Ataxia Telangiectasia Mutated (ATM) Inhibition Transforms Human Mammary Gland Epithelial Cells*

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Carriers of mutations in the cell cycle checkpoint protein kinase ataxia telangiectasia mutated (ATM), which represent 1–2% of the general population, have an increased risk of breast cancer. However, experimental evidence that ATM deficiency contributes to human breast carcinogenesis is lacking. We report here that in MCF-10A and MCF-12A cells, which are well established normal human mammary gland epithelial cell models, partial or almost complete stable ATM silencing or pharmacological inhibition resulted in cellular transformation, genomic instability, and formation of dysplastic lesions in NOD/SCID mice. These effects did not require the activity of exogenous DNA-damaging agents and were preceded by an unsuspected and striking increase in cell proliferation also observed in primary human mammary gland epithelial cells. Increased proliferation correlated with a dramatic, transient, and proteasome-dependent reduction of p21WAF1/CIP1 and p27KIP1 protein levels, whereas little or no effect was observed on p21WAF1/CIP1 or p27KIP1 mRNAs. p21WAF1/CIP1 silencing also increased MCF-10A cell proliferation, thus identifying p21WAF1/CIP1 down-regulation as a mediator of the proliferative effect of ATM inhibition. Our findings provide the first experimental evidence that ATM is a human breast tumor suppressor. In addition, they mirror the sensitivity of ATM tumor suppressor function and unveil a new mechanism by which ATM might prevent human breast tumorigenesis, namely a direct inhibitory effect on the basal proliferation of normal mammary epithelial cells.

Ataxia telangiectasia (A-T)² is an autosomal recessive syndrome characterized by neurodegeneration, oculocutaneous telangiectasia, radiosensitivity, immune deficiency, sterility, strong predisposition to lymphoid cancers, and, at the cellular level, cell cycle checkpoint defects, chromosomal instability, and impaired cell proliferation. ATM, the gene defective in A-T, is localized to chromosome 11q22–23 and encodes a homonymous Ser/Thr protein kinase that regulates cell cycle checkpoints, DNA repair, and apoptosis in response to DNA double-strand breaks (DSBs) by phosphorylating numerous substrates, including p53 (1). Whereas A-T carriers are not affected by the syndrome, conventional and molecular epidemiological studies have consistently shown associations between monoallelic mutations in ATM and breast cancer development, generating the hypothesis that ATM may act as a “low penetrance, high prevalence” breast cancer-predisposing gene (2–4). However, the lack of formal experimental evidence that ATM functions as a human breast tumor suppressor prevented assigning a direct role to ATM deficiency in breast carcinogenesis. In a previous study, mammary gland epithelial cells of irradiated Atm⁺/⁻ mice were shown to develop ductal dysplasia when transplanted into syngenic recipients. However, cells isolated from unirradiated Atm⁺/⁺ mice formed normal ducts (5). In addition, whether exposed to radiations or not, mice carrying genetically engineered Atm inactivation fail to display an increased incidence of mammary gland carcinomas, reflecting potential differences in sensitivity, pathways of tumorigenesis, or mechanisms of ATM activation between the two species (1), thus making the relevance of these findings to the breast cancer susceptibility of A-T carriers unclear. More generally, at the present time, there are no in vitro models available to explore the contribution of ATM loss of function to human tumorigenesis because fibroblasts or lymphocytes isolated from A-T patients or carriers have not been reported to undergo transformation in vitro.

The study of ATM deficiency in human breast carcinogenesis has been hampered by the lack of ATM-deficient human mammary gland epithelial cells. To overcome this limitation, we stably suppressed ATM expression by RNA interference in MCF-10A cells, a spontaneously immortalized and well characterized...
human mammary gland epithelial cell line derived from mastectomy tissue of a 36-year-old woman with fibrocystic disease. MCF-10A cells grow as a contact-inhibited monolayer, form acini-like structures in three-dimensional matrices, do not grow in agar, and are not tumorigenic in immunodeficient mice (6–8). For these reasons, they are a widely accepted model of normal human mammary gland epithelium, where the effects of putative breast cancer genes can be assessed (9, 10). A second human mammary gland epithelial cell line with similar features but derived from reduction mammoplasty tissue of a different female patient, the MCF-12A cell line (8), and human primary mammary gland epithelial cells subjected to pharmacological inhibition of ATM were also investigated.

EXPERIMENTAL PROCEDURES

**Cell Culture**—MCF-10A and MCF-12A cells (6–10) were purchased from ATCC (Manassas, VA) or from the Karmanos Cancer Institute (Detroit, MI). The identity of the two MCF-10A sublines used was verified by DNA fingerprinting. MCF-10A and MCF-12A cells were grown in Dulbecco’s modified Eagle’s medium/F-12 (catalog no. 31331-028, Invitrogen) supplemented with 5% heat-inactivated fetal calf serum (catalog no. 10090-141, Invitrogen) and grown in Dulbecco’s modified Eagle’s medium containing 1.0 g/liter glucose (catalog no. D6046, Sigma). HaCaT spontaneously immortalized human keratinocytes (11) were purchased from Cell Lines Service (Eppelheim, Germany) and grown in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal calf serum (catalog no. 0-20710-8, Invitrogen). C26Ci, or HK-2 cell stable transfectants were generated using Polyfect (catalog no. 301105, Qiagen (Hombrechtikon, Switzerland)) or jetPEI (catalog no. 101-05, Polyplus/Chemie Brunswig AG, Basel, Switzerland) according to the manufacturer’s instructions. Stable transfectants were selected for 2 weeks and grown in normal culture medium thereafter.

**siRNA Transfections**—siRNAs against the human CDKN1A (cyclin-dependent kinase inhibitor 1A) (p21WAF1/CIP1) target sequences CAGTTGTTGTGTCCTTAATTAT (p21_6; catalo
g no. SI00604905, Qiagen), against the human CDKN1B (cyclin-dependent kinase inhibitor 1B) (p27KIP1) target sequence ACCGACGATTCTTCTACTCAA (p27_6; catalo
g no. SI02621997, Qiagen), against the human ATM target sequence AACAGAGACTGCTACCAAGG (ATM0, Qiagen), or two different non-silencing siRNAs (CTRL1 and CTRL2; Qiagen catalog nos. 1022076 and 1027280, respectively) were transfected at a concentration of 5 μM using Hiperfect (catalog no. 301705, Qiagen). Cells were lysed for protein analysis as described under “Western Blotting” at the times indicated.

**Western Blotting**—Cells were lysed using either radioimmune precipitation assay buffer (catalog no. R0278, Sigma) for total protein extraction or the NucBuster protein extraction kit (catalog no. 71183-3, Novagen/Merck Chemicals Ltd. (Nottingham, UK)) for nuclear protein preparation. In either case, Halt protease inhibitor mixture (catalog no. 78410, Pierce/Thermo Fisher Scientific (Lausanne, Switzerland)) and phosphatase inhibitor mixture (catalog no. 78420, Pierce/Thermo Fisher Scientific) were added to the lysis buffer. 50–80 μg of protein were run in SDS-polyacrylamide gels and transferred to nitrocellulose membranes (catalog no. RPM 303 D, GE Healthcare). Blots were incubated with antibodies against ATM (catalog no. ab78, Abcam plc (Cambridge, UK)), ATM Ser(P)-1981 (catalog no. 4526, Cell Signaling Technology/Bioconcept, Allschwil, Switzerland), NBS1 (Nijmegen breakage syndrome 1; catalog no. GTX70224, GeneTex (Irvine, CA)), NBS1 Ser(P)-343 (catalog no. 3001, Cell Signaling Technology/Bioconcept).
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p53 (catalog no. 628201, Biolegend/Lucerna Chem AG (Luzern, Switzerland)), p53 Ser(P)-15 (catalog no. PC386, Merck), p21WAF1/CIP1 (catalog no. M7202, Dako (Baar, Switzerland)), p27kip1 (catalog no. M7203, Dako), cyclin D1 (catalog no. 2926, Cell Signaling Technology/Bioconcept), cyclin D3 (catalog no. 2936, Cell Signaling Technology/Bioconcept), cyclin-dependent kinase 4 (catalog no. 2906, Cell Signaling Technology/Bioconcept), cyclin-dependent kinase 6 (catalog no. 3136, Cell Signaling Technology/Bioconcept), or β-actin (catalog no. ab6276, Abcam plc), according to the manufacturer’s instructions. Horseradish peroxidase-conjugated secondary antibody–primary antibody complexes were revealed on the nitrocellulose membranes using the Lumi-Light (catalog no. 2015200, Roche Applied Science) or Lumi-Light Plus (catalog no. 12015196001, Roche Applied Science) Western blotting substrates. Scanning densitometry was performed using a Bio-Rad Universal Hood II instrument and Quantity One (version 4.5.2) acquisition software (Bio-Rad).

**Cell Cycle Analysis**—For the intra-S phase checkpoint analysis, 5 × 10^5 cells were seeded in 75-cm² bottles. Twenty hours later, the cells were treated with 100 μM etoposide or with DMSO alone as a control. 30 min later, the cells were pulsed with 20 μM EdU for 20 min, trypsinized, and fixed in 4% paraformaldehyde. For G1/S checkpoint assays, MCF-10A cells were seeded at a density of 4 × 10^4 cells/ml. Twenty hours later, samples were treated with 10 μM etoposide or with DMSO alone as a control. After a further 18 h, cells were pulsed with 10 μM EdU for 4 h, trypsinized, and fixed in 4% paraformaldehyde. Fixed cells were stained with the Click-iT EdU Alexa Fluor 488 flow cytometry assay kit (catalog no. C35002, Invitrogen) according to the manufacturer’s instructions and analyzed by flow cytometry using a FACSCalibur machine (BD Biosciences).

**Lactate Measurement**—Lactate was measured in the culture medium as described (17).

**Cell Proliferation Assay**—For the experiments shown in Fig. 2D, cells were maintained for 7 days in medium supplemented with 1% heat-inactivated horse serum and antibiotics without additional supplements. The cells were then trypsinized and seeded at a density of 6.6 × 10^3 cells/cm² in the same medium. 16 h after seeding, the cells were washed with phosphate-buffered saline, the medium was replaced by fresh medium containing 1% heat-inactivated horse serum and antibiotics, and the cells were photographed (six randomly selected fields of 0.54 mm²/75-cm² culture flask) to determine the number of attached cells on day 1. The cells were subsequently grown in medium containing 1% horse serum without additional supplements and photographed each day over a period of 12 days. Parallel cultures were counted with a hemocytometer to confirm the results obtained by photography. As a complementary approach (supplemental Fig. S4), MCF-10A cells were seeded at a density of 5000 cells/16-mm well in complete medium. After 24 h, the cells were transferred in minimally supplemented serum-free medium (Dulbecco’s modified Eagle’s medium/F-12 with 1 μg/ml insulin and 0.5 ng/ml EGF) and grown for 7 days. Cells in triplicate wells were counted with a hemocytometer in three independent experiments.

For the analysis of cell proliferation shown in Fig. 6A, MCF-10A LacZ or MCF-10A ΔATM cells were seeded in triplicate in 6-well plates at 2 × 10^4 cells/well in complete medium and grown for 1 week. Medium was renewed 3 days after seeding. In Fig. 6C, MCF-10A cells were seeded in triplicate in 6-well plates at 5 × 10^3 cells/well in the presence of 1 μM KU-55933 or the same volume of DMSO alone (1:1000) as a control. KU-55933 (1 μM) or DMSO was renewed after 3 days with fresh medium. Cells were counted after 1 week. In Fig. 6D, primary human mammary gland epithelial (HMGE) cells grown for 3 weeks in the presence of 1 μM KU-55933 or the same volume of DMSO alone (1:1000) were seeded in triplicate in 6-well plates at 2 × 10^4 cells/well and grown for a further week in the presence of 1 μM KU-55933 or DMSO as a control. KU-55933 (1 μM) or DMSO was renewed after 3 days with fresh medium. Cells were counted after 1 week. Cells that excluded trypan blue were counted with a hemocytometer.

**Soft Agar Assay**—Cells were suspended at a concentration of 2 × 10^4 cells/ml in low gelling temperature agarose as described (18) and grown for 14 days.

**Matrigel Assay**—Cells were suspended at a density of 2 × 10^4 cells/ml in 20-μl droplets of growth factor-reduced Matrigel (catalog no. 354230, BD Biosciences) as described (19) and grown for 7 or 14 days.

**Transwell Assay**—Transwells (8.0-μm membrane pores; polycarbonate; diameter, 6.5 mm; catalog no. 3422, Costar/Vitaris AG (Baar, Switzerland)) were coated with 100 μl of 1 mg/ml Matrigel for 5 h at 37 °C. 10^5 cells in 100 μl of medium containing 50% of each supplement (2.5% heat-inactivated horse serum, 2.5 μg/ml insulin, 0.5 μM dexamethasone, and 5 ng/ml EGF) were added to the upper chamber and allowed to migrate for 10 h at 37 °C. The bottom chamber was filled with 650 μl of the normal culture medium. Cells on the upper surface of the membrane were removed using a cotton swab, and cells on the lower surface were fixed in methanol for 15 min and stained with 0.2% crystal violet in 2% ethanol. Wells were photographed using a Nikon Eclipse TS100 inverted microscope, and quantification was performed using MetaMorph (version 7.5.6.0) (Molecular Devices, Sunnyvale, CA). Experiments were performed in triplicate.

**53BP1 (p53-binding Protein 1) Immunofluorescence (IF)—**IF was performed as described (20). IF with phospho-Ser-139 histone H2AX antibody JBW301 (catalog no. 05-636, Millipore) was performed as described (20). Immunofluorescence was performed with Ki67 (catalog no. M7201, Dako (Baar, Switzerland)), p53 Ser(P)-15 (catalog no. PC386, Merck), p21WAF1/CIP1 (catalog no. M7202, Dako (Baar, Switzerland)), or β-actin (catalog no. ab6276, Abcam plc), according to the manufacturer’s instructions.

**Array Comparative Genomic Hybridization (CGH)—**DNA was extracted from cells following standard protocols. The array CGH was performed using Agilent human genome CGH microarray kit 44K (Santa Clara, CA). Labeling and hybridization were performed following the protocols provided by the manufacturer. All slides were scanned on an Agilent DNA microarray scanner. Data were obtained by Agilent feature extraction software version 9. Data were analyzed with Agilent CGH analytics version 3.4 software, using the statistical algorithms zscore and ADM-2 according to a sensitivity threshold at 2.5 and 6.0, respectively, and a moving average window of 0.2 Mb. Mapping data were analyzed on the human genome sequence using the NCBI data base Build 35, Hg17 (available on
the World Wide Web). Copy number variations were checked in the data base of genomic variants (May 2004 Assembly) (hg17).

Real-time Quantitative PCR—cDNA was synthesized from 1 μg of total RNA using random hexamers and Superscript III reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. SYBR Green assays were designed using the program Primer Express v 2.0 (Applied Biosystems (Rotkreuz, Switzerland)) with default parameters. Amplicon sequences were aligned against the human genome by BLAST to ensure that they were specific for the gene being tested. Oligonucleotides were purchased from Invitrogen. The efficiency of each primer sequence was as follows: CDKN1A/p21/VAF1/CIP1, TCAGAGAGGAGCACTATGT and TGGTCACTGGCGG AAGA; CDKN1B/p27/KIP1, CGACCTGAACCGACGAT and TGTTCTGTTGGCTCTTTTGTTTTG; ATM, TGCTGACAATCTACCCAAATGTC and TCTTCCCCAGTGT CCTGGA; OAS1, AGGTGTTAAGGTTGCGTCC and ACAACCAGGTCACGAGT. PCRs (10-μl volume) contained diluted cDNA, 2× Power SYBR Green Master Mix (Applied Biosystems), and 300 nm forward and reverse primers. PCR were performed on a SDS 7900 HT instrument (Applied Biosystems) with the following parameters: 50 °C for 2 min, 95 °C for 10 min, and 45 cycles of 95 °C for 15 s and 60 °C for 1 min. Each reaction was performed in three replicates on a 384-well plate. Raw Ct values obtained with SDS version 2.2 (Applied Biosystems) were imported in Excel, and normalization factor and -fold changes were calculated using the GeNorm method (21).

Xenografts—2 × 10^6 transformed MCF-12Akd4 cells or MCF-12ALacZ cells at the same number of population doublings (PDs) after transfection were resuspended in 100 μl of growth factor-reduced Matrigel (catalog no. 354230, BD Biosciences; 4.6 mg/ml) and injected into the mammary fat pad of 6-week-old NOD. CB17/−/Prkdc/scid female mice (Charles River Laboratories, Lenti, France) (three mice per cell line), according to institutional animal work guidelines. Mice were sacrificed 3 months after injection. The same cells were tested in vitro by the agarose gel assay, the Matrigel assay, or conventional two-dimensional cultures as internal controls.

Fluorescence in Situ Hybridization (FISH)—FISH for human HER2/neu or α satellite DNA located at the centromere of human chromosome 17 (17p11.1-q11.1) was performed using the Abbott Molecular PathVysion HER-2 DNA probe kit (catalog no. 30-161060, Abbott) according to the manufacturer’s instructions.

RESULTS

We stably suppressed ATM expression by shRNA in the MCF-10A cell line. MCF-10A cell stable transfectants expressing the ATM shRNAs 4, 5, and 6 (MCF-10Akd4, MCF-10Akd5, and MCF-10Akd6, respectively) consistently showed lower levels of ATM than the cells carrying the LacZ shRNA-expressing vector (MCF-10A LacZ). In MCF-10Akd5 or MCF-10Akd6 cells, scanning densitometry revealed a 55 or 65% reduction, respectively, compared with MCF-10A LacZ cells. The strongest silencing effect was observed in the subline MCF-10Akd4, where ATM levels were virtually undetectable (Fig. 1, A and B). By real-time PCR, ATM mRNA levels were ~2.6-fold lower in MCF-10Akd4 cells than in MCF-10A LacZ cells (supplemental Fig. S1A). 95% inhibition of ATM expression was observed in MCF-12A stable transfectants expressing the ATM shRNA 4 (MCF-12Akd4) (Fig. 1A). Compared with the parental MCF-10A cell line, no up-regulation of OAS1 (2′,5′-oligoadenylate

FIGURE 1. shRNA-mediated stable silencing of ATM and functional analysis of ATM-deficient MCF-10A cells. A, Western blotting for ATM and NBS1 on total protein extracts of MCF-10A, MCF-12A, C26Ci, or HaCaT cells stably transfected with ATM shRNA vector 4, 5, or 6 (kd4, kd5, or kd6, respectively) or with a LacZ shRNA vector as a control (LacZ). The numbers on the left indicate kDa. One of at least two experiments with similar results per cell line is shown. B, MCF-10Akd4 cells (kd4) or MCF-10Akd2 cells (LacZ) were incubated for 30 min in the absence or presence of 20 nm etoposide or DMSO alone as a control, pulsed with 20 μM Edu for 20 min, trypsinized, fixed in 4% paraformaldehyde, and stained with Alexa Fluor 488 azide for replicative DNA synthesis. The graph shows the amount of DNA synthesis in etoposide-treated cells relative to cells treated with DMSO alone with or without S.E. from three independent experiments. *, p < 0.026, t test. C, MCF-10Akd4 cells or MCF-10Akd2 cells were incubated for 30 min in the presence of 100 μM etoposide or DMSO alone as a control, pulsed with 20 μM Edu for 20 min, trypsinized, fixed in 4% paraformaldehyde, and stained with Alexa Fluor 488 azide for replicative DNA synthesis and with 7-aminoactinomycin D for DNA content. The graph shows the number of cells in S phase in etoposide-treated cells relative to cells treated with DMSO alone ± S.E. from three independent experiments. *, p < 0.026, t test.

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1) mRNA levels was observed in MCF-10ALacZ or MCF-10Akd4 cells (supplemental Fig. S1B), demonstrating that the shRNA vector system we used does not induce an interferon response (22).

In response to the DNA-damaging drug etoposide or neocarzinostatin (14, 23) MCF-10Akd4 cells showed a strongly reduced capacity to phosphorylate the well characterized ATM substrates p53 Ser-15 or NBS1 Ser-343 when compared with MCF-10ALacZ cells (Fig. 1B) (data not shown). As expected on the basis of the strong ATM silencing, no residual ATM Ser-1981 phosphorylation could be detected in MCF-10Akd4 cells (Fig. 1D). MCF-10Akd6 cells displayed a partially reduced capacity to phosphorylate p53 Ser-15 in response to etoposide or neocarzinostatin (data not shown), consistent with the partial inhibition of ATM expression (Fig. 1A). Because phosphorylation of NBS1 Ser-343 and p53 Ser-15, in response to DNA damage, contributes to the activation of the intra-S and G1/S checkpoints by ATM, respectively (1, 24), we assessed the capacity of MCF-10A stable transfectants to activate these checkpoints. A small but significant defect of the intra-S checkpoint was observed in MCF-10Akd4 cells compared with MCF-10ALacZ cells in response to etoposide (Fig. 1C). At the G1/S checkpoint, MCF-10Akd4 cells strongly reduced the fraction of cells in S phase in response to etoposide, whereas MCF-10Akd6 cells showed a partially reduced capacity to phosphorylate p53 Ser-15 in response to etoposide or neocarzinostatin (data not shown), consistent with the partial inhibition of ATM expression (Fig. 1A). Because phosphorylation of NBS1 Ser-343 and p53 Ser-15, in response to DNA damage, contributes to the activation of the intra-S and G1/S checkpoints by ATM, respectively (1, 24), we assessed the capacity of MCF-10A stable transfectants to activate these checkpoints. A small but significant defect of the intra-S checkpoint was observed in MCF-10Akd4 cells compared with MCF-10ALacZ cells in response to etoposide (Fig. 1C). At the G1/S checkpoint, MCF-10Akd4 cells strongly reduced the fraction of cells in S phase in response to etoposide, whereas MCF-
Akd4 cell arrest was much less efficient (Fig. 1D and supplemental Fig. S2). Thus, MCF-10Akd4 cells display defective phosphorylation of NBS1 Ser-343 and p53 Ser-15 and defective intra-S and G1/S cell cycle checkpoints in response to DNA damage, expected features of ATM-deficient cells.

In low passage cultures, the MCF-10A and MCF-12A transfectants with stable ATM silencing were similar to LacZ shRNA-expressing controls or to the parental cell lines in terms of cell shape, contact inhibition in postconfluent monolayers, and inability to grow in soft agar (data not shown). However, by ~20 population doublings (PDs), MCF-10A and MCF-12A cultures with stable ATM silencing displayed foci of cellular transformation (Fig. 2A) (data not shown). Upon additional passaging, cells with a transformed phenotype overgrew the morphologically normal population (Fig. 2A, MCF-10Akd6). Lactate concentration was markedly increased in the medium of morphologically transformed MCF-10Akd5 and MCF-10Akd6 cells when compared with MCF-10A LacZ cells (Fig. 2C), demonstrating a switch from oxidative phosphorylation to anaerobic metabolism (Warburg effect), a hallmark of cancer cells (25). Lactate concentration also increased in the medium of morphologically transformed MCF-10Akd5 and MCF-10Akd6 cells (data not shown). Similar results were obtained with MCF-10Akd4kd5 stable transfectants, which stably express an shRNA against a fourth ATM target (CATGAGCCAGCAAATTCTA) giving 65% inhibition of ATM expression (data not shown).

Foci of cellular transformation were observed in six of six transfections of MCF-10A cells with either full (MCF-10Akd4) or partial (MCF-10Akd5, MCF-10Akd6, or MCF-10Akd4kd5) ATM silencing, as well as in MCF-12Akd4 cells where ATM expression remains detectable (Fig. 1A). Partial expression of ATM persisted in morphologically transformed cultures of MCF-10Akd5 and MCF-10Akd6 cells as well as in transformed MCF-12Akd4 cells (Fig. 2B and supplemental Fig. S3), ruling out the possibility that foci in MCF-10Akd5, MCF-10Akd6, and MCF-12Akd4 cells, which have partial ATM silencing, originated from an expanding subset of rare cells with full ATM silencing. Further evidence that morphologically transformed MCF-12Akd4 cells retain partial ATM expression was obtained by analyzing ATM protein levels in cells derived from individual colonies of MCF-12Akd4 cells that had grown in the agarose gel assay (see below).
The capacity to grow in the absence of exogenous growth factors is considered as an early feature of malignant transformation (26). In contrast to the MCF-10A parental cell line, which, in addition to serum, requires several mitogens to achieve growth (6–8), morphologically transformed MCF-10Akd4 cells, but not MCF-10ALacZ cells, proliferated extensively in medium containing 1% serum as the only supplement (Fig. 2D). Alternatively, MCF-10A transfectants were grown in a chemically defined serum-free medium consisting of Dulbecco’s modified Eagle's medium/F-12, 1 μg/ml insulin, and 0.5 ng/ml EGF. Seven days after plating (0.5 × 10⁴ cells/16-mm well), morphologically transformed MCF-10Akd4 cells underwent an ~10-fold increase in cell number, whereas MCF-10Akd6 cells remained virtually quiescent (supplemental Fig. S4). Morphologically transformed MCF-10Akd6 and MCF-12Akd4 cells displayed a similar but less marked capacity to proliferate under the same experimental conditions (data not shown).

The capacity to survive and proliferate in the absence of substrate adhesion is a hallmark of cultured tumor cells (27). Morphologically transformed MCF-10A and MCF-12A cells with stable ATM silencing from six of six transfections formed colonies in agarose gels, whereas LacZ shRNA-expressing controls showed very little or no growth (Fig. 3, A and B). MCF-10Akd4 and MCF-12Akd4 cells formed colonies up to several hundred μm in diameter, thus comparable in size with those formed by MCF-7 breast cancer cells (Fig. 3B). MCF-10Akd4 cells formed smaller colonies (Fig. 3, A and B), and, compared with the latter, MCF-10Akd5 cells displayed little growth in agar (data not shown), mirroring the efficiency of ATM silencing (Figs. 1A and 2B). MCF-10Akd6 cells gave results similar to MCF-10Akd4 cells (data not shown). The results of Fig. 2B and supplemental Fig. S3 show that morphologically transformed MCF-10Akd5, MCF-10Akd6, or MCF-12Akd4 cells retain partial ATM expression, suggesting that partial inhibition of ATM expression is sufficient for the promotion of cellular transformation. To confirm that ATM expression in these cells is not due to residual, non-transformed cells, we analyzed cells derived from individual colonies of MCF-12Akd4 cells that had grown in agarose. Three colonies of ~300 μm in diameter were recovered from agarose gels individually, dissociated by trypsinization, and grown in culture to obtain a number of cells (~2 × 10⁶) sufficient for Western blotting. In the three independent colonies of MCF-12Akd4 cells analyzed, the levels of ATM protein expression were not lower than those of the MCF-12Akd4 total cell population (supplemental Fig. S5), supporting the hypothesis that partial inhibition of ATM expression is sufficient for MCF-10A and MCF-12A cell transformation to occur.

In Matrigel cultures, transformed MCF-10Akd4, MCF-10Akd5, and MCF-10Akd6 cells from three of five tested transfections formed spherical colonies with an invasive behavior (i.e. that formed radial outgrowths or linear chains of cells that extended into the surrounding matrix) (Fig. 3C). This phenomenon was never observed with MCF-10Akd6 cells, which formed exclusively spherical, acini-like structures, as reported (9) (Figs. 3C and 4A). By counting the fraction of colonies that displayed invasion versus colonies that remained spherical after 14 days, we found that transformed MCF-10Akd4 cells were slightly more invasive than MCF-10Akd6 cells, whereas the difference between MCF-10Akd4 and MCF-10Akd5 cells was not statistically significant (Fig. 4A). To assess the invasive potential of morphologically transformed MCF-10A cells with stable ATM silencing by another approach, we tested them in a short term invasion assay using Transwells coated with Matrigel. Ten hours after seeding on the Matrigel coating, morphologically transformed MCF-10Akd4 cells had migrated considerably more than MCF-10Akd6 cells (Fig. 4B), whereas morphologically transformed MCF-10Akd5 or MCF-10Akd6 cells displayed little invasion in the Transwell assay (data not shown).

Cells isolated from A-T patients exhibit genomic instability (28). For array CGH analysis, we selected the transformed MCF-10Akd4 stable transfectants that formed the largest colonies in agarose gels (Fig. 3B) in order to increase the probability...
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Homogeneous genomic changes in transformed MCF-10A cells with stable ATM silencing or pharmacological ATM inhibition (KU-55933) relative to controls as assessed by comparative genomic hybridization

| Chromosome/band | Size Position | Region Size Position |
|-----------------|---------------|----------------------|
| MCF-10A<sup>kd4</sup> | 3pter-q13.12 109 Mb From 224,727 to 109,287,093 | Xpter-p21.3 22.1 From 2,782,031 to 24,943,736 |
|                 | 5q11.1 68.4 Mb From 49,725,929 to 118,203,647 | 12q13.2-q13.3 2.0 From 53,701,785 to 55,730,145 |
|                 | 7q36.1 0.2 Mb From 151,977,025 to 152,183,246 | 12p13.21-q21.3 2.7 From 44,303,563 to 44,305,295 |
|                 | 10pter-p11.21 38.5 Mb From 138,206 to 38,468,053 | 12p13.21-q21.3 2.7 From 44,303,563 to 44,305,295 |
|                 | 11pter15.5 1.2 Mb From 463,677 to 1,733,391 | 12p13.21-q21.3 2.7 From 44,303,563 to 44,305,295 |
|                 | 14qter 2.2 Mb From 103,705,475 to 105,947,193 | 12p13.21-q21.3 2.7 From 44,303,563 to 44,305,295 |
|                 | 17qter15.1 2.6 Mb From 75,982,179 to 78,623,371 | 12p13.21-q21.3 2.7 From 44,303,563 to 44,305,295 |
|                 | 19q13.32 0.5 Mb From 50,357,943 to 50,898,442 | 12p13.21-q21.3 2.7 From 44,303,563 to 44,305,295 |
|                 | 21qter22.3 0.2 Mb From 44,303,563 to 44,305,295 | 12p13.21-q21.3 2.7 From 44,303,563 to 44,305,295 |
|                 | 22q11.21-qter13.33 32.2 Mb From 17,767,772 to 49,522,271 | 12p13.21-q21.3 2.7 From 44,303,563 to 44,305,295 |
|                  | Xq28 0.2 Mb From 152,138,406 to 152,373,855 | 12p13.21-q21.3 2.7 From 44,303,563 to 44,305,295 |
|                  |                        |                        |
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Thus, the MCF-10A cell-specific transforming effect of ATM cell-restricted makes the possibility of shRNA off-target effects unlikely (30).

ATM includes several functional domains, including a phosphoinositide 3-kinase-related protein kinase domain (1). KU-55933 is a highly specific and well characterized inhibitor of ATM catalytic activity. In cultured cells, KU-55933 inhibits ATM at concentrations of 0.3–10 μM (15, 16). To assess whether the transforming effect of ATM shRNA on MCF-10A cells is mediated by the inhibition of ATM catalytic activity, we performed long term cultures of MCF-10A cells in the presence of KU-55933 (1 or 10 μM). Compared with MCF-10A cells cultured in the presence of DMSO (solvent) alone, where only minor alterations of cell shape were observed, MCF-10A cells chronically cultured in the presence of 1 μM KU-55933 developed foci of cellular transformation resembling those observed following ATM shRNA silencing, ~20 PDs after KU-55933 treatment had been initiated (Fig. 2A). They also exhibited evidence of a Warburg effect (Fig. 2C) and, when analyzed by array CGH revealed an extended chromosome 10p deletion similar to that observed in MCF-10A<sup>kd4</sup> cells, as well as telomere losses (Table 1). As in the ATM silencing experiments, HaCaT or HK-2 cells cultured in parallel under the same experimental conditions did not show foci in two-dimensional cultures or growth in agarose gels (supplemental Fig. S7) (data not shown).

By 53BP1 IF, the four cell lines exhibit a similar number of DSBs/nucleus under normal culture conditions (MCF-10A, 3.09 ± 0.61; C26Ci, 2.73 ± 0.36; HK-2, 4.42 ± 0.82; HaCaT, 4.55 ± 0.35; at least 200 nuclei/cell line were counted). Phospho-Ser-139 histone H2AX IF in MCF-10A cells gave similar results (4.48 ± 0.66 DSBs/nucleus; 41 nuclei were counted). Thus, the MCF-10A cell-specific transforming effect of ATM...
inhibition does not correlate with a higher basal level of DSBs/cell in this cell line.

By 35 PDs in culture with 1 mM KU-55933, MCF-10A cells did not grow in agarose gels or in two-dimensional cultures using medium supplemented with 1% serum (data not shown). We speculate that the incompletely transformed phenotype observed in KU-55933-treated cells compared with that achieved by shRNA may be attributed to the more robust and permanent effect of the shRNA that suppresses ATM expression. Despite this limitation, the effect of KU-55933 confirmed the specificity of the ATM shRNA results as well as the cell line-restricted effect of ATM inhibition. It also demonstrated that the transforming effect of ATM silencing is mediated in part by the inhibition of ATM catalytic activity.

As the next step, we wanted to assess the effect of stable ATM silencing on cellular growth in vivo. To obtain representative data, we chose the transformed MCF-12A\textsuperscript{kd4} cells, which show intermediate growth in the agarose assay (Fig. 3B). Cells were resuspended in growth factor-reduced Matrigel and injected into the mammary fat pad of NOD/SCID female mice. The mice were sacrificed after 3 months, a time point at which MCF-7 and MDA-MB-231 cells, two well characterized human breast cancer cell lines, form overt tumors in this model (data not shown). Under the conditions used, the Matrigel was only partially reabsorbed. Histological analysis showed that inoculated cells were still surrounded by abundant extracellular material, representing partially remodeled Matrigel matrix (Fig. 5). Within this environment, transformed MCF-12A\textsuperscript{kd4} cells formed disorganized nests of cells that infrequently contained lumen-like cavities and lacked the typical palisade arrangement of normal ductal or alveolar epithelium. In addition, the cells displayed a heterogeneous nuclear/cytoplasmic ratio (Fig. 5, A–D) characteristic of dysplastic lesions. Formation of palpable tumor masses was not observed during the time period examined. The human origin of these cells was confirmed by FISH with human-specific probes for HER2/neu or \(\alpha\) satellite DNA located at the centromere of chromosome 17 (Fig. 5, E and F). Analysis of mice injected with MCF-12A\textsuperscript{LacZ} cells showed that the Matrigel matrix was exclusively populated by invading fibroblasts and microvessels, whereas nests of epithelial cells were never observed (Fig. 5, G and H). Similar results were obtained with morphologically transformed MCF-10A\textsuperscript{kd5} and MCF-10A\textsuperscript{kd6} cells compared with MCF-10A\textsuperscript{LacZ} cells (data not shown). Thus, although in the 3-month time period investigated morphologically transformed MCF12Akd4, MCF-10A\textsuperscript{kd5}, and MCF-10A\textsuperscript{kd6} cells do not form macroscopic tumors, they are nonetheless endowed with a robust growth advantage that allows them to survive and to form dysplastic lesions in vivo.

As the main drive of selection for mutations that provide a growth advantage, increased proliferative capacity is the clearest hallmark of cancer. To investigate the mechanisms by which ATM inhibition leads to MCF10-A cell transformation, we analyzed cell proliferation in MCF-10A\textsuperscript{kd4} cells before cellular transformation had occurred (absence of foci). Compared with MCF-10A\textsuperscript{LacZ} cells, a 2.2-fold increase in cell proliferation was observed in MCF-10A\textsuperscript{kd4} cells (Fig. 6, A and E). This increase was accompanied by a 2.4-fold parallel increase in the fraction of cells in S phase (Fig. 6B). To corroborate this result by another approach, we incubated MCF-10A parental cells for 1 week in the presence of KU-55933 at the same dose that induces the formation of foci (1 mM) or the equivalent volume of DMSO as a control. Again, we observed a 2.1-fold increase in cell proliferation in KU-55933-treated cells (Fig. 6C). In contrast, a survey of cell proliferation in HaCaT, HK-2, or C26Ci cells did not show an increase in cell proliferation by either stable ATM
silencing or KU-55933 1 μM or 3 μM administration under the same experimental conditions (supplemental Fig. S9). Rather, these cell types showed an overall trend to decreased proliferation (supplemental Fig. S9), which is consistent with the known reduced capacity of ATM-deficient cells to grow (1). To investigate the possibility that the unusual effect of ATM inhibition on MCF-10A cell proliferation was due to proliferation-predisposing mutations in the MCF10-A cell line, we studied cell proliferation in HMGE cells incubated with KU-55933 1 μM or DMSO as a control. ATM inhibition strikingly increased cell proliferation in HMGE cells by 4.3-fold, although, compared with MCF-10A cells, its proliferative effect appeared gradually and peaked after 4 weeks of continuous KU-55933 administration (Fig. 6, D and E) (data not shown). The effect of KU-55933 on HMGE cell proliferation could be not studied beyond 8 weeks, due to a deleterious effect of long term DMSO administration on HMGE cell viability. A survey of non-transformed MCF-10A or HMGE cells with stable ATM silencing or incubated in the presence of KU-55933 for different time periods did not reveal consistent differences in the rate of apoptosis (supplemental Fig. S10), thus ruling out the possibility that increased cell numbers in MCF-10A or HMGE cells with ATM inhibition were due to a decrease in the basal level of apoptosis mediated by ATM. Thus, ATM inhibition has a strong growth-promoting activity in human mammary gland epithelial cells, which is not dependent on the immortalizing mutation(s) of the MCF-10A cell line, because it is also observed in primary cells.

In order to identify potential mediators of the proliferative effect of ATM inhibition, we studied the early response of a panel of cell cycle genes in MCF-10A or HMGE cells incubated with 1 μM KU-55933 forvarious lengths of time. In HMGE cells, KU-55933 massively down-regulated p21WAF1/CIP1 and

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**FIGURE 6.** ATM inhibition increases proliferation in MCF-10A and HMGE cells. A, untransformed MCF-10Akd4 (kd4) or passage-matched MCF-10ALacZ (LacZ) cells (both 10 PDs after transfection) were seeded in 6-well plates at 2 × 10⁴ cells/well and counted after 1 week. Values on the y axis represent the mean number of cells ± S.E. from three independent experiments. *, p = 1.01−15, t test. B, in parallel cultures, untransformed MCF-10Akd4 or passage-matched MCF-10ALacZ cells (both 10 PDs after transfection) were pulsed with 20 μM EdU for 20 min, trypsinized, fixed in 4% paraformaldehyde, stained with the Click-IT Edu Alexa Fluor 488 flow cytometry assay kit, and analyzed by flow cytometry. Error bars, S.D. from two independent experiments. C, MCF-10A cells were seeded in 6-well plates at 5 × 10³ cells/well, incubated with 1 μM KU-55933 (KU) or DMSO alone as a control for 1 week, and counted. Values on the y axis represent the mean number of cells ± S.E. from three independent experiments. *, p = 1.29−08, t test. D, HMGE cells cultured for 3 weeks in the presence of DMSO alone or 1 μM KU-55933 were seeded in 6-well plates at 2 × 10³ cells/well, grown in the presence of 1 μM KU-55933 or DMSO alone as a control for a further week, and counted. Values on the y axis represent the increase in the number of cells ± S.D. from two independent experiments relative to treatment with DMSO alone. E, phase-contrast view of MCF-10Akd4 cells, MCF-10Akd4 cells, and HMGE cells incubated with DMSO alone (D) or with 1 μM KU-55933 at the end of the proliferation assay. Bar, 100 μM.
p27^KIP1 protein expression at 24 h (96 or 85%, respectively, compared with DMSO for 24 h), whereas DMSO had little or no effect (Fig. 7A). In contrast, little or no regulation of p21^{WAF1/CIP1} or p27^KIP1 mRNAs was observed (Fig. 7B). Similarly, in MCF-10A cells, KU-55933 decreased p21^{WAF1/CIP1} by 81% and p27^KIP1 by 50% after 16 h, whereas DMSO alone had little or no effect (Fig. 8A) (data not shown). In contrast, KU-55933 had little or no effect on the levels of p53, cyclin D1, cyclin D3, cyclin-dependent kinase 4, and cyclin-dependent kinase 6 (Fig. 8A). At the mRNA level, 1 µM KU-55933 decreased p21^{WAF1/CIP1} expression by 1.9-fold, whereas it had no effect on p27^KIP1 (Fig. 8B). At 42 h, p21^{WAF1/CIP1} and p27^KIP1 proteins were reduced by 90 or 62%, respectively (Fig. 8C). In contrast, there was little or no effect on the respective mRNAs (Fig. 8D). Similar results were obtained in a 72-h incubation (supplemental Fig. S11A). To confirm the regulatory effect of ATM inhibition on p21^{WAF1/CIP1} and p27^KIP1 protein levels, we treated MCF-10A cells with caffeine, another ATM inhibitor, or transiently transfected them with ATM siRNA. Caffeine reduced p21^{WAF1/CIP1} and p27^KIP1 siRNA on MCF-10A cell proliferation is consistent with the fact that it efficiently inhibits ATM Ser-1981 phosphorylation by a saturating dose of etoposide in C26Ci cells at the same concentrations (supplemental Fig. S12B). This correlated with the lack of effect of ATM inhibition on C26Ci, HK-2, and HaCaT cell proliferation (supplemental Fig. S9). Taken together, these results raised the possibility that the transient down-regulation of p21^{WAF1/CIP1} and p27^KIP1 proteins observed in MCF-10A cells following ATM inhibition might be causally involved in the proliferative effect of ATM inhibition.

To investigate this possibility, we transiently transfected MCF-10A cells with p21^{WAF1/CIP1} or p27^KIP1 siRNAs that efficiently inhibit the expression of these proteins to a similar extent (85–90%; Fig. 10A). A single transfection of two different p21^{WAF1/CIP1} siRNAs (p21_6 or p21_7) slightly decreased MCF-10A cell proliferation when tested alone or when co-transfected with the p21_7 siRNA at equimolar concentrations (Fig. 10B). The slightly negative effect of the p27_6 siRNA on MCF-10A cell proliferation is consistent with the notion that p27^KIP1 facilitates the assembly and nuclear import of cyclin D1-Cdk4 complexes (31). Thus, in MCF-10A cells, simultaneous silencing of p21^{WAF1/CIP1} and p27^KIP1 protein expression that mimics the regulatory effect of ATM inhibition on these cell cycle regulators results in a 2.4-fold net proliferative increase, similar to that observed after ATM inhibition (Fig. 6, A–C) and driven by p21^{WAF1/CIP1} silencing.

**DISCUSSION**

This study provides the first direct experimental evidence that loss of ATM protein or catalytic activity promotes, in a normal human cell type, morphological, functional, and genetic changes that are typical of malignant transformation. Remarkably, among the cell types we tested, this effect was restricted to mammary gland-derived epithelial cells. Although we studied a...
limited number of other cell types, ATM inhibition induced features of cellular transformation or increased cellular proliferation in the three mammary gland epithelial cell types we explored, whereas no such effect was observed in C26Ci fibroblasts, HaCaT keratinocytes, or HK-2 renal epithelial cells despite the presence of known cancer-predisposing mutations in HaCaT and HK-2 cells. These findings corroborate epidemiological data indicating that constitutional ATM alterations selectively predispose to breast cancer (1–4) and reveal an exquisite sensitivity of the human mammary gland epithelium to ATM loss of function. Furthermore, our observations are in agreement with other studies that failed to demonstrate any transforming propensity for fibroblast or lymphoid cells derived from A-T patients or A-T heterozygotes. To our knowledge, our findings also provide a rare or possibly the first example of cellular transformation in vitro induced by the deficiency of a DNA repair gene.

The diverse phenotypic and genetic alterations we observed in MCF-10A and MCF-12A cells by selectively interfering with ATM function (namely increased proliferation, loss of contact inhibition, independence from growth factors, genetic instability, and the capacity to form dysplastic lesions in mice) are compatible with the repertoire of dysregulations generally ascribed to the early stages of tumor formation (26). In that context, the growth as dysplastic lesions in vivo is indicative of a partially tumorigenic phenotype, such as would be expected of cells undergoing the initial steps of the carcinogenic process. This is consistent with the view that ATM is a low penetrance cancer gene conferring a modest increase of breast cancer risk when mutated (1–4). Despite the limited growth potential of transformed MCF-12Akd4, MCF-10Akd6, and MCF-10Akd6 cells in vivo, ATM silencing appears to have conferred a considerable growth advantage to these cells because the LacZ control not only did not grow but appeared not to survive in vivo. Although some of the parameters of cellular transformation we investigated (e.g. the growth in agarose gels) appeared to be dependent on the level of ATM inhibition to some extent, inhibition of ATM expression leading to /\H1101150% reduction of ATM protein levels was sufficient to promote the transformation process in our model. In our view, this result is important because it mirrors the breast cancer susceptibility of A-T carriers, where the loss of the wild type ATM allele in breast tumors appears to be relatively infrequent (32, 33).

The activation of the DNA damage response in precancerous lesions has recently been proposed to act as a widespread barrier to cancer development (34, 35). According to this scenario, DSBs resulting from oncogene-induced DNA replication stress lead to activation of the ATM/p53 pathway in precancerous lesions, thus halting cellular transformation by inducing apoptosis or senescence. In this context, loss of ATM or of a downstream effector would act as a rare second hit that allows tumor progression in cells already engaged in the transformation process. Because MCF-10A cells are immortalized, it could be
argued that they carry oncogenic mutation(s) susceptible to activate the DNA damage response and become transformed when ATM is inhibited. Our findings do not support this hypothesis; when compared with primary human mammary gland epithelial cells by Western blotting, MCF-10A cells did not show signs of an ongoing DNA damage response based on the levels of p53, p53 Ser(P)-15, p21, and, in particular, of their ATM inhibition had little or no effect on the basal levels of apoptosis, total p53 protein, or p21WAF1/CIP1 mRNA levels in the way to cellular transformation, appears to be an upstream event that, in itself, triggers transformation in a normal epithelium, possibly by allowing the random accumulation of unrepaird DSBs, thus generating mutations that are subsequently selected for if they provide a growth advantage. Consistent with this interpretation, MCF-10A and MCF-12A cells with stable ATM inhibition require up to ~20 PDs to become transformed and accumulate mutations as shown by the array CGH. The repertoire of chromosomal abnormalities we found in transformed MCF-10A cells with stable ATM silencing is striking because all of the extended deletions we found (3p, 5q, 10p, and 22q) are frequent in human cancer and, in particular, in breast cancer (36–39). This raises the possibility that these mutations might contribute to the transformation process we observed. It also suggests that each of the parameters of transformation we studied might be dependent on a specific chromosome deletion or rearrangement and that cellular transformation in MCF-10A or MCF-12A cells with stable ATM silencing results from the accumulation of several such mutations. For example, compared with the MCF-10AAtt cells we analyzed by the array CGH, MCF-10A cells chronically cultured in the presence of KU-55933 carry a limited number of similar chromosomal abnormalities (Table 1), which correlates well with their weaker transformed phenotype. However, the limited number of morphologically transformed MCF-10A cell cultures with stable ATM silencing or chronic ATM inhibition we analyzed by the array CGH does not allow us to draw formal conclusions about the potential, specific contribution of individual mutations to the transformation process.

The effect of ATM inhibition on cell proliferation is surprising because fibroblasts and other cell types with genetic inactivation of ATM analyzed so far are known for their reduced proliferative capacity. Although ATM inhibition restores DNA synthesis or cell proliferation in senescent cells by reducing the cell cycle checkpoint response to telomere shortening or DSBs originating from oncogene-induced DNA replication stress (34, 35), we could not detect signs of senescence in the MCF-10A cell line (see above), and HMGE cells were used several passages before the appearance of replicative senescence. Also, ATM inhibition had little or no effect on the basal levels of apoptosis, total p53 protein, or p21WAF1/CIP1 mRNA levels in

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**FIGURE 9.** Proteasome inhibition rescues p21WAF1/CIP1 and p27KIP1 protein down-regulation mediated by ATM inhibition. MCF-10A cells were incubated for 23 h in the presence or absence of KU-55933 1 μM, followed by 1 h in the presence or absence of epoxomicin 1 μM as indicated (p21WAF1/CIP1, top); or for 20 h in the presence or absence of KU-55933 1 μM, followed by 4 h in the presence or absence of epoxomicin 1 μM as indicated (p27KIP1, bottom). KU-55933 or epoxomicin were diluted 1:1000 in the culture medium starting from 1 mM stocks in DMSO. The same volume (1:1000) of DMSO was added to controls as indicated (−). Total protein extracts were analyzed by Western blotting for p21WAF1/CIP1 or p27KIP1. One of two experiments with similar results is shown.

**FIGURE 10.** Transient RNA interference for p21WAF1/CIP1 increases MCF-10A cell proliferation. A, MCF-10A cells were transfected with p21WAF1/CIP1 (p21_6 or p21_7) or p27KIP1 (p27_6) siRNAs or with two different control siRNAs (CTRL1 or CTRL2). 48 h after transfection, the cells were lysed and analyzed by Western blotting for p21WAF1/CIP1 or p27KIP1. B, 5 × 10⁴ MCF-10A cells/well in 6-well plates were transfected with p21WAF1/CIP1 (p21_6 or p21_7) siRNAs, p27KIP1 (p27_6) siRNA, p21_7 and p27_6 siRNAs in combination or with two different control siRNAs (CTRL1 or CTRL2). The cells were counted after 1 week with a hemocytometer. The graph shows the number of cells ± S.E. from three independent experiments performed in quadruplicate, except for p21_6, for which two experiments were performed and where the error bar indicates S.D. * CTRL2 versus p21_7 + p27_6, p = 0.00017, t test.
MCF-10A or HMGE cells, which is not what one would expect if the ATM/p53 axis was actively responding to endogenous DNA damage. Importantly, the experiments with primary mammary gland epithelial cells rule out the possibility that the positive effect of ATM inhibition on MCF-10A cell proliferation is attributable to concomitant immortalizing mutation(s). Finally, we found it striking that ATM inhibition promotes proliferation in primary mammary gland epithelial cells but not in HaCaT or HK-2 cells that contain known oncogenic mutations. Taken together, our findings suggest that in the human mammary gland epithelium, ATM might have a hitherto unrecognized antiproliferative function in addition to its recognized contribution to the DNA damage response. We propose that the increased proliferation we observe in human mammary gland epithelial cells following ATM inhibition is an important component of the transformation process by driving the selection for mutations, originating from defective cell cycle checkpoints or DNA repair, that provide further growth advantage.

In our experiments, the increase in cell proliferation induced by ATM inhibition correlates with a major decrease of p21\(^{\text{WAF1/CIP1}}\) and p27\(^{\text{KIP1}}\) protein levels, whereas their respective mRNAs showed no or little down-regulation. Although the mechanisms by which ATM decreases p21\(^{\text{WAF1/CIP1}}\) and p27\(^{\text{KIP1}}\) remain to be defined, the marked uncoupling of p21\(^{\text{WAF1/CIP1}}\) protein and mRNA levels suggests that ATM inhibition down-regulates p21\(^{\text{WAF1/CIP1}}\) at the post-transcriptional level, perhaps by regulating protein stability. Consistent with this interpretation, basal p53 Ser-15 phosphorylation is almost undetectable in MCF-10A cells, and p53 shows little or no decrease at the protein level in response to KU-55933. Down-regulation at the level of protein stability by ATM inhibition might also apply to p27\(^{\text{KIP1}}\), whose mRNA is not regulated by KU-55933 despite a massive decrease of the protein. Consistent with this hypothesis, experiments using the proteasome inhibitor epoxomycin showed that ATM inhibition decreases the levels of p21\(^{\text{WAF1/CIP1}}\) and p27\(^{\text{KIP1}}\) through the activity of the proteasome. Interestingly, ATM inhibition did not lead to decreased p21\(^{\text{WAF1/CIP1}}\) or p27\(^{\text{KIP1}}\) protein levels in C26Ci, HaCaT, or HK-2 cells, which correlated with the observation that ATM inhibition does not increase proliferation in these cells.

Because the decrease of p21\(^{\text{WAF1/CIP1}}\) and p27\(^{\text{KIP1}}\) following ATM inhibition is transient, it is possible that this phenomenon represents part of the activation of a proliferation pathway whose main player(s) remains to be identified. Nonetheless, the observation that the silencing of p21\(^{\text{WAF1/CIP1}}\) recapitulates the proliferative effect of ATM inhibition in MCF-10A cells provides a new functional link between ATM loss of function and increased proliferation that might contribute to the transformation process we observed.

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