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Foxa1 is Essential for Mammary Duct Formation

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Abstract

Introduction—The transcription factor forkhead box protein A1 (FOXA1) plays a critical role in the proliferation of human breast cancer cells, particularly estrogen receptor alpha (ERα)-positive luminal breast cancer cells. However, genetic studies of the requirement for Foxa1 in mammary tumor formation in mice have been hampered by the lack of a conditional gene ablation.

Methods—We examined three mouse models of mammary-specific ablation of Foxa1 in ductal epithelial cells to identify the best system for complete and mammary-specific ablation of Foxa1.

Results—We found that *MMTV-Cre* and *MMTV-rtTA;Tet-On-Cre* led to partial deletion of Foxa1 and attenuated mammary duct formation, whereas *Krt14-Cre* led to complete ablation of Foxa1 and abolished mammary duct formation, in *Foxa1^{loxP/loxP}* mice.

Conclusion—These results demonstrate that Foxa1 is essential for mammary duct formation, and reveal a series of mouse models in which mammary expression of Foxa1 can be attenuated or completely blocked. Our study also suggests a potentially powerful model for complete ablation of Foxa1 in mammary epithelial cells using Krt14-driven Cre expression in an inducible manner, such as *Krt14-rtTA;Tet-On-Cre*. This model system will facilitate further *in vivo* functional studies of Foxa1 or other factors in mammary gland development and tumor formation and progression.

Keywords

mammary gland; transgenic mice; MMTV; Krt14

INTRODUCTION

Breast cancer is the most common cancer and the second leading cause of cancer death in women all over the world (Torre et al., 2015). Breast cancer originates from the mammary gland epithelial cells and can be classified into several distinct transcriptional subtypes, of which the most common is the luminal subtype (Eroles et al., 2012). Luminal breast cancer cells are given rise from the amalgamation of epigenetic and genetic changes on luminal epithelial cells. It has been shown that the expression of estrogen receptor alpha (ERα) and

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forkhead box protein A1 (FOXA1) are strongly associated with luminal subtype of breast cancer, and that FOXA1-dependent ER α -mediated estrogen signaling promotes the growth of ER α -positive breast cancer cells (Badve et al., 2007; Carroll et al., 2005; Habashy et al., 2008; Hu et al., 2014; Laganieri et al., 2005). FOXA1, previously known as hepatocyte nuclear factor 3 α (HNF3 α), belongs to a large family of transcription factors, characterized by a common 'winged helix' DNA binding domain. In the past three decades, many studies have shown that Foxa factors were shown to control gene regulation during organogenesis, such as liver (Kaestner, 2010; Lee et al., 2005; Li et al., 2009), pancreas (Gao et al., 2008b; Monaghan et al., 1993), lung (Besnard et al., 2005; Kaestner et al., 1999b; Shih et al., 1999), prostate (Besnard et al., 2004; Mirosevich et al., 2005), kidney (Behr et al., 2004), renal pelvis, ureters, bladder, and testis (Peterson et al., 1997). High expression of FOXA1 has been found in human luminal subtype of breast cancer cell lines. Mammary gland ductal development depends upon growth and morphogenesis of luminal mammary epithelial cells (Asselin-Labat et al., 2007; Ewald et al., 2008), which occur in a hormone-dependent fashion, through the interaction of ER α and FOXA1 (Briskin and O'Malley, 2010). The regulatory network of ER α and FOXA1 in human breast cancer cells has been functionally evaluated using suppression of FOXA1 but with two different sets of results (Bernardo et al., 2013; Hurtado et al., 2011). Hurtado et al showed that suppression FOXA1 in human breast cancer cell lines, MCF7 and T47D, led to the inhibition of cell growth (Hurtado et al., 2011), whereas Bernardo et al found that suppression of FOXA1 in MCF7 and T47D cells not only inhibited their growth but also converted these cancer cells from the luminal to the basal subtypes (Bernardo et al., 2013). However, a defined role of Foxa1 in the early stage of mammary tumorigenesis has never been investigated, due to in part the fact that *Foxa1*^{-/-} mice die within two weeks after birth (Kaestner et al., 1999a). Furthermore, an *ex vivo* culture of explanted *Foxa1*^{-/-} mammary gland tissue has shown that Foxa1 is required for mammary duct morphogenesis (Bernardo et al., 2010). Thus, to better understand the role of Foxa1 in mammary tumorigenesis *in vivo*, a mouse model of mammary-specific ablation of Foxa1 is needed.

In the past several decades, genetically engineered mouse models have provided many powerful tools for improving our understanding in breast cancer biology, including initiation, progression, and metastasis. Conditional gene ablation in the mammary gland has frequently employed the mouse mammary tumor virus (MMTV) long terminal repeat (LTR) promoter-driven Cre recombinase (*MMTV-Cre*) (Wagner et al., 1997). Additionally, inducible MMTV-driven Cre expression, using the *MMTV-rtTA;Tet-On-Cre* transgene, has also been employed for mammary gland-specific manipulation of targeted genes to achieve tissue-specific gene expression or to avoid perinatal lethality (Gunther et al., 2002). Moreover, the whey acidic protein (WAP) gene promoter-driven Cre recombinase (*WAP-Cre*) was developed to modify mammary gland-specific gene expression, but the use of this model has been limited by the fact that *WAP-Cre* expression was only detected in alveolar epithelial cells of mammary tissue during lactation (Wagner et al., 1997). Cytokeratin proteins are markers of many epithelial cells, including mammary epithelial cells; *Krt5-Cre* and *Krt8-Cre* models have been used for tracing embryonic development of mammary glands (Van Keymeulen et al., 2011), and *Krt14-Cre* has shown to drive Cre expression in many epithelial cells, including mammary epithelia (Ahn et al., 2013; Bowman-Colin et al.,

2013; Caffarel et al., 2012; Cang et al., 2007; Chakrabarti et al., 2012a; Chakrabarti et al., 2012b; Choi et al., 2009; Dassule et al., 2000; Gritli-Linde et al., 2007; Lindley et al., 2015; Mitchell and Serra, 2014).

Here, we compare gene ablation efficacy and the consequences on mammary duct formation in three mouse models with mammary gland-specific ablation of *Foxa1*, *Foxa1^{loxP/loxP};MMTV-Cre*, *Foxa1^{loxP/loxP};MMTV-rtTA;Tet-On-Cre*, and *Foxa1^{loxP/loxP};Krt14-Cre*. We found that the novel *Krt14-Cre* system resulted in complete ablation of *Foxa1* in mammary glands and concomitant abrogation of mammary duct formation, whereas the MMTV-driven Cre models only showed partial ablation of the loxP flanked *Foxa1* gene with more limited effects on mammary gland development.

RESULTS

Incomplete suppression of *Foxa1* in mammary glands leads to partial defects in mammary ductal formation

To achieve mammary-specific ablation of *Foxa1*, we mated the *Foxa1^{loxP/loxP}* mice with *MMTV-Cre* mice. H&E staining showed that mammary glands in *Foxa1^{loxP/loxP};MMTV-Cre* mice had fewer mammary ducts compared to those in wild-type (WT) control *Foxa1^{loxP/loxP}* mice (Fig. 1). Further staining for a ductal epithelial cell marker, cytokeratin 19 (CK19), confirmed that mammary glands in *Foxa1^{loxP/loxP};MMTV-Cre* mice indeed had significantly fewer ductal epithelial cells than the control mice (Fig. 1 and 2). When we examined *Foxa1* expression in mammary glands by immunohistochemical staining, we found that *Foxa1*-positive staining was only found in ductal epithelial cells; however, MMTV-Cre only led to partial deletion of *Foxa1* in *Foxa1^{loxP/loxP};MMTV-Cre* mice compared to controls ($18.10 \pm 3.69\%$ vs $28.07 \pm 4.18\%$ positivity in Fig. 1 and 2). This was accompanied by partial reduction of ER α expression in *Foxa1^{loxP/loxP};MMTV-Cre* mice ($11.32 \pm 8.45\%$ vs $22.06 \pm 2.51\%$ positivity in Fig. 1 and 2). Further whole-mount staining of mammary glands showed that partial ablation of *Foxa1* in *Foxa1^{loxP/loxP};MMTV-Cre* mice also led to reduced branching of ductal trees and terminal end bud formation compared to the control WT mice (Fig. 3).

As the partial ablation of *Foxa1* disturbed the development of mammary ducts, we also examined an inducible model of mammary-specific ablation of *Foxa1* using *Foxa1^{loxP/loxP};MMTV-rtTA;Tet-On-Cre* mice with treatment of 2 $\mu\text{g/ml}$ doxycycline for 6 weeks. These mice only showed minimal ablation of *Foxa1* and the frequency of CK19-positive and ER α -positive epithelial cells was not changed significantly ($26.46 \pm 2.75\%$ *Foxa1* and $20.32 \pm 2.80\%$ ER α positivity in Fig. 1 and 2). Interestingly, while the structures of ductal trees in these mice appeared normal histologically, the ductal branching and terminal end buds were slightly reduced in the mammary glands of the inducible transgenic model compared to the control WT mice (Fig. 3). Thus, these results suggest that MMTV-driven Cre recombinase may be a good model system for the genetic analysis of partial deletion of *Foxa1* in mammary development and tumorigenesis.

Foxa1 is essential for ductal epithelium formation

To achieve complete ablation of Foxa1 in mammary epithelial cells, we mated the *Foxa1^{loxP/loxP}* mice with *Krt14-Cre* mice. H&E staining showed that mammary fat pads in *Foxa1^{loxP/loxP};Krt14-Cre* mice were formed normally, but lacked mammary ducts, which was a striking difference when compared with the well developed mammary ducts and alveoli in wild-type (WT) control *Foxa1^{loxP/loxP}* mice (Fig. 1). This observation was confirmed by CK19 staining (Fig. 1) that no mammary epithelial cells were present in *Foxa1^{loxP/loxP};Krt14-Cre* mice (Fig. 1 and 2). In addition, Foxa1 and ER α expression was absent from the mammary glands of *Foxa1^{loxP/loxP};Krt14-Cre* mice as expected if ductal epithelial cells are lacking completely (Fig. 1 and 2). Whole-mount staining of mammary glands confirmed complete ablation of Foxa1 in *Foxa1^{loxP/loxP};Krt14-Cre* mice, causing complete depletion of ductal tree formation (Fig. 3). Thus, Foxa1 is required and essential for mammary duct formation, and the *Foxa1^{loxP/loxP};Krt14-Cre* mouse model will be a useful tool to study Foxa1 functions in mammary tumorigenesis *in vivo* in the future.

Coordinative regulation of Foxa1 and ER α in mammary duct formation

Partial or complete ablation of Foxa1 in mammary glands in three mouse models produced corresponding reductions in ER α expression (Fig. 1 and 2), with high correlation ($r = 0.91$) (Fig. 4A). Additionally, the expression of these two factors, Foxa1 and ER α , was also highly correlated with CK19 expression of the ductal epithelial cell marker, $r = 0.73$ for Foxa1 and CK19, and $r = 0.76$ for ER α and CK19, respectively (Fig. 4B and 4C). Moreover, principal component analysis showed that Foxa1, ER α , and CK19 expression was clustered closely in mice with four groups of different genotypes, while the variance within each group was minimal (Fig. 4D), indicating that expression of these three genes is highly correlated. These results suggest that Foxa1 and ER α are required for coordinative regulation in mammary duct formation.

DISCUSSION

Our study evaluated the roles of Foxa1 in mammary ductal branching and morphogenesis and provides the first fully *in vivo* demonstration that Foxa1 is essential for mammary ductal formation. A hallmark for luminal breast cancer is that FOXA1 is required for the growth of human ER α -positive breast cancer cells (Carroll et al., 2005; Laganier et al., 2005). The fact that Foxa1-dependent and ER α -mediated estrogen signaling is required for mammary ductal development and progression of luminal breast cancer suggests that luminal breast cancer cells are still in a mammary lineage. Indeed, anti-estrogen therapy, such as tamoxifen, has been used to reverse the growth of luminal breast cancer cells. However, lack expression of both FOXA1 and ER α is a molecular signature for basal subtype of breast cancer. Thus, our study on the requirement of Foxa1 for the formation of mammary ductal epithelial cells indicates that suppression of FOXA1 and ER α in basal subtype of breast cancer might lead to cell differentiation away from the mammary lineage. This suggests that treatment of basal subtype of breast cancer should not target estrogen signaling.

Our observations of the highly correlated expression of Foxa1, ER α , and CK19 in mammary epithelial cells of the different models (Fig. 4) suggests the coordinative regulation of Foxa1

and ER α is essential for the development of mammary epithelial cells and ductal trees. Paralleled changes of ER α following partial and complete ablation of Foxa1 in mammary ductal epithelia suggest that Foxa1 is also required for the maintenance of ER α signature in mammary ducts (Figure 1, 2, and 4). Furthermore, we found that these gene expression changes were consistent to the changes in the ability of ductal tree formation in the mammary fat pad (Figure 1–4). However, genetic deficiency of *FOXA1* has been barely seen in humans, thus, the investigation of *Foxa1* functions in mouse mammary duct formation and tumorigenesis remains invaluable to provide key insights into the mechanisms of FOXA1 regulation in human breast cancer development.

Moreover, our study provides a model of duct-less mammary glands in *Foxa1^{loxP/loxP};Krt14-Cre* mice, suggesting that mammary fat pad formation is independent of Foxa1, as only the ductal epithelia were affected. *Foxa1^{loxP/loxP};Krt14-Cre* mice live normally without mammary ducts, suggesting that removing or terminating the formation of mammary ducts in females at high risks of breast cancer could be a potential therapy.

In conclusion, our study shows that Foxa1 is essential for mammary duct formation *in vivo*. Although Krt14-Cre abrogated the mammary duct formation in *Foxa1^{loxP/loxP}* mice, it also completely deleted Foxa1 in mammary epithelial cells. Thus, ablation of Foxa1 in a matured and well developed mammary glands using Krt14-driven inducible Cre expression, *Foxa1^{loxP/loxP};Krt14-rtTA;Tet-On-Cre*, would be promising for the future investigation of Foxa1 in mammary tumorigenesis *in vivo*, which will also benefit other genetic studies of mammary development and tumorigenesis.

METHODS

Animals

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the Mayo Clinic. The experiment was carried out under controlled conditions with a 12-h light/dark cycle. Cages with filters were used along with sterile bedding, ad libitum diet, and water. Animals were maintained on a normal chow. The derivation of *Foxa1^{loxP/loxP}* mice (from Dr. Klaus H. Kaestner, University of Pennsylvania) has been reported previously (Gao et al., 2008a). *Foxa1^{loxP/loxP}* mice were mated with *MMTV-Cre* (from Jackson Laboratory), *MMTV-rtTA;Tet-On-Cre* (from Dr. Lewis A. Chodosh, University of Pennsylvania) (Gunther et al., 2002), or *Krt14-Cre* (from Jackson Laboratory) mice to obtain *Foxa1^{loxP/loxP};MMTV-Cre*, *Foxa1^{loxP/loxP};MMTV-rtTA;Tet-On-Cre*, or *Foxa1^{loxP/loxP};Krt14-Cre* mice. *Foxa1^{loxP/loxP}* mice were used as control wild-type (WT) mice. Genotypes of *Foxa1^{loxP/loxP}* and Cre were determined by PCR. 2 μ g/ml doxycycline in drinking water was given to *Foxa1^{loxP/loxP};MMTV-rtTA;Tet-On-Cre* mice from age of 6 weeks old to 12 weeks old to induce Cre expression. Mammary glands of all female mice (3 to 10 mice per group) were collected at 12 weeks old for the following analysis.

Whole-mount staining

Dissected mammary glands were placed on slides and dried for at least 30 minutes, and then fixed in Carnoy's solution (60% ethanol, 30% chloroform, and 10% acetic acid) for over 4

hours and stored in 70% ethanol. Samples were then gradually rehydrated to 100% water, stained with carmin alum, dehydrated and cleared with xylene. The images of all slides were captured using AmScope microscope (Irvine, CA).

Immunohistochemical Staining

Excised mammary gland tissues were fixed by immersion in 10% buffered formalin overnight and then transferred to 70% ethanol for long-term fixation. Representative sections of fixed tissue were trimmed and embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E), cytokeratin 19 (CK19) antibody (clone, TROMA-3), Foxa1 antibody (Santa Cruz, Dallas, TX), or ER α antibody (Santa Cruz) for histological examination. The TROMA-3 antibody was purchased from The University of Iowa, Iowa City, IA. All of the stained sections were imaged using Aperio ScanScope XT (Vista, CA, USA) and analyzed using Aperio ImageScope (v11.1.2.752) to determine the total positivity.

Statistical analysis

Student's *t* test was applied to compare two groups. Data are expressed as means $\pm$ STD. A probability (*p*) value of 0.05 or less was considered to be the criterion for a significant difference by asterisks: *, *p* 0.01; **, *p* 0.001. Principal component analysis (PCA) (Pearson, 1901) was used to analyze the correlations among Foxa1, ER α , and CK19.

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Fig. 1.

Foxa1 is required for the development of mammary ductal epithelial cells. Structures of mammary glands by H&E staining and expression of cytokeratin 19 (CK19), Foxa1, and ER α in the entire mammary glands from control wild-type (WT), *Foxa1*^{loxP/loxP}; *MMTV-Cre* (*MMTV-Cre*), *Foxa1*^{loxP/loxP}; *MMTV-rtTA*; *Tet-On-Cre* (*MMTV-rtTA*; *Tet-On-Cre*), and *Foxa1*^{loxP/loxP}; *Krt14-Cre* (*Krt14-Cre*) mice by immunohistochemical staining.

Fig. 2.

Foxa1 is required for mammary ductal branching and formation. (A) Whole-mount staining of mammary glands from control wild-type (WT), *Foxa1*^{loxP/loxP};MMTV-Cre (MMTV-Cre), *Foxa1*^{loxP/loxP};MMTV-rtTA;Tet-On-Cre (MMTV-rtTA;Tet-On-Cre), and *Foxa1*^{loxP/loxP};Krt14-Cre (Krt14-Cre) mice. (B) Quantitative analysis of mammary ductal branching and terminal end bud (TEB) formation measured by the number of branch points and TEB per mm from control wild-type (WT, n=10), *Foxa1*^{loxP/loxP};MMTV-Cre (MMTV-Cre, n=3), *Foxa1*^{loxP/loxP};MMTV-rtTA;Tet-On-Cre (MMTV-rtTA;Tet-On-Cre, n=5), and

Foxa1^{loxP/loxP};Krt14-Cre (*Krt14-Cre*, n=3) mice. Values are expressed as means $\pm$ STD, * p <0.01, ** p <0.001.

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Fig. 3. Quantitative analysis of CK19, Foxa1, and ERα expression in ductal epithelial cells. (A) Expression of CK19, Foxa1, and ERα in the ductal epithelial cells of mammary glands from control wild-type (WT), *Foxa1^{loxP/loxP};MMTV-Cre* (MMTV-Cre), *Foxa1^{loxP/loxP};MMTV-rtTA;Tet-On-Cre* (MMTV-rtTA;Tet-On-Cre), and *Foxa1^{loxP/loxP};Krt14-Cre* (Krt14-Cre) mice by immunohistochemical staining. (B) The total positivity of CK19, Foxa1, and ERα expression in ductal epithelial cells in the entire mammary glands from control wild-type (WT, n=10), *Foxa1^{loxP/loxP};MMTV-Cre* (MMTV-Cre, n=3), *Foxa1^{loxP/loxP};MMTV-rtTA;Tet-*

On-Cre (*MMTV-rtTA*; *Tet-On-Cre*, n=5), and *Foxa1*^{*loxP/loxP*}; *Krt14-Cre* (*Krt14-Cre*, n=3) mice. Values are expressed as means $\pm$ STD, * $p < 0.01$, ** $p < 0.001$.

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Fig. 4.

Correlation analysis of CK19, Foxa1, and ERα expression in mammary ductal formation. (A–C) correlation analysis of Foxa1 and ERα expression (A), Foxa1 and CK19 expression (B), and ERα and CK19 expression (C) from the positivity data (Fig. 2B) in the ductal epithelial cells of mammary glands in control wild-type (WT, n=10), *Foxa1^{loxP/loxP};MMTV-Cre* (MMTV-Cre, n=3), *Foxa1^{loxP/loxP};MMTV-rtTA;Tet-On-Cre* (MMTV-rtTA;Tet-On-Cre, n=5), and *Foxa1^{loxP/loxP};Krt14-Cre* (Krt14-Cre, n=3) mice. D, The principal component

analysis (PCA) of CK19, Foxa1, and ER α expression in mammary ductal formation of total 21 WT, *MMTV-Cre*, *MMTV-rtTA;Tet-On-Cre*, and *Krt14-Cre* mice.

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