Ataxia-telangiectasia mutated is a protein kinase that is critical for the maintenance of genomic stability and proper cellular response to redox imbalance. Previous studies indicate that ataxia-telangiectasia mutated is also required for expression of the insulin-like growth factor-1 receptor in human fibroblasts. As insulin-like growth factor-1 receptor is critical in mammary gland development, we sought to determine the relationship between ataxia-telangiectasia mutated and insulin-like growth factor-1 receptor using mouse as a model system. Knockdown of ataxia-telangiectasia mutated in cultured mouse mammary epithelial cells resulted in a significant reduction in insulin-like growth factor-1 receptor mRNA levels. Using a conditional knockout mouse model, we observed that loss of ataxia-telangiectasia mutated resulted in a severe decrease in both insulin-like growth factor-1 receptor mRNA and protein expression. These results support the observation that insulin-like growth factor-1 receptor expression is ataxia-telangiectasia mutated-dependent in the mammary epithelium, and given the critical role that insulin-like growth factor-1 receptor plays in mammary gland development, suggests that ataxia-telangiectasia mutated may be critical in the development of this organ as well.

**Keywords**

Mammary development; signaling; mammary epithelium; growth factors

1. Introduction

Ataxia-telangiectasia mutated (ATM) is a central protein kinase activated in response to DNA damage and redox imbalance [1]. Germ-line mutation of the ATM gene causes a rare, pleiotropic, recessive genetic disorder termed Ataxia-Telangiectasia (A-T) [1, 2]. The most consistent A-T phenotype is early-onset ataxia caused by progressive neurodegeneration within the cerebellum [3, 4]. Other symptoms of A-T are ocular telangiectasia, immunodeficiency, radiosensitivity, premature aging, and increased cancer predisposition, principally lymphoid tumors. Also, obligate female ATM heterozygotes have a modest increase in the relative risk of breast cancer development [5], and reduced ATM expression is commonly observed in sporadic breast cancers [6, 7].

Insulin-like growth factor (IGF) is a small peptide hormone fundamental for growth and survival. During puberty, release of growth hormone (GH) from the pituitary, stimulates the expression of both isoforms of IGF (i.e. IGF1 and IGF2) by the liver as well as numerous cell types within the body [8]. IGF1 and IGF2 are both synthesized by the stroma and ductal epithelium of the developing mammary gland and are necessary for normal gland development including formation of terminal end buds and ductal branching [9]. The effects of IGF are mediated through the IGF1 receptor (IGF-1R), a membrane-associate tyrosine kinase. Insulin receptor substrates 1 and 2 (IRS1 and IRS2), which act as signaling adaptors for both IGF-1R and the insulin receptor (InsR), dock with tyrosine phosphorylated IGF-1R at the cytosolic face of the receptor and activate numerous downstream signaling cascades such as the PI3K-AKT and RAS-MAPK-ERK1 signaling axes [10].

Owing to its central role in promoting mammary gland growth and development, it is unsurprising that IGF-1R is commonly dysregulated in breast cancer with approximately 50% of breast tumors expressing an activated form of IGF-1R [10]. Thus, unsurprisingly, studies have documented that IGF signaling is commonly associated with pro-oncogenic activities (reviewed in [11]). Paradoxically, other groups have documented that loss of IGF-1R is linked to tumor progression and less differentiated breast tumors, suggesting that IGF-1R may serve to constrain tumor development [12]. More recently, Obr and co-workers found that, using both mouse models and human breast cancer cell lines, reduction of IGF-1R function increases cell stress and cytokine production which promote a more pro-tumorigenic tumor microenvironment [13]. Further, IGF-1R signaling has been linked to resistance to anti-estrogen therapy [14].

The Glaser laboratory initially reported that, using A-T patient cell lines, that IGF-1R expression was markedly suppressed in fibroblasts that do not express functional ATM [15]. Moreover, this study showed that IGF-1R expression could be complemented in an A-T line by ectopic expression of full-length ATM, indicating that IGF-1R expression is ATM-dependent. Transcriptional reporter assays indicated that ATM was driving IGF-1R expression at the transcriptional level, and that forced ectopic expres-
ssion of IGF-1R in A-T cells could complement the inherent radio-
sensitivity of these cells. Of note, other studies [16] indicated
that IGF-1R inhibition results in the accumulation of DNA dou-
ble strand breaks (DSBs) in cells as scored by accumulation of
ATM-dependent phosphorylation of histone H2AX (γH2AX), a
biochemical marker of DSBs [17]. Taken together, the current lit-
erature clearly suggests that a relationship, albeit still not well un-
derstood, exists between ATM and IGF-1R.

To study ATM in mammary gland development and function,
we generated a mouse line with conditional deletion of ATM in
the mammary epithelium [18]. We documented that these mice
display an associated defect in lactation as evidenced by a reduc-
tion in litter weight, reduced lobulo-alveolar structure, and signif-
ically diminished expression of several milk protein genes. We
also demonstrated that expression of the critical anti-oxidant gene
Mn-dependent superoxide dismutase (MnSod or Sod2) was sup-
pressed in ATM-deficient mammary glands and that the loss of
Sod2 expression was likely responsible for, at least in part, the
lactation defect as mice harboring Sod2-deficient mammary gland
phenocopied lactation and structural defects observed in mice with
ATM-deficient mammary glands. Given the importance of ATM and
IGF-1R in mammary gland development and tumorigenesis,
we sought to examine the relationship between ATM and IGF-1R
expression within this critical organ.

2. Materials and methods

2.1 Cell culture

NMuMG (CRL-1636) cells were obtained from ATCC (Man-
assas, VA). Cells were cultured in DMEM supplemented with 10%
fetal bovine serum at 37 °C in a humidified 5% CO2 environment.

2.2 RNA interference

For RNAi-mediated knockdown of ATM in cultured mouse
cells, a shRNA lentiviral vector (Clone V2LHS_89366) was ob-
tained from Open Biosystems (Huntsville, AL). Lentivirus encod-
ing shRNA or pLKO.1 empty vector were packaged in Lenti-X
cells (Takara, Mt. View CA) by co-transfection with the packaging
plasmids psPAX2 and pMD2.G (Addgene, Cambridge, MA). Two
days after transfection, spent medium was collected, polybrene (fi-
nal concentration of 10 μg/ml) added, and applied to cultured cells.
Selection with 2 μg/ml puromycin was conducted for 2-3 weeks
prior to analysis of the resultant polyclonal cell populations.

2.3 Q-PCR analysis

Total RNA was isolated from cultured cells or dissected mouse
mammary glands using TRI Reagent (Ambion, Austin, TX) per
manufacturer's instructions. Two μg of total RNA was then used
in 20 μl first strand reactions with the High Capacity RNA-to-
cDNA Kit (Life Technologies, Grand Island, NY). For Q-PCR, 1.0
μl of cDNA reaction was added to the following: 1.0 μl of 10 mM
stock each of indicated forward and reverse primer, 7.5 μl of SYBR
Green master mix (Applied Biosystems, Norwalk, CT), and 6.5 μl
H2O for a total volume of 15 μl. PCR was carried out in an Applied
Biosystems StepOnePlus thermocycler, fold changes in transcript
abundance were calculated by the 2(-ΔΔCt) method [19] using
GAPDH as the internal standard. Primers used for ATM Q-PCR
are: (Forward) 5'-GTGCTGATGAGATGCTCTT-3', (Reverse)
5'-AAAAGGACTGACGACCAAC-3'. Primers used for IGF-1R
Q-PCR are: (Forward) 5'-ACAGCAACCCAGAGCATGTA-3', (Re-
verse) 5'-GCATCTTGGAGCATTGGAG-3'. Primers used for
GAPDH are: (Forward) 5' AACGACCCCTTCATTGAC-3', (Re-
verse) 5'-GTGCTGAGATGCTGAGGA-3'. Primers used for
KRT18 are: (Forward) 5'-GCTGAGATGCTGAGGATTT-3', (Reverse)
5'-CCTCCCTCTGCTCCAGTGT-3'.

2.4 Immunoblot analysis

SDS-PAGE and immunoblotting was performed using estab-
lished protocols [20]. Following electrophoresis, nitrocellulose
membranes were probed with anti-ATM (Cat# 07-1286, Millipore,
Billerica MA) or monoclonal anti-β tubulin (E7) obtained from
the Developmental Studies Hybridoma Bank (Univ. of Iowa).
Immunoblot signals were developed using Pierce ECL chemi-
luminescence substrate (Thermo Scientific, Waltham, MA) and
recorded on X-Ray film.

2.5 Mouse care and genotyping

A mouse line with LoxP sites flanking exon 58 of the mouse
ATM gene was developed using conventional gene targeting as pre-
viously described [18]. Transgenic mice harboring a Cre recom-
binicase transgene under control of the whey-acidic protein (WAP)
promoter [B6.Cg-Tg(WAP-Cre)11738Mam; strain#01XA8] were
obtained from the NCI Mouse Repository (mouse.ncifcrf.gov).
Mice were maintained in a C57Bl/6 genetic background in an
AAALAC approved facility managed by University of Florida An-
imal Care Services. All mouse husbandry and experimentation
were conducted in accordance with protocols approved by the Uni-
versity of Florida IACUC.

DNA was isolated from tail snips (0.5-1.0 cm) taken from 3-
week old pups and placed in tubes with buffer containing 100 mM
NaCl, 20 mM Tris (pH 8.0), 25 mM EDTA (pH 8.0), 0.5% SDS,
and 100 μg/mL of freshly added protease K (Sigma Aldrich,
St. Louis, MO). Tubes were incubated for 4 hr or overnight at
50 °C. Five hundred μL of phenol-chloroform pH 7.6 (Fisher
Scientific, Pittsburgh, PA) was added to each tube, mixed, and
centrifuged for 10 min at top speed. The clear aqueous phase
was transferred to new tubes supplemented with 100% ethanol,
inverted a few times and centrifuged for 5 min at top speed.
The ethanol was removed from the tubes and 70% ethanol was
added, vortexed gently and centrifuged for a final time. The
70% ethanol was removed and the DNA pellet was allowed to air
dry. 50-200 μL of dH2O or TE buffer containing 10 mM Tris
pH 8.0, 1mM EDTA, was added to each tube and incubated at
50 °C until resuspended. Before PCR, the DNA was vortexed
and centrifuged for 5 min at top speed to pellet any insoluble
material and was diluted to a final concentration of 100 ng/μL.
PCR primers used for genotyping the ATM gene were: P1 5'-
CCAGTGTATATGCGAGACTGTTACATCC-3' and P2
5'-AACACTGAGAAGACCGCTTCCG-3'. PCR primers used
to genotype the WAP-cre transgene were (Forward)
5'-ACAGCCAGCTATTCAACTGTTACA-3' and (Reverse)
5'-TTGGTCCAGCCACCC-3'.

2.6 Immunohistochemistry

Mammary tissue was dissected and fixed in 4% paraformalde-
yde overnight. The next day, tissue was placed in 70% ethanol,
processed and sectioned. Sectioned tissues were then depara-
finized in xylene (5 min × 2), 100% ethanol (2 min × 2), 95% ethanol (3 min), 70% ethanol (1 min) and H2O (1 min × 2). ATM
antigen retrieval was performed by treating the sections with proteinase K (20 µg/mL) for 2 min at RT. Antigen retrieval for IGF-1R was performed in citrate buffer pH 6.0 in a steaming water bath (90º-100º). Slides were rinsed with 1x Tris-Buffered Saline Tween-20 (TBST) and tissue staining was performed by using VECTASTAIN® Elite ABC system (Vector Labs, Burlingame, CA). Briefly, to quench endogenous peroxidase activity, slides were incubated for 30 min in 0.3% H₂O₂ in methanol, washed and incubated for 20 min with diluted normal blocking serum prepared from the species in which the secondary antibody was made. After blocking, slides were washed and incubated with primary anti-ATM (Cat# 07-1286, Millipore, Billerica MA) anti-IGF-1R antibody (Cat# 14534S, Cell Signaling, Danvers MA) diluted in 1X TBST buffer at 4 ºC overnight. The next day slides were washed and biotinylated secondary antibody was added and incubated for 30 min at RT. After incubation, the slides were washed, ABC reagent added, and incubated for 30 min at RT. The slides were then washed and incubated with 3,3’-Diaminobenzidine (Vector Labs, Burlingame, CA) until desired staining intensity was obtained. Where indicated, tissues were stained with Hematoxylin and eosin using a TissueTek automated slide stainer.

2.7 Statistical analysis

All graphs are plotted as the mean with error bars representing ± standard deviation (SD). A "***" is used to denote P ≤ 0.001, exact P values are provided in the figure legends. When examining Q-PCR analyses, a Student’s t-test was used when comparing two samples, and ANOVA test was used when three or more populations were tested. Statistical analyses were performed using SPSS ver 20 software (IBM, Armonk, NY)
Fig. 2. ATM expression and conditional deletion in the mouse mammary gland. (A). Mammary glands from a mature female virgin C57BL6 mouse were dissected, fixed, sectioned and stained with anti-ATM (left) or non-immune (control) rabbit IgG (right) followed by incubation in diaminobenzidine (DAB) and subsequent H&E staining. Note the strong, largely nuclear immunoreactivity (brown staining) observed in gland incubated with anti-ATM. Scale bar = 250 μm. (B). Schematic diagram showing location of LoxP sites flanking ATM exon 58 and the location of PCR primers P1 and P2 used in genotyping reactions. (C). Genomic DNA (gDNA) was isolated from mouse tail snips and used in PCR reactions with P1 and P2 primers. Where indicated, gDNA was also analyzed for the WAP-cre transgene by PCR. (D). Relative ATM transcript levels were analyzed in mammary glands from lactating control (Atm<sup>flox/flox</sup>) or AtmcKO (Atm<sup>flox/flox</sup>; WAP-cre) females by Q-PCR using primers for the GAPDH or KRT18 transcripts as internal standards. Error bars = ± SD, *P* = 0.00023 for GAPDH, **P** = 0.00051 for KRT18, Student’s *t*-test. All Q-PCR reactions were run, at a minimum, in triplicates; histograms represent the average relative mRNA abundance.

Mary parenchyma. Moreover, staining was principally noted in the cell nuclei, consistent with the previously reported localization of ATM within cultured cells and expression of ATM within all cell types in the mammary gland is in keeping with other reports indicating the largely ubiquitous nature of ATM expression in various tissues and cell types within the body [21].

Previous work from our laboratory outlined that female mice with germline deletion of ATM exhibit severely aberrated development of their mammary glands and that to study ATM function in this organ, required the development of a novel mouse line with conditional deletion of ATM in the mammary epithelium [18]. These mice were developed by insertion of LoxP sites flanking exon 58 of the mouse ATM gene (Fig. 2B). To obtain deletion of ATM in the mammary epithelium we mated these mice with mice harboring a transgene which places the cre recombinase cDNA under the control of the Whey Acidic Protein (WAP) promoter [22]. Fig. 2C displays the results of PCR genotyping analysis of female mice containing various combinations of wild type and ATM alleles containing LoxP sites (i.e. floxed allele) and the WAP-cre transgene. Mice with the conditional ATM knockout genotype (ATM<sup>flox/flox</sup>; WAP-Cre) are referred throughout this manuscript as AtmcKO mice, while mice lacking the WAP-Cre transgene (i.e., ATM<sup>flox/flox</sup> genotype) are consistently used as controls (Fig. 2C).

Transcription from the wap promoter does not occur until pregnancy day 13, persists through lactation and ceases as the mammary gland undergoes involution [22]. We previously observed that deletion of exon 58 from genomic dna harvested from atm<sup>flox/flox;wap-cre</sup> dams was detectable at lactation day 1 (L1) [18]. Using both gapdh and an epithelium-specific cytokeratin, krt18, as internal standards for q-pcr analysis we detected a statistically significant decrease in atm in the #4 mammary gland harvested from 11 atmcko mice (Fig. 2D). When mammary glands from control (Atm<sup>flox/flox</sup>) and atmcko dams were subjected to the staining with anti-atm or control rabbit igg, we observed a notable decrease in atm immunoreactivity in atmcko mice [18] consistent with diminished atm mrna and protein in atmcko dams.

We next assayed the expression of IGF-1R in mammary glands dissected from three control and two AtmcKO dams using Q-PCR. We observed that IGF-1R mRNA abundance was significantly reduced in mammary glands from lactating AtmcKO glands compared to glands dissected from lactating control dams (Fig. 3A). Finally, immunohistochemical analysis was conducted to examine IGF-1R protein expression in an AtmcKO and a control dam. As was clearly evident, IGF-1R staining was prominent in the luminal epithelium of the control dam but dramatically reduced in the gland dissected from the lactating AtmcKO dam (Fig. 3B), supporting the conclusion that ATM is required for IGF-1R expression in the mammary epithelium of mouse.

4. Discussion

4.1 ATM is required for IGF-1R expression

The Glaser laboratory was first to report that, in fibroblasts cultured from a human A-T patient, expression of the IGF-1R protein and mRNA were notably diminished [15]. Moreover, this defect
that links ATM with IGF-1R gene transcription remains unknown.

NFκB, a critical regulatory subunit of IκB kinase (IKK), is required for basal activity of the transcription factor complex NFκB [28, 29]. We found that pharmacological inhibition or genetic knockdown of ATM resulted in reduced constitutive NFκB transcriptional activity, presumably through blocking nuclear export of NEMO/IKKγ, a critical regulatory subunit of IκB kinase [26]. We are unaware of any reports that directly link NFκB to the regulation of IGR-1R, however, others have shown that the NFκB-regulated microRNAs miR195 and miR497 directly target the IGF-1R transcript [30, 31]. Of note, NFκB regulates the expression of two key IGF-1 binding proteins, IGFBP-1 [32] and IGFBP-2 [33], further establishing cross-talk between IGF-1 signaling and NFκB. Nevertheless, the exact nature of the molecular mechanism that links ATM with IGF-1R gene transcription remains unknown.

4.2 ATM in mammary gland development

During puberty, a surge of the ovarian hormone estrogen synergizes with pituitary growth hormone (GH) to stimulate the mammary stroma to produce IGF-1 [34]. In support of this, Igf-1-/-/ mice and growth hormone receptor knockout (Ghr-/-) mice have impaired ductal development during puberty and treatment of Igf-1-/-/ mice with exogenous estrogen and growth hormone did not restore ductal outgrowth; however, this phenotype was rescued with the addition of IGF-1 and estrogen [35], demonstrating IGF-1 action is downstream of growth hormone. Also, administration of IGF-1 to these animals did not stimulate development illustrating a requirement for synergistic actions between estrogen, growth hormone and IGF-1 [12, 34]. Richards et al. [36] confirmed the local production of IGF-1 is necessary for ductal outgrowth by utilizing mice with a liver-specific deletion of the IGF-1 gene. This caused a reduction in overall IGF-1 serum levels, but IGF-1 transcript levels were normal in the mammary gland and mammary gland development progressed as normal [36].

Consistent with IGF-1 being a mediator of ductal morphogenesis, the IGF-1R is similarly critical for normal pubertal development of the mammary gland [37]. Given that ATM is required for IGF-1R expression in mammary epithelium, logic follows that ATM would similarly be required for mammary gland development. Likely stemming from severely blunted ovarian development in ATM-/-/ mice, females are anovulatory and display a clear lack of estrous cycling [38]. Unsurprisingly, we documented that the mammary glands in ATM-/-/ females also show severe developmental defects [18]. To limit off-target deletion of ATM, we used the WAP-cre model to delete ATM within the mammary gland. As WAP expression is limited to late pregnancy and lactation, this model does not allow us to analyze a requirement for ATM in mammary gland development during or prior to puberty. Clearly, development of new conditional mouse models that allow an analysis of the requirement for ATM in pubertal mammary gland development is required to adequately address this issue.

Fig. 3. ATM knockout results in reduced IGF-1R abundance in mouse mammary epithelium. (A). Total RNA was harvested from lactating control dams (#141,143,144) and AtmcKO dams (#145,150) and relative IGF-1R transcript abundance was determined by Q-PCR (GAPDH used as internal standard). Q-PCR reactions were run, at a minimum, in triplicate; histograms represent the average relative mRNA abundance (error bars = ± SD). ANOVA analysis was used to compare IGF-1R transcript levels in the control glands to each of the AtmcKO glands P = 0.0000000032 (dam 145) and P = 0.00000065 (dam 150). (B). Mammary glands from a lactating control (left and center) or AtmcKO female 145 (right) were stained with anti-IGF-1R (left and right panels) or control rabbit IgG (center) followed by DAB and H&E staining.
In sum, our findings clearly indicate that ATM is required for the expression of IGF-1R in cultured mouse mammary epithelial cells and the lactating mouse mammary gland. As this defect is linked to reduced steady-state levels of IGF-1R mRNA, it is likely that ATM is positively affecting transcription of the IGF-1R gene through a mechanism that is currently unknown. Given the importance of IGF-1R in mammary gland development, and the requirement for ATM in IGF-1R expression, future studies aimed at determining the requirement for ATM in mammary gland development are needed to further understand the nature of ATM/IGF-1R signaling within the developing mammary gland.

Acknowledgments
The authors are grateful to Drs. Dr. Lingbao Ai and Eugene Izumchenko for technical assistance and suggestions. LMD was supported by funding from NIH (R03 CA125824), and the Florida Department of Health to KDB.

Conflict of Interest
The authors declare no competing interests.

Submitted: August 10, 2020
Accepted: September 16, 2020
Published: September 20, 2020

References


