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; Lagniere 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...
that requires ER\text{\alpha} and GATA3. ER\text{\alpha} 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 ER\text{\alpha} 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 excess 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 ER\text{\alpha}, GATA3 and FOXA1, loss of Gata\text{\alpha} in the normal mammary gland decreases the ER\text{\alpha}-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 ER\text{\alpha} 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 ER\text{\alpha}, 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 ER\text{\alpha} 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; ER\text{\alpha}, 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, ER\text{\alpha}, 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 ER\text{\alpha} 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 Ex3aERKO and MMTV-cre;Gata3\text{\alpha} mice, were approved by the Case Western Reserve University IACUC. Ex3aERKO 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)Amc/J; Jackson Labs]. DNA was evaluated by PCR using P1 and P3 primers as described (Dupont et al., 2000). The MMTV-cre;Gata3\text{\alpha} alleles were generated at the Walter and Eliza Hall Institute of Medical Research as described (Asselin-Labat et al., 2007). Foxa1\text{\alpha} males (Kaestner et al., 1999) were bred with wild-type C57BL/6 females generating Foxa1\text{\alpha} progeny that were intercrossed to generate Foxa1\text{\alpha} 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% CO2 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\text{\alpha} anlage was inserted into the cleared fat pad, and a Foxa1\text{\alpha} anlage was inserted into the contralateral cleared fat pad of the same mouse. The incision was sutured and infiltrated with marcame (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. The inguinal fat pad from a Foxa1\text{\alpha} 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\text{\alpha} pup on to the contralateral kidney. The incisions were closed using wound clips, and the wound infiltrated with marcame (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 alcian, 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-TTGTAGTGAAGATC-3' and 5'-TTTACTTGTCGCTTTA; 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-Streptomycin (Invitrogen). Cells were seeded in 100 mm dishes to be 30-50% confluent upon transfection. siRNA targeting firefly luciferase mRNA (siCONTROL Nontargeting 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 20 nM. 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 nontargeting 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'-AGGAGGGGGAATCAAACAGA-3' and 5'-TTTACCCTTCTGCTGCTGCTG-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...
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 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).

**FOX1A is required for mammary ductal outgrowth in an orthotopic transplantation model.**

(A-C) Representative whole mounts of ductal outgrowths arising from mammary anlage 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.
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, mammmary 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.

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. 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.

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 (ERKO) mouse (Feng et al., 2007; Mallepalli et al., 2006; Mueller et al., 2002), and FOXA1 expression colocalizes with ERα within mammary fat pad tissues (assayed by immunohistochemistry). 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 glands 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 (Koulos-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 expression 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−/−) (Asselin-Labat et al., 2007). FOXA1 expressing cells in MMTV-cre;Gata3−/− (null) versus Gata3+/+ (intact) controls were indistinguishable (Fig. 5F).

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.
FOXA1 regulates mammary morphogenesis

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; Kourosh-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; Eckhoute 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 (Eckhoute et al., 2007). However, Gata3
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 (Kourous-Mehr et al., 2006) and induce expression of Foxa1 in mammary tumors (Kourous-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; Mc Bryan 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-alveolar. 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.
FOXA1 regulates mammary morphogenesis

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Competing interests statement

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