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The estrogen-responsive Agr2 gene regulates mammary epithelial proliferation and facilitates lobuloalveolar development

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ABSTRACT

Agr2 is a putative protein disulfide isomerase (PDI) initially identified as an estrogen-responsive gene in breast cancer cell lines. While Agr2 expression in breast cancer is positively correlated with estrogen receptor (ER) expression, it is upregulated in both hormone dependent and independent carcinomas. Several *in vitro* and xenograft studies have implicated Agr2 in different oncogenic features of breast cancer; however, the physiological role of Agr2 in normal mammary gland development remains to be defined. *Agr2* expression is developmentally regulated in the mammary gland, with maximum expression during late pregnancy and lactation. Using a mammary gland specific knockout mouse model, we show that Agr2 facilitates normal lobuloalveolar development by regulating mammary epithelial cell proliferation; we found no effects on apoptosis in *Agr2*$^{-/-}$ mammary epithelial cells. Consequently, mammary glands of *Agr2*$^{-/-}$ females exhibit reduced expression of milk proteins, and by two weeks post-partum their pups are smaller in size. Utilizing a conditional mouse model, we show that *Agr2* constitutive expression drives precocious lobuloalveolar development and increased milk protein expression in the virgin mammary gland. *In vitro* studies using knock down and overexpression strategies in estrogen receptor positive and negative mammary epithelial cell lines demonstrate a role for Agr2 in estradiol-induced cell proliferation. In conclusion, the estrogen-responsive Agr2, a candidate breast cancer oncogene, regulates epithelial cell proliferation and lobuloalveolar development in the mammary gland. The pro-proliferative effects of Agr2 may explain its actions in early tumorigenesis.

Introduction

Agr2 is the mammalian homolog of the previously identified *Xenopus laevis* cement gland protein XAG-2, which promotes cement gland differentiation and ectodermal patterning (Aberger et al., 1998). It is a secreted protein and, based on homology, a putative member of the protein disulfide isomerase family; it was recently shown to bind to nascent proteins and direct them to the endoplasmic reticulum (Higa et al., 2011; Park et al., 2009; Persson et al., 2005; Zhao et al., 2010). The mammalian *Agr2* was initially identified in a screen for estrogen-responsive genes in breast cancer cell lines (Fletcher et al., 2003; Thompson and Weigel, 1998). Correspondingly, it is expressed at a higher level in estrogen receptor (ER) positive breast cancers, and within ER positive breast cancers *Agr2* expression correlates with poor prognosis (Barraclough et al., 2009; Fletcher et al., 2003; Innes et al., 2006; Thompson and Weigel, 1998). However, in one study where ER status was not taken into account Agr2 was shown to associate with good prognosis (Fritzsche et al., 2006). *In vitro* work in breast cancer cell lines has implicated Agr2 in transformation, metastasis, and proliferation (Liu et al., 2005; Vanderlaag et al., 2010). Clinical studies demonstrating overexpression of *Agr2* in a number of other adenocarcinomas including esophagus, pancreas, ovary, lung and prostate, further support the oncogenic role of Agr2 (Bu et al., 2011; Fritzsche et al., 2007; Maresh et al., 2010; Park et al., 2011; Pizzi et al., 2012; Ramachandran et al., 2008; Riener et al., 2009; Wang et al., 2008; Zhang et al., 2005). Functional *in vitro* studies point to Agr2's oncogenic features, also supporting the role of this protein in tumor biology (Brychtova et al., 2011; Dumartin et al., 2011; Fletcher et al., 2003; Maslon et al., 2010; Pohler et al., 2004; Ramachandran et al., 2008; Vanderlaag et al., 2010; Wang et al., 2008).

Despite strong suggestions for a pro-tumorigenic role of Agr2 in breast cancer, no published studies have defined its role in normal mammary gland development; such *in vivo* studies may provide important clues to the mechanism of action for this candidate breast cancer oncogene. To this end, we generated mammary gland specific

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Agr2 knockout and conditional overexpression mouse models. We show that *Agr2* expression is developmentally regulated in the mammary gland, and that the gene promotes epithelial cell proliferation and milk protein production, facilitating normal alveolar development. *Agr2* knockout and constitutive expression mice exhibit decreased and increased mammary epithelial cell proliferation, respectively, without changes in apoptosis rates. This study is the first to utilize transgenic mouse models to show that Agr2 regulates cell proliferation and differentiation in the mammary gland epithelium. We speculate that the pro-proliferative role of Agr2 in normal mammary gland development may mirror its oncogenic role in breast tumorigenesis. Combined with genetically engineered breast tumorigenesis mouse models, the two mouse models developed in the current study will be useful for testing the *in vivo* role of Agr2 in tumorigenesis and metastasis in breast and other adenocarcinomas.

Materials and methods

All experiments performed on animals were conducted under approved IACUC protocol # 2001–2239, following strict guidelines as provided by IACUC.

Generation of inducible Agr2$^{flox/flox}$MMTV-Cre mice

Agr2 flox mice were generated as previously described (Zhao et al., 2010). In order to produce inducible deletion of *Agr2* we bred Agr2 mice to MMTV-LTR-Cre Transgenic Mice obtained from the Jackson Laboratory (JAX 003553). In the bigenic mice, *Agr2* is deleted in Mouse Mammary Tumor Virus Long Terminal Repeat (*MMTV-LTR*) expressing tissues, including primarily mammary gland (virgin, pregnant and enhanced expression during lactation) as well as salivary gland, seminal vesicles and skin. The specificity of the Cre-induced recombination was confirmed by crossing MMTV-LTR mice to ROSA26 reporter (R26R) mice (Soriano, 1999). Tissues from virgin *R26R*/*MMTV-LTR-Cre* female mice were stained with X-Gal to confirm tissue specific expression. We observed abundant staining in the mammary epithelium but not in the brain or heart (Supplemental Fig. 2), confirming faithful Cre recombinase expression.

Generation of TRE-hAgr2/MMTV-LTR-rtTA (hAgr2-Tet-On) mice

pCMV5-Sport6-hAGR2 vector was purchased from ATCC (10701095). Full length cDNA was amplified from the vector template using forward primer (with ClaI restriction sequence) designed to anneal to nucleotides 44–63 and reverse primer to nucleotides (with SpeI restriction sequence) 598–617. The PCR product was then cloned into the pTMILA vector, downstream of an inducible tetracycline promoter (tetop) using ClaI and SpeI sites. Correct insertion of the human Agr2 (hAgr2) transgene into the pTMILA plasmid was verified by sequencing. Tetop-hAgr2-IRES-Firefly-Luc cassette was excised from the vector using PvuII restriction site and injected into fertilized ova from FVB/N background donors. The founders were identified by genotyping. The *TRE-hAgr2* homozygous females were crossed to *MMTV-LTR-rtTA* (Gunther et al., 2002) males to generate bitransgenic *TRE-hAgr2*/*MMTV-LTR-rtTA* progeny.

Genotyping

Genomic DNA was extracted from mouse tail using Quick Genotyping DNA Preparation Kit (Bioland Scientific).

TRE-hAgr2/MMTV-LTR

MMTV-LTR forward primer—TGCCGCCATTATTACGACAAGC, reverse primer—ACCGTACTCGTCAATTCCAAGGG; TRE-hAgr2 forward primer—CGTCAGATCGCCTGGAGAC, reverse primer—TTTCTTTAAAGCTTGACTGTGTGG.

Agr2$^{flox/flox}$/MMTV-Cre

Agr2 flox: primer 1—ATCCAACAAGCATCCACTGA, primer 2—CTTTGGCCAAGGTACCAGAA, primer 3—CTGGATCTAATTTGTGCTGAAT. Wild type allele represented by 228 bp product, floxed allele by 335 bp product, and recombined allele by 519 bp product. MMTV-Cre forward primer—TGAGGTTCGCAAGAACCTGAT, reverse primer—GCCGCATAACCAGTGAAACAGC.

Mammary gland whole mounts and quantification of lobuloalveolar development

The estrous cycle was synchronized by putting male bedding in female cages. Synchronization of estrous cycle was confirmed by vaginal smears prior to any analysis of virgin mice. Fourth or ninth inguinal mammary glands were spread on a glass slide. Mammary glands were fixed in carnoy's fixative, and stained in carmine alum overnight. The mammary glands were dehydrated by gradual change in graded ethanol and were cleared in xylene. After clearing in xylene the glands were mounted in permount. The images were captured on Nikon SMZ1500 dissecting microscope. Mammary glands number 4 and 9 were analyzed for quantification of lobuloalveolar development. Quantification was performed by calculating the percent of area filled with epithelial tissue in ImageJ and by counting the number of alveolar buds per millimeter of duct. To count the number of alveolar buds per mm of duct: for each mammary gland five ducts were selected at random and for each duct the number of alveolar buds were counted in ImageJ for at least 5 mm of duct tissue. This number was normalized to the length of the duct (measured in ImageJ), and the mean number of alveolar buds per mm of duct tissue is reported for each mouse. A minimum one mammary gland from at least three mice were used to calculate the mean and SEM, where each mouse represents one biological replicate.

Quantitative real-time PCR (qPCR) analysis

RNA was extracted from fourth or ninth inguinal mammary gland using trizol Reagent (Invitrogen). cDNA was synthesized using iScript cDNA reverse transcriptase (Bio-Rad Laboratories). *Agr2* expression in *Agr2* KO and over-expression mice was measured in triplicates using pre-designed taqman assay for mouse (TaqMan Probe #4331182, Applied Biosystems) and human Agr2 (TaqMan probe #4331182, Applied Biosystems) respectively. The expression level was normalized to GAPDH expression measured using GAPDH TaqMan probe (TaqMan probe #4331182, Applied Biosystems) or Keratin 8 (Krt8) expression. The relative gene expression level was calculated using delta-delta-cT method (Livak and Schmittgen, 2001). Primers for mouse Agr3: forward—CATGCTCGCAAAAGTAACAAACC, reverse—GAAGGGTCAACAAACATGATCCT; human Agr3: forward—ATCACCTGATGGGCAATATGTG, reverse—GAGTATCTTCCAGCTATGTCAGC; mouse Krt8: forward—GAAGTTCGTGCCCAGTACGAG, reverse—CGGTTGATGTTGCGGTTCAT; mouse α-casein: forward—GCCCTTCCCACAAATCTTCCA, reverse—GGGAGTAAGGTACTGCATATCCT; mouse β-casein: forward—GGCACAGGTTGTTCAGGCTT, reverse—AAGGAAGGGTGCTACTTGCTG; mouse κ-casein: forward—AACTGCCGTGGTGAGAAGAAT, reverse—AAAGATGGCCTGTAGTGGTAGTA; and mouse lactalbumin: forward—GTTCCTTTGTTCCTGGTGTGT, reverse—TGCCTTGATAGCCATCTATGTCT; human AREG: forward—GTGGTGCTGTCGCTCTTGATA, reverse—CCCCAGAAAATGGTTCACGCT; mouse AREG: forward—GGTCTTAGGCTCAGGCCATTA, reverse—CGCTTATGGTGGAAACCTCTC.