Abstract. Long non-coding RNAs (lncRNAs) serve critical roles in regulating cellular homeostasis, and their deregulated expression/activity is associated with neoplastic transformation. The maternally expressed gene 3 (MEG3) has been extensively described as a tumor suppressor gene in different types of cancer, including breast cancer. Interestingly, using a panel of seven different breast cancer cell lines, the present study revealed that MEG3 is highly expressed in the triple negative metastatic human Hs578T breast cancer cell line, which is refractory to different therapeutic approaches. Therefore, the present study aimed to investigate the phenotypic impact of MEG3 deletion in this cell line. Using the CRISPR/Cas9 system, complete knockout (KO) of MEG3 was achieved. Deletion was confirmed by genomic PCR and reverse transcription-quantitative PCR. The MEG3_KO cell population displaying the highest efficiency of genomic editing was selected for phenotypic in vitro assays, including wound scratch and Transwell assays, flow cytometry and immunofluorescence. The results demonstrated that MEG3 deletion increased cell proliferation, anchorage-independent cell growth and cell motility, which was consistent with its well-known tumor suppressor function. However, the present study revealed that MEG3_KO also lead to decreased cell invasiveness ability, supporting previous evidence that MEG3 modulates epithelial-to-mesenchymal inducing factors. The present study demonstrated that deletion of MEG3 promoted an increase in transforming growth factor β and N-cadherin protein levels and significant reduction in matrix metallopeptidase 2, zinc-finger E-box binding homeobox 1 and collagen type III α1 chain gene expression levels. Additionally, MEG3_KO cells displayed significant resistance to doxorubicin treatment, demonstrating the role of this lncRNA in cancer cell survival by regulating apoptosis. The present study highlighted the utility of CRISPR/Cas9 for anticancer studies of intergenic lncRNAs and demonstrated that, although Hs578T cells express MEG3 at high levels, these cells display mechanisms to escape the growth suppression effects of this lncRNA. Notably, the detailed pathological mechanisms of MEG3 concerning tumor metastasis remain to be elucidated prior to applying MEG3 expression/activation in future therapeutic approaches for breast cancer treatment.

Introduction

Due to increasingly sophisticated Molecular Biology analytical methods, new groups of non-coding RNAs (ncRNAs) have been described over the past few years (1). These molecules were revealed to be more than simple inert sequences, emerging as functional regulatory components, which play critical roles in modulating chromatin architecture, transcription and RNA splicing, translation and turnover (2). Due to their biological relevance, these ncRNAs were also found to be involved in different human diseases (3), including cancer (4).

Long non-coding RNAs (lncRNAs) are noncoding transcripts of around 200 nucleotides or more (5). The number of annotated sequences greatly improved with third-generation sequencing methods (6), however, the functional characterization of most of these IncRNAs is still lacking (7). Various functional mechanisms have been described for IncRNAs including recruitment of transcription factors for promoter regions, guiding chromatin modifiers to specific genomic loci, allosteric modulation of transcriptional regulatory proteins,
alteration of nuclear domains, modulation of translation or mRNA stability and working as a natural competing endogenous RNAs (5,8,9).

The maternally expressed gene 3 (MEG3) was the first lncRNA proposed to have a tumor suppressor function (10,11). Using cDNA-representational difference analysis, MEG3 expression was not detected in either pituitary tumors, when compared to normal human pituitary tissue, nor in several human cancer cell lines (10). Moreover, ectopic expression of MEG3 RNA suppresses cell growth in different tumor cells (12-14), further supporting the tumor suppressor role of this gene.

Despite all the great advances in the field, breast cancer remains to be the leading cause of cancer death among women between 20 to 59 years old (15,16). The most lethal type of breast cancer is the triple negative breast cancer (TNBC), which lacks the expression of cell receptors for estrogen, progesterone and do not show amplification of the human epidermal growth factor receptor 2 (HER2) gene (17). These characteristics prevent the use of conventional drug therapies and account for approximately 15% of all diagnosed breast cancers (18), highlighting the urgent need for well-defined molecular targets for treatment of this type of cancer.

In silico analysis has suggested that MEG3 could be a valuable prognostic factor and a potential therapeutic target for breast cancer patients, with an impact on disease-free survival, relapse-free survival and progression-free survival (19-21). Consistently, functional studies have shown that overexpression of MEG3 decreases breast cancer cell lines growth rate, invasion capacity, and tumor angiogenesis through downregulation of AKT signaling (22) and by enhancing p53 transcriptional activity (23).

The CRISPR/Cas9 system provides a revolutionary genome-editing tool for all areas of Molecular Biology (24-26). Some techniques have been previously applied to achieve lncRNA deletion, however, the CRISPR/Cas9 approach to target lncRNAs has scarcely been explored in the literature (27-29). Similarly to protein-coding genes, Cas9 nucleases may be used to delete the entire lncRNA gene or to introduce RNA-destabilizing elements into their loci, particularly in their promoter region. Here, using a panel of seven breast cancer cell lines, which are representative of tumor progression and aggressiveness in vitro, we found that MEG3 has a discrepant expression in the triple negative metastatic human Hs578T cell line. To better understand the contribution of the lncRNA MEG3 in breast tumorigensis, we developed a protocol to knockout MEG3 expression by CRISPR/Cas9 and analyzed the phenotypic impact of MEG3 KO using in vitro assays.

Materials and methods

MEG3 expression profiling in breast cancer derived cell lines. Expression profiling was carried out using a panel of breast cancer derived cell lines representing tumor progression, ranging from non-tumorigenic to highly metastatic tumor cells. The following cell lines were obtained from ATCC (American Type Culture Collection): Non-tumoral cell lines MCF10A (CRL-10317; ER-/PR-/AR-/HER2-); MCF12A (CRL-10782; ER-/PR-/AR+/HER2-); T47D (HTB-22; ER+/PR+/AR+/HER2+); ZR-75-1 (CRL-1500; ER+/PR+/AR+/HER2+); and tumor cell lines estrogen-negative SK-BR-3 (HTB-30; ER-/PR-/AR+/HER2+); MDA-MB-231 (HTB-26; ER-/PR-/AR+/HER2-); and Hs578T (HTB-126; ER-/PR-/AR-/HER2-). Replicate experiments were carried out with cells at increasing sequential passage number. 293T cells (CRL-3216) were used for the production of the lentiviral particles. All cell lines were maintained at 37°C with 5% CO2 in specific culture mediums following recommendations suggested by the ATCC.

RNA extraction, cDNA synthesis and reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated from cultured cells with Trizol (Life Technologies) and purified with an RNAspin Mini kit (GE Healthcare) according to the manufacturer’s instructions, with an extended treatment with DNase I for 1 h. Total RNA was quantified using the ND-1000 (NanoDrop) and its integrity was assessed on a Bioanalyzer (Agilent Technologies). For measuring lncRNAs, reverse transcription was performed with the SuperScript III (Life Technologies) followed by qPCR. PCR with 40 cycles and 1 µg of the resulting purified total RNA (without reverse transcription), using different pairs of primers for tubulin TUBA1C gene and Histone H3 (multiple copy gene) were used to confirm absence genomic DNA in all samples previously. For all genes, oligo-dT primer reverse transcription was performed using 1 µg of total RNA in 20 µl of RT reaction with SuperScript III (Life Technologies), followed by qPCR using 5 µl of the 8-fold diluted RT reaction in 20 µl of qPCR (ViiA 7 Real-Time PCR System, Thermo Fisher Scientific). Transcript levels were normalized to HMBS, and represented as relative abundance using the delta Ct method (30). Two controls for the RT step, one without primer (-primer) and the other without reverse transcriptase (-RT) were performed, followed by qPCR with the pair of primers, in order to confirm the absence of RNA self-priming and of genomic DNA contamination in the RT, respectively. Graph design and statistical analyses were carried out using the Graphpad Prism V6 (Graphpad Software). Conditions for PCR reactions were: 40 cycles of 95°C/15 sec, 60°C/1 min, using the following primers: MEG3 forward 5’-TGAAGAACTGCGGATGGAAG-3’ and reverse 5’-CAGTGGCCTACAGGTG-3; HMBS forward 5’-TGACCAGTGGTTGCTACTC-3’ and reverse 5’-CAACACGATCATGAGGTTTTC-3’; TUBA1C forward 5’-TCAACACTTCCTATAGGAAG-3’ and reverse 5’-AGTCCGAGTCGAC-3; CAGAC-3’ and reverse 5’-CGCCTGAACCTTGTCTTC-3’. Knockout of MEG3 in Hs578T breast cancer cells. Small guide RNAs (sgRNAs) were designed using an online CRISPR Design Tool (http://tools.genome-engineering.org) and then cloned (guide sequences Table I) into the pL-CRISPR.EFS.GFP (Addgene #57818, Massachusetts, USA) or lentCRISPR-v2 (Addgene #52961, Massachusetts, USA). Viral particles were produced in 293T cells using fourth-generation lentiviral packaging. Hs578T cells were co-transduced in a ratio 1:1 with sgRNAs targeting the 5’ and 3’ ends of the gene locus. Cell populations were first selected with 6 µg/ml puromycin (Life Technologies) for 48 h and after cell expansion, GFP positive cells were sorted by FACS (BD FACSARIA II, BD Bioscience).

To verify genome-editing efficiency of the different sgRNAs combinations (1+ 3; 1+4; 2+3 and 2+4), transduced
cell populations were further expanded for genomic DNA and total RNA extraction. Genomic DNA was extracted with QIAamp DNA Kit (Qiagen, Venlo, Netherlands) and MEG3 deletion was detected by PCR (30 cycles of 94˚C/1 min, 60˚C/30 sec) using the following primers: forward: 5'-ACCCGAAGTGGGCCGAGTCTAAGGTAAACACCTAGACTCGGCCCACTTC-3'. Total RNA extraction and RT-qPCR were carried out following the same procedure for MEG3 expression profiling characterization. To verify the stability of the MEG3_KO population, we collected samples from passages 0, 5, 10 and 15 to perform FACS sorting (BD FACSARIA II, BD Bioscience) and RT-qPCR. Hs578T wild type (WT) cells were used as control.

**Growth curve and anchorage-independent clonal growth.** 5x10^3 cells from the Hs578T_WT and MEG3_KO 2+4 cell population, were collected samples from passages 0, 5, 10 and 15 to perform FACS sorting (BD FACSARIA II, BD Bioscience) and RT-qPCR. Hs578T wild type (WT) cells were used as control.

**Migration and invasion assays.** For wound-healing scratch assay, 1x10^5 cells were plated in 24 well plates in triplicates. On the following day, the cell layer was scratched using a 200 µl sterile pipette tip. The wound location was marked and images of the same field were captured to record the wound width at 0.8 and 12 h. The area of migrating cells was measure by ImageJ software.

**Immunofluorescence.** For immunofluorescence, cells were seeded onto 13 mm diameter glass coverslips and maintained under usual culture conditions until sub-confluence (less than 80%). Each sample was then fixed in 4% formaldehyde for 10 min, permeabilized with 0.5% Triton X-100 for 10 min and blocked in 1% Bovine Serum Albumin for 60 min, all at room temperature. Primary antibody anti-GFP (1:500, ab6556, Abcam, Cambridge, UK) was incubated overnight at 2-8˚C and washed three times with PBS buffer for 10 min. Alexa Fluor 594-phalloidin (1:100, A12370, Thermo Fisher Scientific, Massachusetts, USA) and secondary antibody AlexaFluor 647 goat anti-mouse IgG (H + L) (1:100) (A21236 Thermo Fisher Scientific) or AlexaFluor 647 goat anti-mouse IgG (H + L) (1:100) (A21236 Thermo Fisher Scientific) were incubated 1 h at room temperature. Coverslips were mounted using VECTASHIELD Anti-fade Mounting Medium with DAPI (H-1200, Vector Laboratories, CA) and images were acquired with a confocal Zeiss LSM 780-NLO microscope.

**Immunofluorescence by high-content screening assays.** After seeding the cells in 96-well plates (Greiner Bio-One, 655986), the cultures were washed with PHEM buffer (2 mM HEPES, 10 mM EGTA, 2 mM MgCl₂, 60 mM PIPES-pH 6.9) and fixed for 1 h with cold 4% PFA. Cells were permeabilized with 0.1% Triton X-100 for 5 min, blocked with 1% bovine serum albumin (BSA) for 30 min, and then incubated with primary antibody overnight at 4˚C. After PHEM-glycine washing (3X), the cells were incubated with the fluorescent dye at room temperature for 1 h and plates were subjected to high-content imaging analysis on MetaXpress High-Content Image Acquisition & Analysis Software (Molecular Devices). The primary antibodies used were mouse anti-N-Cadherin (DAKO, M3613) (1:50) and rabbit anti-TGF-β (Santa Cruz, sc-146) (1:100). After PHEM washing (3X), the cells were incubated with fluorescent dye, namely, FITC-labeled goat anti-rabbit IgG (H + L) (1:100) (A11008 Thermo Fisher Scientific) or AlexaFluor 647 goat anti-mouse IgG (H + L) (1:100) (A21236 Thermo Fisher Scientific) at room temperature for 1 h. Protein expression was determined and quantified through fluorescence signal changes (MFI-Median Fluorescent Intensity) using the Multi Wavelength Cell Scoring module. Cell counts were assessed using Hoechst 33342 (5 µM-Life Technologies, Thermo Fisher Scientific) staining for 1 h, and the stained samples were subjected to fixation in cold methanol for 10 min and staining with Mayer's alum hematoxylin for 20 min. Inserts were mounted in glass slides and six fields per sample were counted, with duplicates for each cell line in each experiment.
high-content imaging analysis. In parallel, the cells were labeled with rabbit anti-GFP (1:1,000) (ABCAM, ab6556) to confirm expression of the reporter protein in MEG3_KO cells. Cytoskeletal F-Actin was marked with AlexaFluor-555 Phalloidin for imaging (1:2,000) (Life Technologies, Thermo Fisher Scientific). The image acquisition and fluorescence intensity measurements were conducted by automatic scanning through the MetaXpress software, using a 40X objective. For each treatment condition and channel, 20 images per well in triplicate, were acquired and analyzed.

Doxorubicin-induced apoptosis. To assess the endogenous modulation of MEG3 expression upon doxorubicin (DXR) treatment (D1515, Sigma-Aldrich, Missouri, USA), 1x10^5 Hs578T parental cells were plated in six-well plate and treated with DMSO or with a sub-lethal concentration of 0.25 µg/ml DXR for 24 h. Cells were then harvested for RT-qPCR analysis. The chemosensitivity of Hs578T_WT and MEG3_KO cells to DXR was determined by MTT assay. Briefly, 10^4 cells/well were seeded onto 96-well plates. On the next day, cells were treated with different concentrations of DXR (range, 0-5 µg/ml). At 24 h post-DXR application, cell metabolism was assessed using 0.5 mg/ml MTT (Sigma-Aldrich, St. Louis, MO, USA) solution. Following 4 h incubation at 37°C, the medium was replaced with 150 µl dimethyl sulfoxide (Sigma-Aldrich) and vortexed for 10 min. The absorbance of each well at 490 nm was measured using a microplate reader (SpectraMax Paradigm, Molecular Devices, California, USA). For apoptosis assay, 10^4 cells/well were seeded onto 6-well plates and incubated overnight under culture conditions. Culture medium was replaced by fresh medium containing DXR (0.5 and 1 µg/ml) and incubated for 24 h. The cells were then harvested, washed with PBS and resuspended in Annexin-V binding buffer (BioVision; #1006). APC conjugated Annexin-V (ThermoFisher Scientific; A35110) was added according to manufacturer's instructions and propidium iodide (ThermoFisher Scientific; P3566) was added at final concentration of 1.0 µg/ml. Samples were kept in the dark for 5 min and the analyses was carried out using the FACSARIA II cytomter. For analysis of BAX and BCL2 protein levels, 1x10^5 cells/ml were trypsinated, centrifuged and fixed in 4% paraformaldehyde. Cells were washed twice with PBS and pelleted at 400 g for 4 min. For permeabilization, cells were incubated for 20 min with 0.05% Triton X-100 in PBS and blocked for 1 h at room temperature in 2% bovine serum albumin. Primary rabbit BAX (#2772S, Cell Signaling) and BCL2 (#2876S, Cell Signaling) antibodies (1:200 dilution) were incubated for 1 h at 4°C. Unbound antibodies were washed out through two cycles of washing with PBS. Rabbit secondary antibody (1:250, Invitrogen AlexaFluor Dye) was incubated for 30 min at 4°C. Cells were washed again and the analyses were performed on a FACS (BD FACSARIA II, BD Bioscience). Data were analyzed using FlowJo 7.6 software and statistical analysis was carried out using the GraphPad Prisma Software.

Statistical analysis. Results are expressed as the mean and standard error of the mean (SEM) of triplicate determinations. Statistical significance was calculated using a two-way ANOVA followed by the Bonferroni test as a post-test or using a two-tailed paired Student t-test. P<0.05 was considered to be significant and statistical results are denoted in the graphs with one asterisk (P<0.05), two asterisks (P<0.01) or three asterisks (P<0.001). All statistical analyses were carried out using Prism 5 (GraphPad Software, La Jolla, CA).

Results

MEG3 is highly expressed in the Hs578T human triple negative mammary cell line. Using a panel of seven breast cancer cell lines, which display different invasive and metastatic potential in vitro, we found that expression of the maternally expressed gene 3 (MEG3) is strikingly increased in the triple negative and highly metastatic Hs578T cell line (average Ct value of 21.5), when compared to the expression levels in the non-tumorigenic MCF12A cell line (average Ct value of 32.9) (Fig. 1A). Interestingly, this gene has been extensively described as a tumor suppressor gene (31). Due to this apparent controversy, we next carried out loss-of-function experiments using the CRISPR/Cas9 knockout system.

MEG3 CRISPR/Cas9-mediated knockout. To obtain the complete knockout (KO) of the MEG3 gene, we used four sgRNAs: Two targeting the 5’-end (GFP reporter) and two targeting the 3’-end (puromycin resistant cassette) of this gene's locus (Fig. 1B). The cells were then co-transduced with a combination of viral particles coding for the different regions of the gene, thus generating four MEG3_KO cell populations designated according to the sgRNAs used: 1+3, 1+4, 2+3 and 2+4.

We observed that different combinations of sgRNAs result in distinct efficiency of genomic editing, although all KO populations showed significant reduction in lncRNA expression (Fig. 1C). The combination 2+4 displayed a striking reduction of around 98% in MEG3 expression when compared to the Hs578T_WT cells. Likewise, the PCR set up to detect MEG3 deletion, revealed a higher rate of genomic edition in this cell population.

To better test our CRISPR/Cas9 protocol, we next aimed to investigate the knockout stability in this cell population. To that end, we collected samples from sequential passage numbers in order to verify the percentage of GFP-positive cells and MEG3 IncRNA expression levels. FACS sorting confirmed that the number of GFP expressing cells remained above 95% over time (Fig. 1D) while MEG3 expression levels was consistently reduced (Fig. 1E). Therefore, we selected the MEG3_KO 2+4 cell population to investigate the phenotypic effects of MEG3 deletion in the Hs578T cell line.

MEG3 knockout cells exhibit increased proliferation and anchorage-independent cell growth. First, we examined the proliferative potential of the MEG3_KO cells, observing that cells display increased proliferative rates upon MEG3 deletion, when compared to the Hs578T_WT cells (Fig. 2A). Next, we evaluated the anchorage-independent cell growth ability in soft agar assay. As shown in Fig. 2B, MEG3_KO cells exhibit significant increase in the ability to form colonies in semi-solid substrate.

MEG3 knockout increases cell migration through cytoskeleton changes but reduces the cell invasion capacity. Given the increased anchorage-independent growth capacity of KO_MEG3 cells, we next sought to investigate the effect of MEG3 knockout in cell motility in vitro. Wound healing assays showed that MEG3_KO cells migrate significantly
more, when compared to the Hs578T_WT cells (Fig. 2C). We further validated their migration potential using transwell inserts. Consistently, the number of cells which migrated through the membrane was 50% higher in MEG3_KO cells than in Hs578T_WT cells (Fig. 2D). Since cytoskeleton reorganization affects cancer cell motility (32), we investigated whether F-Actin polymerization was changed upon MEG3 deletion. Interestingly, staining of F-Actin showed evident changes in the cytoskeleton organization of KO_MEG3 cells (Fig. 2E).

Next, we used Matrigel-coated inserts to analyze KO_MEG3 cells invasiveness capacity. Surprisingly, we found that these cells invade significantly less, when compared to the WT cells, suggesting a reduced ability of these cells to digest the extracellular matrix and migrate through the membrane (Fig. 2F).

**MEG3 regulates the expression of invasiveness factors.** To better understand the effects of MEG3 on cell migration and invasion, we investigated the expression of different markers...
related to cell motility and invasiveness by RT-qPCR and immunofluorescence. Interestingly, we found significant reduction of MMP2, ZEB1 and COL3A1 in KO cells, when compared to WT (Fig. 3A). Since MEG3 has previously been described to be involved in regulation of EMT factors, we next sought to analyze epithelial-mesenchymal transition (EMT) factors by immunofluorescence (33). We detected an increase, of at least two fold, in TGF-β and N-Cadherin protein expression in KO cells (Fig. 3B-D), but we did not observe significant differences for Vimentin, Fibronectin or E-Cadherin (data not shown).

Effect of MEG3 knockout on doxorubicin-induced apoptosis. MEG3 has previously been shown to contribute to cisplatin resistance of lung cancer cells (34,35). To verify whether MEG3 is involved in Hs578T cells chemo-resistance, we treated parental cells with a sub-toxic doxorubicin (DXR) concentration (0.25 µg/ml). We found that MEG3 expression is significantly downregulated upon this treatment, suggesting that this lncRNA may be involved with the effect of DXR (Fig. 4A). Moreover, it has been suggested that MEG3 exerts a pro-apoptotic function in different cancer cell lines (12,14,36,37). To extend these findings and determine how MEG3 may affect breast cancer cell sensitivity to chemotherapy, we analyzed the metabolic activity, using the MTT assay, upon cells exposure to different concentrations of DXR for 24 h. We observed that MEG3_KO cells were less sensitive...
to DXR treatment, when compared to control cells, with significant differences at the concentration of 1.25 µg/ml (Fig. 4B).

We further analyzed the rate of appearance of apoptotic cells following treatment with DXR using the Annexin-V binding assay, confirming that this rate is not significantly different when cells are treated for 24 h with 0.5 µg/ml DXR. However, we found a significant resistance in DXR-induced apoptosis when cells were treated with 1 µg/ml (Fig. 4C). The rate of cells undergoing apoptosis was around 28% in Hs578T_WT cells, whereas no more than 18% of MEG3_KO cells were sensitive to this treatment, as represented by the dot plot shown in Fig. 4D.

In parallel, we aimed at investigating the possible relation of BAX (an apoptosis promoter) to BCL2 (an apoptosis inhibitor) ratios with the DXR chemoresistance mediated by MEG3. We found that MEG3_KO cells display a significant reduction in BAX/BCL2 endogenous levels when compared to WT cells, emphasizing the relationship between this IncRNA and the death phenotype (Fig. 4E). We also observed a significant increase of BAX/BCL2 ratio upon treatment in MEG3_KO cell, nevertheless, not sufficient to reach BAX/BCL2 protein levels in WT cells.

Discussion

The versatile role of IncRNAs highlight their relevance and the complexity of the signaling network in which they are involved. Various functional mechanisms have been described for IncRNAs, including recruitment of transcription factors, guiding chromatin modifiers to specific genomic loci, allosteric modulation of transcriptional regulatory proteins, alteration of nuclear domains, modulation of translation and mRNA stability as well as natural competing endogenous RNAs (5,8,9).

MEG3 is a maternally expressed imprinted IncRNA gene, comprised of 10 exons located at chromosome 14q32 (38).
Under physiological conditions, it is found to be expressed in many normal tissues and maternal deletion of the MEG3 gene in mice was shown to cause skeletal muscle defects and perinatal death (39). Interestingly, MEG3 gene expression is significantly reduced or completely lost in several human cancers (10,12-14,23) as a result of different events, including promoter hypermethylation, hypermethylation of the intergenic differentially methylated region as well as gene deletion (31). MEG3 downregulation has been previously suggested to be a potential prognostic factor for breast cancer patients, representing an unfavorable risk factor with significant correlation to patient survival (19-21). Mechanistically, overexpression of MEG3 in MDA-MB-231 and MCF-7 breast cancer cell lines promoted downregulation of AKT signaling, which is pivotal for breast cancer cell growth, invasion and tumor angiogenesis (22). Additionally, MEG3 was shown to act through activation of the p53 pathway, playing a role as a growth suppressor factor (40,41). Importantly, these authors showed that the secondary RNA folding structure of each MEG3 isoform is essential to its function, promoting significant increase in p53 protein levels and stimulating the expression of p53 downstream targets (40).

LncRNAs may regulate cancer cell migration by targeting the Rho/ROCK signaling pathway (42). In our model, we found that MEG3 deletion promoted cell migration, potentially through induction of cytoskeleton rearrangement. Curiously, downregulated MEG3 was previously associated with lymph node metastasis in primary thyroid cancer (43). This study showed that MEG3 suppresses the expression of Rac1 through a specific site in the 3’ UTR. This small signaling GTPase exerts a critical regulatory role driving cell motility by formation of lamellipodia (44), confirming the involvement of MEG3 as a key regulator of F-actin polymerization dynamic.

Here, we used the CRISPR/Cas9 system to analyze the effects of MEG3 deletion in the TNBC Hs578T cell line. Consistently, we found that KO cells display increased proliferation rate and anchorage-independent growth, suggesting
greater tumorigenic potential of these cells. In parallel, using Matrigel-coated inserts, we observed a decrease in cell invasion capacity upon MEG3 deletion. Interestingly, integrated analysis has identified MEG3 overexpression and their gene regulation network as an important player in ovarian cancer EMT (45). By genome-wide mapping, it was shown that 73% of MEG3-regulated genes are EMT-linked pathway factors. However, survival analysis showed no significant correlation of MEG3 overexpression with overall patient survival. Another study also described MEG3 as a regulatory RNA for EMT in lung cancer cell lines (33). These authors showed that MEG3 knockdown inhibited EMT by antagonizing TGF-β-dependent changes in the expression of EMT-related genes. Additionally, MEG3 overexpression caused significant changes in the expression of CDH1, E-cadherin, ZEB1, ZEB2, miR-200a, and miR-200c but had no effect in the expression of FN1/fibronectin, vimentin or JARID2. In fact, Mondal and collaborators demonstrated that MEG3 represses the expression of TGF-β pathway genes through formation of RNA-DNA triplex structures (46). Our results show that MEG3 deletion alone leads to increased TGF-β and N-cadherin protein levels and also promotes the reduction of MMP2, ZEB1 and COL3A1 gene expression. These data highlight the role of MEG3 in inhibiting cell migration by regulating the TGF-β pathway however, the exact mechanisms by which MEG3 regulates cell invasion in Hs578T cells and why this effect may differ among different cell lines requires further investigation.

The potential mechanisms involved in chemotherapy drug resistance are largely unclear. Different studies have already suggested that epigenetic alterations, such as histone methylation and acetylation, may play a role in the development of drug resistance (47). In a very elaborate study, Li and collaborators have recently demonstrated that MEG3 promoter methylation correlates with Chronic Myeloid Leukemia tumor stages (48). In that study, MEG3 expression was found to be reduced in advanced phase of the disease, while the expression of methylation related genes, such as DNMT1, DNMT3B, MBD2, MECP2 and HDAC1, was found to be increased, when compared to controls. We speculate that reduced MEG3 expression resulting from DXR treatment may be due to induced promoter hypermethylation. Moreover, it will be interesting to investigate whether the promoter methylation status might be responsible for the discrepant expression levels of MEG3 found in the Hs578T cell line.

MEG3 has been previously implicated in cell death response, regulating intracellular signals triggering apoptosis pathways (12,14,34-37). Thus, MEG3 was shown to inhibit the intrinsic cell survival pathway both in vitro and in vivo by reducing Bcl-2 protein expression, enhancing BAX protein levels and activating caspase-3 in prostate cancer cells (14). Consistently, lung adenocarcinoma patients with lower levels of MEG3 expression displayed worse responses to cisplatin-based chemotherapy (35). MEG3-mediated chemosensitivity enhancement in lung cancer cells was associated with induction of cell-cycle arrest and increased apoptosis, through regulation of p53, β-catenin and survivin, which are target genes of the WNT/β-catenin signaling pathway (34). Here, we demonstrate that Hs578T MEG3_KO cells display reduced ratios of BAX/BCL-2, consistent with a higher resistance to a given apoptotic stimulus. Moreover, MEG3_KO cells showed a slight increase in the ratio of these factors upon treatment with DXR, however, this response is not sufficient to restore the physiological BAX/BCL-2 levels, further supporting a role for MEG3 as an important apoptosis regulator.

In conclusion, IncRNAs are key components involved in diverse biological processes and MEG3 has been previously shown to exert regulatory functions in cell proliferation, apoptosis, migration/invasion and angiogenesis. We confirmed the overall tumorigenic effect of MEG3 deletion by CRISPR/Cas9 system in Hs578T cells, nevertheless, MEG3 was found to be highly expressed in this cell line, suggesting that escape mechanisms are used to counteract its growth suppressor functions, but this need to be further investigated.

Taken together our results indicate that reduced MEG3 expression in breast cancer tissues may contribute to drug resistance in DXR-containing chemotherapy, nevertheless, future therapeutic approaches to promote MEG3 expression/activation should be carefully considered given the ability of MEG3 to modulate EMT factors, which may in turn, promote metastasis. A more conclusive assessment of MEG3 function could benefit from patient derived samples. Also, determining the sensitivity to apoptotic induction and MEG3 expression levels in different models should provide a better understanding of the pathways involved and contribute to decision making regarding patient treatment.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

CDP and RACM developed the concept, designed and performed experiments and wrote the article. TM cloned the CRISPR/Cas9 constructs, generated the KO cell lines and performed functional experiments. HCJF and ACOC performed functional experiments. TFP performed Annexin-V/PI experiments and FACS analysis. MCS contributed to the conception and design of the study, data interpretation, and drafting and critical revision of the manuscript, and approved the manuscript for publication. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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