).

Depletion of epithelial ERα with deficiency of *Foxa1* could result from regulation of ERα expression by FOXA1 or a loss of differentiated cells that can express ERα, as seen in GATA3-depleted mammary glands (Asselin-Labat et al., 2007; Kouros-Mehr et al., 2006). Complementing our observations in the developing mammary gland, transient suppression of FOXA1 results in decreased transcription of *ESR1* and protein expression of ERα in breast cancer cells. Hence, FOXA1 not only mediates ERα activity as has been described (Carroll et al., 2005; Laganiere et al., 2005), but is also essential for sustained ERα expression. These data reveal that FOXA1 tightly regulates ERα activity through two distinct mechanisms, i.e. basal expression and functional activity.

Previous reports examining a role for FOXA1 in mediating ERα binding to target gene promoters did not observe a decrease in ERα expression upon transient knockdown of *FOXA1* (Carroll et al., 2005; Eeckhoute et al., 2006; Laganiere et al., 2005). The disparity between these results might be explained by variation in experimental conditions. For the studies reported herein, changes in ERα in response to transient knockdown of *FOXA1* were observed using media containing hormone-replete serum. By contrast, previous studies in which sustained ERα occurred following *FOXA1* silencing were performed under hormone deprivation. The presence of estradiol substantially decreases the stability of *ESR1* mRNA and protein (Reid et al., 2002). Thus, the experimental paradigm used herein likely maintains a higher turnover rate of *ESR1* mRNA and ERα protein, and thus, is permissive to detecting changes in expression as a result of *FOXA1* silencing.

Expression of *Gata3* is independent of FOXA1 and ERα

FOXA1, ERα and GATA3 are positively correlated in breast cancer, and ERα appears necessary for *GATA3* expression in breast cancer cell lines (Eeckhoute et al., 2007). However, *Gata3*

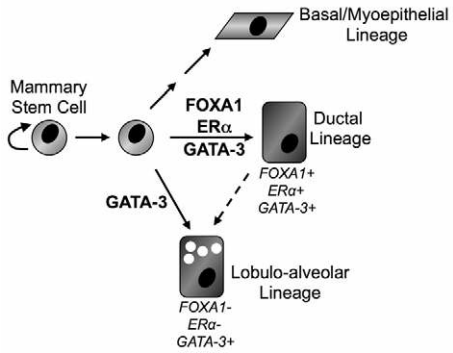

Fig. 7. Schematic of the mammary epithelial cell hierarchy. FOXA1 is expressed in and required for ductal development. GATA3 is expressed in and required for both ductal and alveolar development and is independent of FOXA1 expression. ERα is required for ductal and alveolar development, but is only expressed in ductal cells. This supports intercellular communication and/or lineage progression from ERα-positive ductal to ERα-negative alveolar cells (broken arrow).

expression is sustained in *Foxa1* null mammary glands that also lack detectable ERα. In addition, *Gata3* mRNA is maintained in mammary glands that lack functional ERα, providing further evidence that ERα is not necessary for *Gata3* expression in normal mammary epithelium. These data reveal that expression of *Gata3* occurs independently of FOXA1 and ERα during lineage specification. We confirmed these data by silencing *FOXA1* in vitro and found that GATA3 remains constant even with a reduction in ERα. These results contrast with previous analyses of breast cancer cell lines (Eeckhoute et al., 2007). To reconcile these data, we propose that although ERα may not be required for *Gata3* expression under normal conditions, it may become necessary during tumorigenesis. It is also important to note that the *FOXA1* knockdown in breast cancer cells presented herein resulted in only a 50% reduction in ERα expression, which may be sufficient to sustain GATA3.

It has also been suggested that GATA3 regulates ERα expression in breast cancer cell lines (Eeckhoute et al., 2007). Although our results do not refute this conclusion, they do indicate that GATA3 alone is insufficient to maintain ERα in the absence of FOXA1. This conclusion stems from the loss of ERα, but not GATA3, that occurs both in *Foxa1*-null glands and with transient silencing in breast cancer cells. Lastly, GATA3 was previously reported to bind to the *Foxa1* promoter in primary mammary cells (Kouros-Mehr et al., 2006) and induce expression of FOXA1 in mammary tumors (Kouros-Mehr et al., 2008) and a kidney cell line (Usary et al., 2004). We found that FOXA1 expression is maintained in glands deficient for *Gata3*, indicating that GATA3 is not necessary for *Foxa1* expression during normal development.

Development of the mammary ductal, but not alveolar lineage is dependent on FOXA1

Both orthotopic and renal transplantation models used herein revealed that *Foxa1*-null glands were unable to invade the mammary fat pad in response to pregnancy-associated hormones. However, the rudimentary ductal epithelium that was grafted into the renal capsule developed differentiated alveoli in response to pregnancy. Although these alveoli arose from a rudimentary duct and were substantially fewer in number, they were otherwise indistinguishable from wild-type glands. These data reveal that *Foxa1* is unnecessary for lobulo-alveolar lineage specification (see model, Fig. 7) and provide additional evidence that ductal expansion and alveolar lineage specification are independent processes. A similar phenotype has been observed in murine mammary glands lacking amphiregulin (Ciarloni et al., 2007), ERBB3 (Jackson-Fisher et al., 2008) and FGFR2b (Parsa et al., 2008), or in glands exposed to exogenous TGFβ1 (Daniel et al., 1989; Silberstein and Daniel, 1987). Interestingly, TEB development is also disrupted in all of these models. Thus, it is possible that FOXA1 participates in a signaling network that includes one or more of these mediators of breast development and cancer progression (Holbro et al., 2003; McBryan et al., 2008; Wakefield et al., 2001).

Previous studies have shown that ERα and PR are independently required for alveologenesis (Brisken et al., 1998; Feng et al., 2007; Mallepell et al., 2006). Thus, the loss of ERα and PR in *Foxa1*-null glands along with the sustained ability to form alveoli was unanticipated. A trivial explanation for these data is that although we cannot detect ERα and PR by IHC, low levels still occur and are sufficiently functional. Supporting this notion, *Pgr* mRNA is still present, albeit only at ~10% of normal levels. Like FOXA1, ERα is not expressed in lobulo-alveoli. Thus it is not clear whether ERα acts in a cell-autonomous manner to regulate alveologenesis, or if intercellular communication or lineage progression involving ERα silencing is involved (broken arrow in Fig. 7). It is also possible that FOXA1 maintains the ductal epithelium in an undifferentiated state, thus inhibiting alveologenesis. The loss of FOXA1 could then induce alveolar differentiation in response to pregnancy-associated hormones even in the absence of ERα. This hypothesis is supported by the enhanced alveologenesis observed in *Foxa1* heterozygotes when treated with pregnancy-level hormones. Notably, both orthotopic and renal capsule transplantation models preclude investigating lactational differentiation in detail because the transplanted glands undergo involution post-partum due to the lack of suckling (Li et al., 1997). Hence, conditional knockout of *Foxa1* is necessary to directly examine the function of FOXA1 in lactation and involution, and these studies are currently underway.

Conclusions and implications

FOXA1 is essential for development and specification of cell fate in the prostate, liver, kidney, pancreas and lung (Behr et al., 2004; Besnard et al., 2005; Gao et al., 2005; Kaestner et al., 1999; Shih et al., 1999). We now describe an indispensable role for FOXA1 in mammary ductal morphogenesis (Fig. 7). Our studies also reveal that FOXA1 is necessary for expression of ERα in the normal mammary epithelium, and modulates transcription of *ESR1* in vitro. Approximately 75% of breast cancers are ERα-positive, hence these findings have implications in hormone receptor-positive disease because *FOXA1* expression occurs in most, if not all ERα-positive breast cancers. It is likely that the positive correlation seen between FOXA1 and the differentiated luminal breast tumor subtype stems from this previously undefined role of FOXA1 in regulating the differentiation of the mammary ductal lineage and controlling *ESR1* transcription. We also suggest that FOXA1 may also modulate other well-known pathways of tumorigenesis (e.g. amphiregulin-EGFR, heregulin-ERBB3, TGFβ1) providing a possible explanation and function for FOXA1 in breast tumors lacking ERα.

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Competing interests statement
The authors declare no competing financial interests.

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