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Original Research

ATM is necessary for IGF-1R expression in the murine mammary gland

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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 telangiectasias, 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-associated 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-

sion of IGF-1R in A-T cells could complement the inherent radiosensitivity of these cells. Of note, other studies [16] indicated that IGF-1R inhibition results in the accumulation of DNA double 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 literature clearly suggests that a relationship, albeit still not well understood, 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 reduction in litter weight, reduced lobulo-alveolar structure, and significantly 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 suppressed 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 (Manassas, VA). Cells were cultured in DMEM supplemented with 10% fetal bovine serum at 37 °C in a humidified 5% CO_2 environment.

2.2 RNA interference

For RNAi-mediated knockdown of ATM in cultured mouse cells, a shRNA lentiviral vector (Clone V2LHS_89366) was obtained from Open Biosystems (Huntsville, AL). Lentivirus encoding 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 (final 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 ml 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 5 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 H_2O for a total volume of 15 μl. PCR was carried out in an Applied Biosystems StepOnePlus theromocycler, fold changes in transcript abundance were calculated by the 2(-$\Delta\Delta$Ct) method [19] using GAPDH as the internal standard. Primers used for ATM Q-PCR are: (Forward) 5'-GTCCATCGTCCACTGGTCTT-3', (Reverse) 5'-AAAGGACTCATGGCACCAAC-3'. Primers used for IGF-1R Q-PCR are: (Forward) 5'-ACAGCACCCAGAGCATGTA-3', (Reverse) 5'-GCATCCTTGGAGCATTTGAG-3'. Primers used for GAPDH are: (Forward) 5' AACGACCCCTTCATTGAC-3', (Reverse) 5'- GTGCTGAGTATGTCGTGGA-3'. Primers used for KRT18 are: (Forward) 5'-GCTGGAG GATGGAGAAGATTT-3', (Reverse) 5'-CCTCCTTCTCTGCCTCAGTG-3'.

2.4 Immunoblot analysis

SDS-PAGE and immunoblotting was performed using established protocols [20]. Following electrotransfer, 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 chemiluminescence 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 previously described [18]. Transgenic mice harboring a Cre recombinase 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 Animal Care Services. All mouse husbandry and experimentation were conducted in accordance with protocols approved by the University 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 proteinase K (Sigma Aldrich, St. Louis, MO). Tubes were incubated for 4 hr or overnight at 50 ºC. 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 dH_2O or TE buffer containing 10 mM Tris pH 8.0, 1mM EDTA, was added to each tube and incubated at 50 ºC 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'-CCCAGTGTATATGCCACCGACTGAGTTACATCC-3' and P2 5'-ACCACTCGAAGAAC AACCGCTTCGC-3'. PCR primers used to genotype the WAP-cre transgene were (Forward) 5'-ACCAGCCAGCTATCAACTCGTTACA-3' and (Reverse) 5'-TTGGTCCAGCCACC-3'.

2.6 Immunohistochemistry

Mammary tissue was dissected and fixed in 4% paraformaldehyde overnight. The next day, tissue was placed in 70% ethanol, processed and sectioned. Sectioned tissues were then deparaffinized in xylene (5 min × 2), 100% ethanol (2 min × 2), 95% ethanol (3 min), 70% ethanol (1 min) and H_2O (1 min × 2). ATM

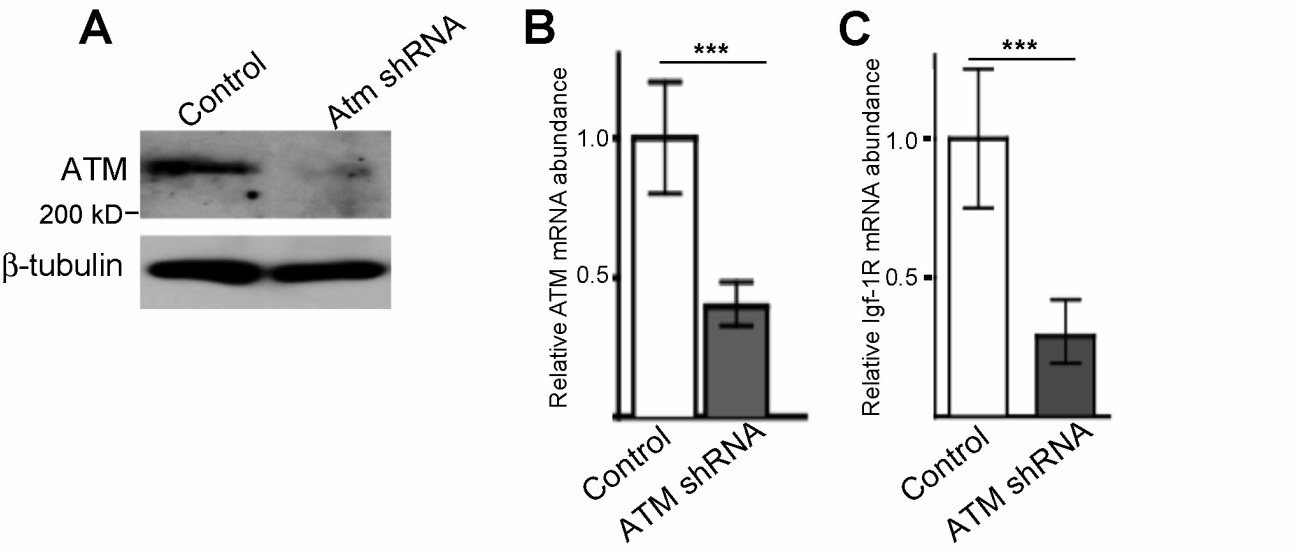

Fig. 1. ATM knockdown results in reduced IGF-1R mRNA levels. The mouse mammary epithelial cell line NMuMG was transduced with lentivirus developed from either an empty (control) plasmid (pLKO.1), or a plasmid encoding an ATM-specific shRNA transcript followed by puromycin selection. (A). Extracts from ATM shRNA and control NMuMG cells were immunoblotted with anti-ATM (*top*) or anti-β-tubulin (*bottom*) to assure even protein loading. (B). Total RNA was harvested from ATM shRNA and control NMuMG cells, and analyzed for relative ATM mRNA abundance using quantitative PCR (Q-PCR). Error bars = $\pm$ SD, $P = 0.000055$, Student's *t*-test. (C). ATM shRNA and control NMuMG cells were analyzed for IGF-1R mRNA abundance using Q-PCR. Error bars = $\pm$ SD, $P = 0.00093$, Student's *t*-test. All Q-PCR reactions were run in triplicate, graphed is the average relative mRNA abundance.

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% H_2O_2 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 $\pm$ standard deviation (SD). A "***" is used to denote $P \leq 0.001$, 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)

3. Results

3.1 ATM knockdown results in decreased IGF-1R expression in cultured mouse mammary epithelial cells

As outlined above, ATM is required for IGF-1R expression in cultured human fibroblasts [15]. To further explore this relationship in the mouse mammary gland, we scored IGF-1R expression by Q-PCR in the normal mouse mammary line NMuMG with shRNA-mediated knockdown of ATM expression. Immunoblot analysis of these cells with anti-ATM indicated that transduction of these cells with a lentivirus encoding ATM-specific shRNA resulted in a multi-fold decrease in ATM expression compared to cells transduced with a control (empty vector) lentivirus (Fig. 1A). Using Q-PCR we found a ~4-fold decrease in ATM transcript in ATM knockdown NMuMG cells (Fig. 1B). Consistent with previous findings, we measured a similar (~4-fold) reduction in IGF-1R mRNA in NMuMG cells with knocked down ATM expression (Fig. 1C).

3.2 Conditional deletion of ATM in mouse mammary epithelium results in reduced expression of IGF-1R

As previously outlined, we sought to investigate the role of ATM within functional mammary glands and, to this end, we chose mouse as a model system for the study. To examine ATM expression within the mammary gland of mice, a 10-week old virgin C57/BL6 female was sacrificed and an abdominal (#4) mammary gland was embedded, sectioned and stained with an anti-ATM antibody or control (non-immune) rabbit IgG, and counterstained using hemotoxylin/eosin (Fig. 2A). As can be readily seen, ATM was detectable throughout the mammary gland with positive staining noted in the luminal and basal epithelium, stromal cells surrounding the ductal structures and adipocytes within the mam-

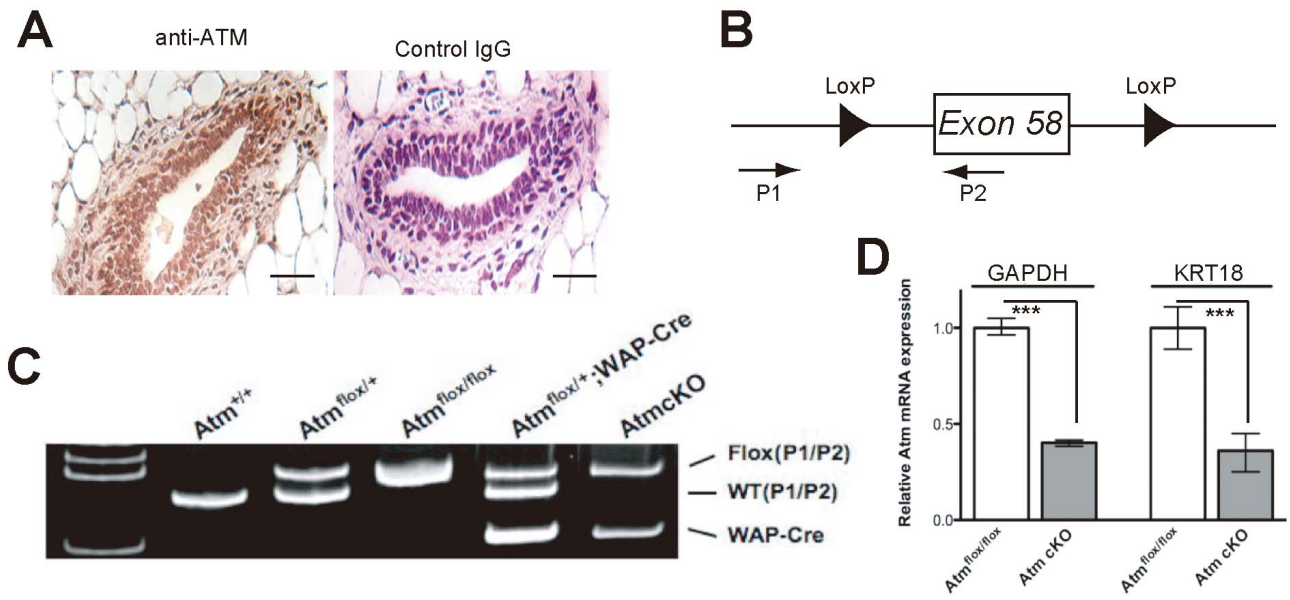

Fig. 2. ATM expression and conditional deletion in the mouse mammary gland. (A). Mammary glands from a mature female virgin C56/BL6 mouse were dissected, fixed, sectioned and stained with anti-ATM (*left*) or non-immune (control) rabbit IgG (*right*) followed by incubation in diaminobenzidine (DAB) and 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 ($Atm^{flox/flox}$) or AtmcKO ($Atm^{flox/flox}$; WAP-cre) females by Q-PCR using primers for the *GAPDH* or *KRT18* transcripts as internal standards. Error bars = $\pm$ 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 abrogated 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 ($ATM^{flox/flox}$;*WAP-Cre*) are referred throughout this manuscript as AtmcKO mice, while mice lacking the WAP-Cre transgene (i.e., $ATM^{flox/flox}$ 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 atmflox/flox;wap-cre 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 l1 atmcko mice (Fig. 2D). when mammary glands from control (Atmflox/flox) and atmcko dams were subjected to ihc 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

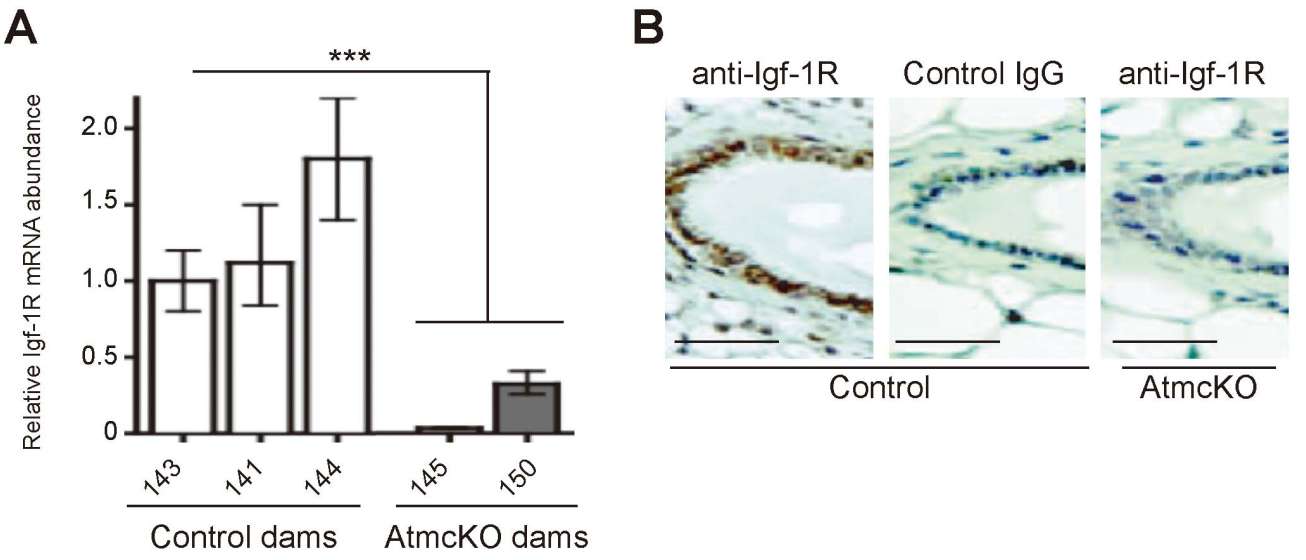

Fig. 3. ATM knockout results in reduced IGF-1R abundance in mouse mammary epithelium. (A). Total RNA was harvested from lactating control dams (#141,143,144) and AtmcKO dams (#145,150) and relative IGF-1R transcript abundance was determined by Q-PCR (GAPDH used as internal standard). Q-PCR reactions 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 grands 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.

was complemented by ectopic expression of recombinant ATM, clearly indicating that IGF-1R expression in this cell type is dependent upon ATM function. We also observed that loss of ATM, either by RNAi in cultured mouse mammary epithelial cells or genetic disruption in the mouse mammary gland, led to a dramatic disruption in IGF-1R mRNA and protein levels. Using reporter assays, Peretz *et al*. [15] concluded that ATM loss was negatively impacting transcription of the *IGF-1R* gene. Similarly, we found that diminished ATM led to reduced steady-state mRNA abundance, strongly supporting the notion that ATM positively promotes transcription of the *Igf-1R* gene in mouse cells as well.

Studies have established that ATM, either directly or indirectly, influences gene transcription [23, 24, 25, 26, 27]. For example, our group as well as other groups documented that ATM 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 anovuatory 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.

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 effecting 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.

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Conflict of Interest

The authors declare no competing interests.

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