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Research paper

ADAM9 silencing inhibits breast tumor cell invasion *in vitro*

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Kelli Cristina Micocci [a,1], Ana Carolina Baptista Moreno Martin [a,1],
Cyntia de Freitas Montenegro [a,1], Araceli Cristina Durante [a,1], Normand Pouliot [b,c,d,2],
Márcia Regina Cominetti [a,*], Heloisa Sobreiro Selistre-de-Araujo [a,1]

[a] Departamento de Ciências Fisiológicas, Rodovia Washington Luís, Km 235, CEP 13565-905, São Carlos, SP, Brazil
[b] Metastasis Research Laboratory, Peter MacCallum Cancer Centre, The University of Melbourne, Melbourne, VIC, Australia
[c] Sir Peter MacCallum Department of Oncology, The University of Melbourne, Melbourne, VIC, Australia
[d] Pathology Department, The University of Melbourne, Melbourne, VIC, Australia

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ABSTRACT

ADAM9 (A Disintegrin And Metalloproteinase 9) is a member of the ADAM protein family which contains a disintegrin domain. This protein family plays key roles in many physiological processes, including fertilization, migration, and cell survival. The ADAM proteins have also been implicated in various diseases, including cancer. Specifically, ADAM9 has been suggested to be involved in metastasis. To address this question, we generated ADAM9 knockdown clones of MDA-MB-231 breast tumor cells using silencing RNAs that were tested for cell adhesion, proliferation, migration and invasion assays. In RNAi-mediated ADAM9 silenced MDA-MB-231 cells, the expression of ADAM9 was lower from the third to the sixth day after silencing and inhibited tumor cell invasion in matrigel by approximately 72% when compared to control cells, without affecting cell adhesion, proliferation or migration. In conclusion, the generation of MDA-MB-231 knockdown clones lacking ADAM9 expression inhibited tumor cell invasion *in vitro*, suggesting that ADAM9 is an important molecule in the processes of invasion and metastasis.

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1. Introduction

Attachment of cells to the extracellular matrix (ECM) depends mainly on a family of glycoproteins known as integrins [1], which are expressed on the cell surfaces of many cultured cell types at specialized adhesion sites known as focal contacts [2]. A number of structural and signaling proteins, such as integrins, cytoskeletal proteins, and kinases are concentrated at these sites and are known to initiate signal transduction pathways [3,4]. The aggregation of integrin receptors, ligand occupancy and tyrosine kinase-mediated phosphorylation are the key events that result in different processes, including cell migration, differentiation, tissue remodeling, cell proliferation, angiogenesis, tumor cell invasion and metastasis [1,5].

Members of the ADAM (an acronym for A Disintegrin And Metalloprotease) protein family are involved in several human diseases such as inflammatory disorders, neurological diseases, asthma and cancer metastasis [6,7]. ADAM9 is a transmembrane protein with a number of characteristic domains, including a pro-domain, a metalloproteinase domain, a disintegrin-like domain, a cysteine-rich region, a transmembrane domain, and a short cytoplasmic tail [8]. The ADAM9 disintegrin domain binds to numerous integrins, such as $\alpha_6\beta_1$ integrins in fibroblasts [9], $\alpha_V\beta_5$ in myeloma cells [10] and $\alpha_V\beta_3$ in MDA-MB-231 breast tumor cells [11]. Mahimkar et al. [12] and Zigrino et al. [13] have demonstrated that the recombinant disintegrin and cysteine-rich domains from human ADAM9 mediate cellular adhesion through β_1 integrins. Furthermore, the disintegrin-like and cysteine-rich domains of ADAM9 mediate interactions between melanoma cells and fibroblasts [14].

Over-expression of ADAM9 has been reported in several human carcinomas, including kidney [15], prostate [16], breast [17], liver [18,19], pancreatic [20], gastric [21], cervix [22] and oral [23]. Expression of ADAM9 is elevated in skin melanoma but is restricted to the invading front [24]. Peduto et al. [25] found a correlation between ADAM9 titer and cancerous changes in mouse models of prostate cancer, especially in well-differentiated tumors. Increased expression of ADAM9 led to increased structural abnormalities and growth of early-stage tumors compared to controls.

* Corresponding author. Present address: Departamento de Gerontologia, Rodovia Washington Luís, Km 235, CEP 13565-905, São Carlos, SP, Brazil. Tel.: +55 16 33066663; fax: +55 16 33519628.
E-mail address: mcominetti@ufscar.br (M.R. Cominetti).
[1] Tel.: +55 16 33518333; fax: +55 16 33518328.
[2] Tel.: +61 3 96561285; fax: +61 3 96561411.

The ADAM9 protein also appears to interfere with various cell signaling systems. In prostate cancers, the fibroblast growth factor (FGF) signaling pathway is believed to be particularly important [26], with down-regulation of the fibroblast growth factor receptor 2 isoform IIIb (FGFR2IIIb) being a feature of prostate tumor progression [27]. Transfection of FGFR2IIIb into malignant tumors is enough to inhibit their growth [28]. Therefore, it is potentially significant that over-expression of ADAM9 increases shedding of FGFR2IIIb from cells, which is expected to disrupt FGFR2IIIb signaling and reduce its function [25]. Additionally, over-expression of ADAM9 leads to increased release of epidermal growth factor (EGF) [25], a factor known to induce prostate cancer growth in rat pups [29].

Although ADAM9 is normally considered a transmembrane protein, a soluble form ADAM9-S has been described [30], which is derived from alternative splicing of the gene [31]. The ADAM9-S protein promotes the invasive phenotype of carcinoma cell lines, and ADAM9 is strongly expressed at the invading front of hepatic metastases, although the authors did not distinguish ADAM9 and ADAM9-S [31]. Taken together, these studies suggest that ADAM9 has a significant role in tumorigenesis and metastasis.

To better understand the role of ADAM9 in breast cancer progression, we generated knockdown clones lacking ADAM9 using RNAi in the MDA-MB-231 human breast tumor cell line. As far as we know, this is the first demonstration that decreased ADAM9 expression impaired the invasiveness of this cell line. In addition, the present work demonstrated, for the first time, ADAM9 silencing in a breast tumor cell line and provided evidence that ADAM9 may play an important role in the metastatic progression of human breast cancer.

2. Material and methods

2.1. Cell culture

MDA-MB-231 breast tumor cells were cultivated in DMEM medium (Invitrogen) containing 10% bovine fetal serum (FBS), L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml) and amphotericin B (250 μg/ml) (Invitrogen) in 5% CO_2 at 37 °C. The anti-ADAM9 antibody was from Abcam (anti-RP2ADAM9) and the anti-β-actin antibody was from Santa Cruz Biotech (sc-1616).

2.2. Design of siRNA primers

Primer set #104056, which targets exon 13 (disintegrin domain) (Silencer® Pre-designed siRNA, Ambion), was selected to ensure that other ADAMs would not be silenced simultaneously. The primer sequences were: sense (5′-rCrCrArGrArGrUrArCrUrGrCrArArUrGrGrUrUrCrUrUrCTC-3′) and antisense (5′-rGrArGrArArGrArArCrCrArUrUrGrCrArGrUrArCrUrCrUrGrGrArA-3′). The negative control (scrambled) used in the assays was Silencer® Select Negative Control No. 1 siRNA (Ambion). This sequence does not target any gene product and have no significant sequence similarity to human gene sequences, being essential for determining the effects of siRNA delivery.

2.3. ADAM9 RNA silencing

On the day before transfection, 2×10^5 MDA-MB-231 cells were plated in 5 ml of DMEM medium supplemented with 10% FBS without antibiotics. Ten microliters of lipofectamine were mixed with 490 μl OPTI-MEM serum-free medium (Invitrogen) and incubated at room temperature for 5 min. A total of 10 nM of RNA silencing primer was diluted in OPTI-MEM, added to the lipofectamine/OPTI-MEM mixture and incubated for 20 min at room

temperature. This mixture was then added to the cells. The medium was changed 24 h after transfection. Controls comprised of non-treated cells, cells treated with transfection reagent only (lipofectamine), and cells treated with a scrambled primer. Cells were washed in phosphate buffered saline, harvested with Trizol reagent (Invitrogen) according to the manufacturer's protocol, and frozen immediately. For western blotting assays, cells were lysed with Triton X-100 in Hepes buffer [150 mM NaCl, 50 mM Hepes, 1.5 mM $MgCl_2$, 1% Triton X-100, 0.1% SDS, protease inhibitor cocktail (Sigma), 100 mM NaF and 100 mM Na_3VO_4]. Protein concentrations in the lysed samples were determined by the BCA method (Pierce), and 30 μg of each sample was resolved by SDS-PAGE [32]. Protein bands were transferred to nitrocellulose membranes and probed with anti-RP2ADAM9 and anti-β-actin antibodies. Western blots were scanned on an Image Scanner (GE − General Electric). All the assays using ADAM9 knockdown MDA-MB-231 cells were performed after the third day of siRNA transfections.

2.4. Extraction of RNA and synthesis of cDNA

Total RNA was extracted from cells using Trizol reagent (Invitrogen) following the manufacturer's instructions. All samples were treated with DNase I (Deoxyribonuclease I, Amplification Grade, Invitrogen). After quantification, a total of 1 μg of RNA was mixed with 0.5 μl of oligo dT (0.5 μg/μl) (Promega) and nuclease-free water to a volume of 7 μl and incubated at 70 °C for 5 min, followed by 5 min on ice. Next, 0.5 μl of 200 units/μl of Moloney Monkey Leukemia virus (MMLV) reverse transcriptase (Promega), 2.5 μl of 5× MMLV buffer (Promega), and 2.5 μl of 10 mM dNTP mix was added to the reaction. The whole mixture was incubated at 37 °C for 1 h, and was used posteriorly for qPCR.

2.5. Design of qPCR primers

ADAM9 primers targeting the disintegrin domain were designed using Primer3Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). Primers spanned exon boundaries so that only mRNA sequences would be amplified. The primers were ADAM9DF1 (5′CTT GCT GCG AAG GAA GTA CC); and ADAM9DR1 (5′AAC ATC TGG CTG ACA GAA CTG A). Primers targeting HPRT1F1 (5′TGA CAC TGG CAA AAC AAT GCA), HPRT1R1 (5′GGT CCT TTT CAC CAG CAA GCT), GAPDHF1 (5′GAT GCT GGT GCT GAG TAT GT) and GAPDHR1 (5′GTG GTG CAG GAT GCA TTG CT) were used as endogen controls.

2.6. Gene expression

ADAM9 mRNA expression was measured in a Corbett Rotorgene RG 3000 (Corbett Research) using the following thermocycling conditions: 95 °C for 10 min, followed by 40 cycles of amplification at 95 °C for 15 s, 55 °C for 5 s and 72 °C for 20 s. The master mix in each well consisted of 12.5 μl ABsoluteTM QPCR SYBR Green mix (6 mM $MgCl_2$, reaction buffer, DNA polymerase and SYBR green dye) (Advanced Biotechnologies), 1.25 μl each of 5 μM forward and reverse primer and 10.5 μl of nuclease-free water in a total volume of 25 μl.

2.7. Proliferation assays

To measure the effect of RNAi-mediated ADAM9 silencing on cell proliferation the transition of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan was used [33]. Cells without any treatment, cells treated only with lipofectamine, and cells transfected with siRNAs were seeded in 96-well plates and left at 37 °C for 24 and 48 h. The cells were then washed with

PBS and incubated in 50 μl of 0.5 mg/ml MTT in culture medium at 37 °C for 4 h. Following the addition of 100 μl of isopropanol, the absorbance was read at 595 nm in an ELISA plate reader. The mean proliferation of cells without any treatment was expressed as 100%.

2.8. Adhesion assays

The effect of RNAi-mediated ADAM9 silencing on the adhesion of MDA-MB-231 cells was analyzed in 96-well plates (Corning). A solution of type I collagen (10 μg) was immobilized on the plates in 0.1% acetic acid. Fibronectin and laminin (10 μg) were dissolved in adhesion buffer (20 mM HEPES, 150 mM NaCl, 5 mM KCl, 1 mM MgSO$_4$ and 1 mM MnCl$_2$ pH 7.35), overnight at 4 °C. On the next day, the wells were blocked with a solution of 1% BSA diluted in adhesion buffer for 1 h. Cells were counted and their concentration was adjusted in proportion to 5×10^6/ml. The blocking solution was removed from the wells and they were washed twice with adhesion buffer (100 μl). After this period the cells were incubated for 45 min on the coatings and subsequently wells were washed in order to remove non-adherent cells. A solution of 70% ethanol (100 μl) was added to the wells and the plate was incubated for 10 min at room temperature. Subsequently, the ethanol was removed and 60 μl of crystal violet (0.5%) was added and incubated for 20 min at room temperature. After this time, the solution containing the crystal was removed and the wells were washed with PBS to remove excess. Finally 100 μl of 1% SDS was added and incubated for 30 min at room temperature. The reading of absorbance was performed at a wavelength of 595 nm, and three treatments were compared, including cells without any treatment, cells treated only with lip-ofectamine, and cells transfected with siRNAs. The adhesion of control cells to each substrate was determined as 100%.

2.9. Wound healing assay

Wound-healing migration assay is based on the repopulation of wounded cultures. The cells were seeded into 24-well culture plates at 1×10^5 cells/well and the cell monolayer were cultured in medium containing 10% FBS until reach 100% of confluence. The monolayers were carefully wounded using a yellow pipette tip, and any cellular debris present was removed by washing twice with DMEM medium. The wounded monolayers were then incubated in DMEM medium containing 10% FBS. Photographs of the exact wound areas taken initially (0 h) were again taken after 16 and 24 h. The images were compared between three treatments, including cells without any treatment, cells treated only with lipofectamine, and cells transfected with siRNAs, and with or without incubation with the ADAM9D protein [11]. Photographs were analyzed using ImageJ software and the formula of % of wound closure [34].

2.10. Cell migration

Cell migration was assessed in 24 well Boyden chambers (BD Biosciences). MDA-MB-231 (5×10^4) cells were seeded on the up-per chamber in FBS-free DMEM medium. DMEM containing FBS (10%) was added to the bottom chamber and acted as a chemo-attractant. Tumor cells were allowed to migrate for 22 h at 37 °C and 5% CO$_2$ in a humidified environment. Then, the cells that remained in the upper chamber were removed using a cotton swab. The cells that migrated to the other side of the upper chamber membrane were fixed with methanol and stained with 1% toluidine blue in 1% borax. Cells were counted using the ImageJ software (public domain software) in 5 fields ($100\times$ magnification) per well that essentially covered 80% of the well surface. The average number of cells from each of the triplicates represents the average number of cells that migrated in the different groups. Each experiment had triplicate wells for every treatment group and we repeated each experiment three times. The mean of all results from controls was considered as 100%. After that, the images were compared among three treatments, including cells without any treatment, cells treated only with lipofectamine, and cells trans-fected with siRNAs.

2.11. Matrigel invasion assay

Cellular invasion assays were carried out using BioCoat Matrigel Invasion Chambers (BD Biosciences) with 8-μm pores in 6-well plates. A total of 2.5×10^4 cells were added to each chamber. Complete medium was used as a chemoattractant in the lower chamber. After incubation for 22 h at 37 °C and 5% CO$_2$, cell invasion was measured in the same way as performed for migration assay (item 2.10). The invasion of cells without any treatment was determined as 100%.

2.12. Gelatin zymography

The effect of ADAM9 silencing on the proteolytic activity of MDA-MB-231 cells was determined by zymography [35]. MDA-MB-231 cells (2×10^6) in FBS-free DMEM medium were seeded in 6-cm dishes. After incubation for 24 h at 37 °C and 5% CO$_2$, cells were lysed with a buffer containing Tris–HCl (0.2 M) (pH 7.4) and Triton X-100 (0.2%). The cell lysates were centrifuged (10 min at $13,000 \times g$ and 4 °C), and the supernatants were separated. The total protein concentration in each sample was measured using the BCA color-imetric detection kit (BCA Protein Assay, Pierce). Protein samples (20 μg) were subjected to electrophoresis under non-reducing conditions in 10% SDS polyacrylamide gels containing 1 mg/ml gelatin. After electrophoresis, gels were washed twice in 2.5% of Triton X-100 to remove SDS and incubated in substrate buffer [50 mM Tris–HCl (pH 8.0); 5 mM of CaCl$_2$ and 0.02% NaN$_3$] at 37 °C for 20 h. To confirm the metalloproteinase activity, EDTA in a final concentration of 15 mM was added to the samples and substrate buffer. Proteins were stained with Coomassie brilliant blue for 1.5 h and destained with an acetic acid, methanol and water mixture (in a 1:4:5 v:v:v ratio). Gels were photographed with a Canon G6 Po-wer Shot 7.1 machine. Gelatinase activity was visualized as clear bands in the stained gels, and the average band intensities was measured using the Gene Tools v3.06 software (Syngene). MMP-2 and MMP-9 activity were quantified as arbitrary units and compared between three treatments, including cells without any treatment, cells treated only with lipofectamine, and cells trans-fected with siRNAs.

2.13. Statistical analysis

For all assays, each experiment was repeated three times in triplicate (independent experiments), and standard errors of the mean were calculated. The results were compared statistically us-ing a one-way analysis of variance (ANOVA) and Tukey's test was applied for multiple comparisons. All statistical tests used $p \leq 0.05$ as a cut-off for significance. Cases where $p < 0.05$ were marked as follows: $^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

3. Results

3.1. ADAM9 silencing is detected at the mRNA and protein levels

ADAM9 gene expression was dramatically decreased in MDA-MB-231 cells treated with siRNAs at both the mRNA and protein (Fig. 1A and B, respectively) levels when compared to the controls (MDA-MB-231 cells without treatment and lipofectamine or
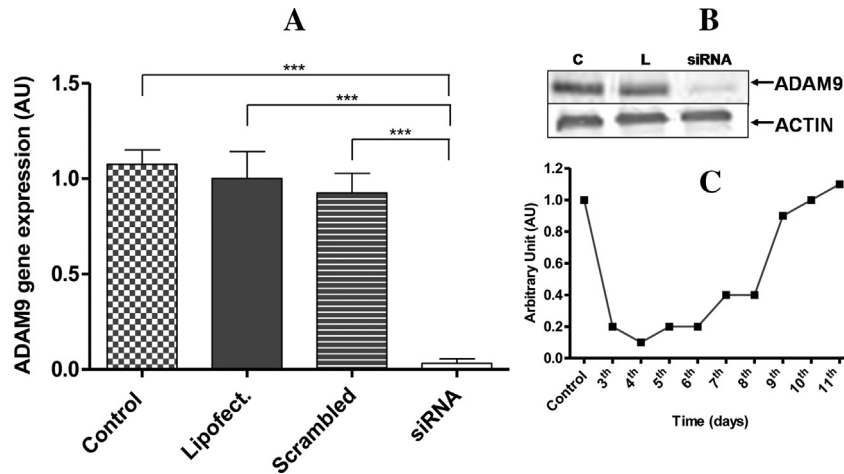
Fig. 1. Silencing of ADAM9. (A) Expression of ADAM9 mRNA in RNAi-mediated ADAM9 silenced MDA-MB-231 cells was analyzed by qPCR. The values are in arbitrary units (AU) and the *p* value was determined using ANOVA with a Tukey's test comparing control, cells treated with lipofectamine, negative control (scrambled) and cells treated with ADAM9 siRNAs (***$p < 0.001$). (B) Western blotting analysis of MDA-MB-231 cell lysates, with three treatments: cells alone (C), lipofectamine alone (L), and ADAM9 siRNAs using anti-ADAM9RP2 primary antibody and goat anti-rabbit IgG as a secondary antibody. β-actin was used as endogen control. (C) The silencing of ADAM9 using siRNAs is transient because it is effective only from the third to the sixth day. The values are in arbitrary units (AU).

scrambled-treated cells). qPCR analysis showed a down regulation of $91.35 \pm 6.32\%$ of ADAM9 expression in RNAi-mediated knockdown MDA-MB-231 cells when compared to control cells (Fig. 1A). At the protein level, western blotting analysis using anti-ADAM9 antibody presented similar results (Fig. 1B).

Gene silencing using synthetic duplexes siRNA is transient. As a result, 10 plates (6-cm) containing 2×10^5 MDA-MB-231 cells were silenced. One plate was removed randomly each day to determine the efficiency of ADAM9 silencing over time. RNA knockdown was measured from the third to the eleventh day after transfection and the kinetics of ADAM9 silencing in MDA-MB-231 cells are shown in Fig. 1C. The highest efficiency of ADAM9 silencing was observed from the third to the sixth days, although on the seventh and eighth days, the gene expression still remained low. From the ninth and tenth days onwards, the expression of ADAM9 expression increased exponentially and reached similar levels to those obtained by control cells (Fig. 1C).

3.2. ADAM9 silencing does not affect MDA-MB-231 cell proliferation or adhesion

ADAM9 silencing had no effect on the proliferation of MDA-MB-231 cells on the third (Fig. 2A) or sixth (Fig. 2B) days after silencing

in 24 or 48 h of incubation. No significant differences were observed among the groups. ADAM9 silencing also had no effect on the adhesion of MDA-MB-231 cells to different ECM proteins, such as collagen type I, fibronectin or laminin (Fig. 3).

3.3. ADAM9 silencing strongly inhibits MDA-MB-231 cell invasion without affecting cell migration

RNAi-mediated ADAM9 silencing was not able to significantly inhibit the migration of MDA-MB-231 human breast tumor cells when compared with non-transfected cells or with lipofectamine-transfected cells after 16 (Fig. 4A) or 24 h that the wounds were made (Fig. 4B). ADAM9 knockdown cells were incubated with different concentrations of ADAM9D, the disintegrin domain of ADAM9 [11]. ADAM9D in concentrations of 500, 1000 and 2000 nM had no effects in inhibiting ADAM9 knockdown MDA-MB-231 cell migration after 16 (Fig. 4A) or 24 h (Fig. 4B) of incubation. Photographs were taken after 0, 16 or 24 h after wound (Fig. 4C).

To ensure these results we also performed migration assays using Boyden chambers. RNAi-mediated ADAM9 silencing had had no effect on MDA-MB-231 cell migration when compared with control cells or lipofectamine-treated cells after 22 h of incubation (Fig. 5A and B).
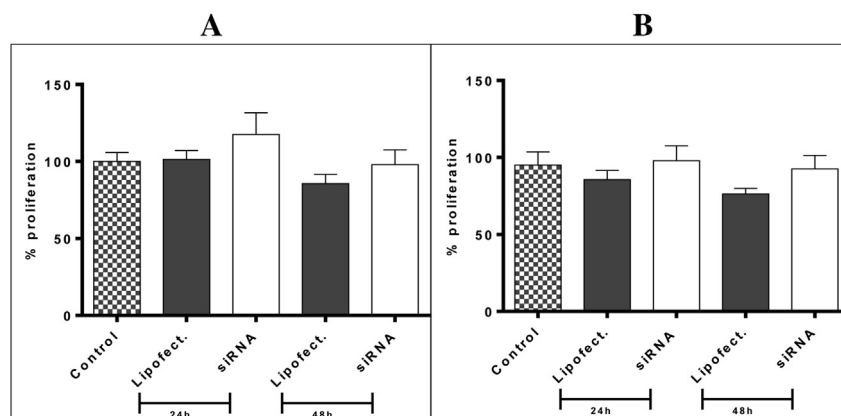

Fig. 2. Proliferation assay. Silencing of ADAM9 had no effect on the proliferation of the MDA-MB-231 cells after three (A) or six days (B) of transfection. After the different times, the cells were incubated with MTT for 24 h or 48 h and compared with control cells or with lipofectamine-treated cells. The absorbance of the samples was measured at 595 nm and the proliferation of control cells was determined as 100%.
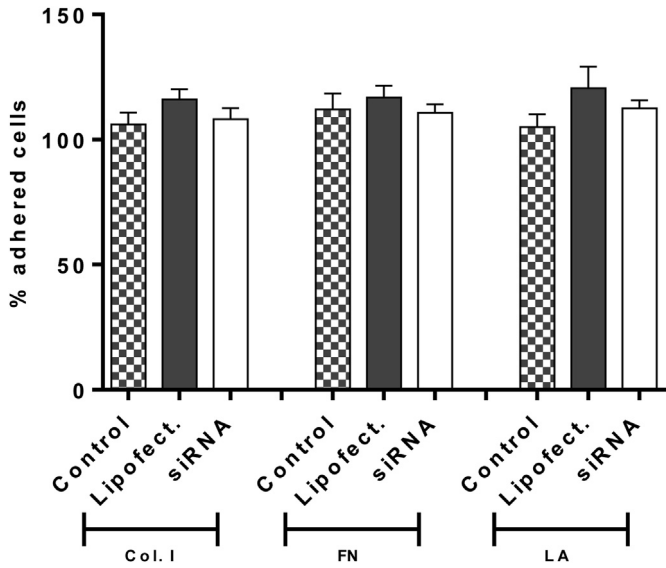
Fig. 3. Adhesion assay. RNAi-mediated ADAM9 silencing had no effect on the adhesion of the MDA-MB-231 under different extracellular matrix proteins, such as collagen I (Col. I), fibronectin (FN) and laminin (LA). The extracellular matrix proteins were coated on the wells of the plate, and on the following day, after the blocking of wells, the cells were allowed to adhere for 45 min. The percentage adhesion was determined as described in the materials and methods. The results were compared using a one-way analysis of variance (ANOVA), followed by a Tukey's post-hoc analysis.

On the other hand, RNAi-mediated ADAM9 knockdown MDA-MB-231 cells strongly inhibited the invasion in an *in vitro* matrigel assay by $71.51 \pm 8.02\%$ when compared to control untransfected cells (Fig. 6A). Lipofectamine and negative control-transfected cells (scrambled) remained invasive and no statistically significant differences were observed when compared to untransfected cells (Fig. 6A). Photographs were taken after 22 h of incubation (Fig. 6B).
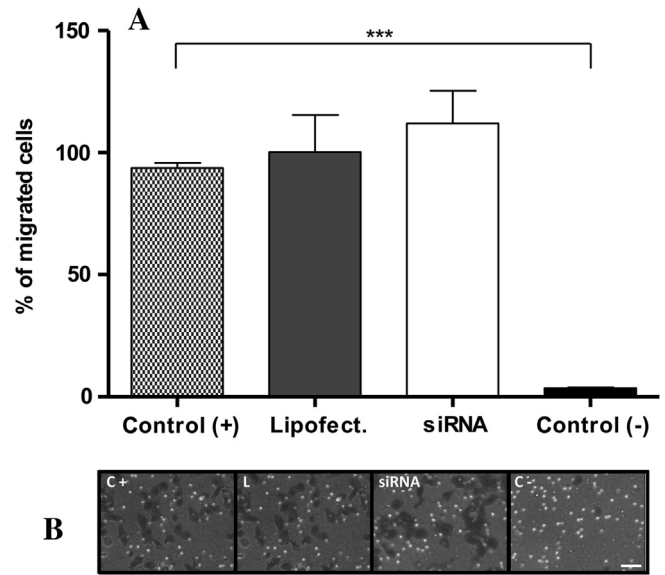

Fig. 5. Effect of RNAi-mediated ADAM9 silencing on migration of MDA-MB-231 cells. (A) A transwell migration assay was used to determine the effect of ADAM9 silencing migration of MDA-MB-231 cells. Control cells, lipofectamine-treated cells or siRNA-ADAM9 cells were allowed to migrate toward medium containing 10% FBS for 22 h. Graphs are representative of three independent experiments. The results were compared using a one-way analysis of variance (ANOVA), followed by a Tukey's post-hoc analysis ($***p < 0.001$). (B) Morphology of cells in the three different treatments: control cells (C+), lipofectamine-treated cells (L), and siRNA-ADAM9 treated cells (siRNA) migrating toward a 10% FBS containing medium. The negative control (C−) was control cells migrating toward a FBS-free medium. Bar represents 10 μm.

3.4. MMP-2 and MMP-9 concentration and activity

In order to investigate the mechanisms involved in the inhibition of the invasion ability of RNAi-mediated ADAM9 silencing of
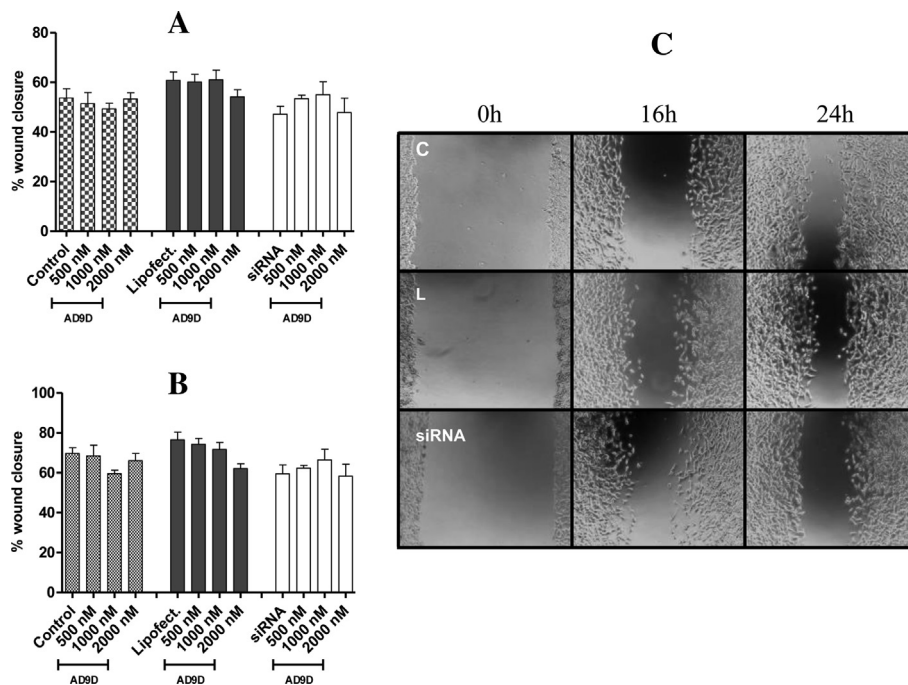

Fig. 4. Migration of MDA-MB-231 cells after wounding. Effects of ADAM9 silencing and ADAM9D recombinant protein on MDA-MB-231 cells migration were plotted as a percentage of wound closure in 16 (A) or 24 h (B) after wounding. Representative photos of wounds were taken at time zero, 16 h, and 24 h after wounding, (C). In these photos we show pictures representing control (C), lipofectamine (L) and siRNA-ADAM9 (siRNA) cells without previous treatment with ADAM9D recombinant protein.
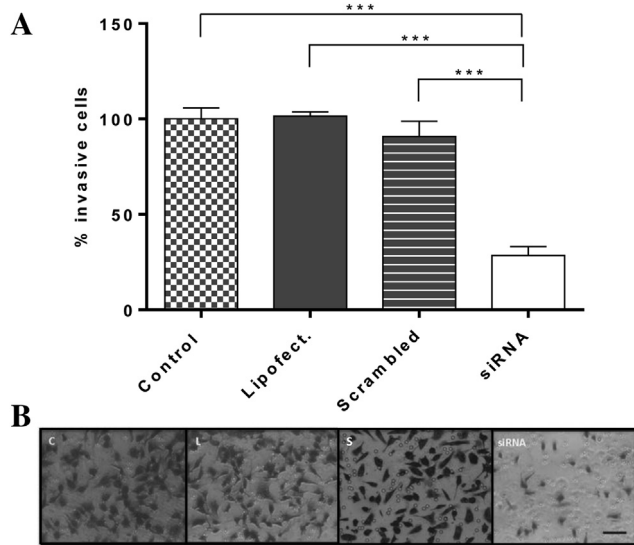
Fig. 6. Effect of RNAi-mediated ADAM9 silencing on the invasion of MDA-MB-231 cells. ADAM9 silencing significantly inhibits the invasion of MDA-MB-231 human breast tumor cells through matrigel compared to the invasion of control cells (A). The cells were plated in wells containing matrigel and FBS was used as a chemoattractant in the lower chamber. The invasive cells were fixed and counted (an average of eight fields from each treatment). The assay was performed in triplicate. The results were compared using a one-way analysis of variance (ANOVA), followed by a Tukey's post-hoc analysis (***$p < 0.001$). (B) Cell morphology in the four different treatments: untreated control (C), lipofectamine-treated cells (L), scrambled siRNA-treated control (S), and siRNA-ADAM9 treated cells (siRNA). Bar represents 10 μm.

MDA-MB-231 cells, we performed zymography assays to evaluate the activity of MMP-2 and MMP-9. There was no variation in the total concentration of MMP-2 or MMP-9 among the three treatment types analyzed in this study, as demonstrated by 1% gelatin-SDS-PAGE (Fig. 7A). The incubation with EDTA resulted in the inhibition of MMP-2 and 9 activities confirming the nature of metalloprotease activity (Fig. 7B). The average activity of MMP-2 and MMP-9 was measured as indicated in Section 2.12 and plotted on a

graph (Fig. 7C and D, respectively). This result suggests that RNAi-mediated ADAM9 silencing does not affect the activity of MMP-2 or MMP-9.

4. Discussion

The progression of malignant tumors results from the invasion of the primary tumor to a secondary site, causing metastasis in a multi-step process that requires cell–cell and cell–matrix interactions within the host tissue. These steps can be summarized as follows: cell detachment from the primary tumor, migration into the ECM, intravasation into a blood or lymphatic vessel, survival within the vasculature, adherence of these tumor cells in the endothelium, extravasation, and formation of secondary tumors [36,37]. These interactions lead to the production, release and activation of a variety of cytokines and growth factors and subsequent generation of signals to directly or indirectly promote tumor growth and survival [24]. Different proteases have been implicated in these processes, such as MMPs, ADAMs and ADAMTSs [7,24,38].

Due to the strong involvement of ADAM9 in the metastatic process, in this study we have generated knockdown clones of MDA-MB-231 human breast tumor cells that lack ADAM9 expression and then tested these clones to their ability to adhere, migrate, proliferate and invade through ECM using *in vitro* assays. The RNAi-mediated silencing in MDA-MB-231 cells was very successful, with more than 90% of ADAM9 knocked down, as estimated by quantitative PCR and western blotting analysis. The expression of ADAM9 was easily silenced using a relatively small (10 nM) concentration of ADAM9 siRNAs. A similar result was obtained by other investigators in highly invasive SCC68 cells, a squamous cell carcinoma cell line, but with tenfold higher concentration of siRNAs (100 nM) [39].

Using a matrigel invasion assay, we showed that the ADAM9 silencing significantly inhibited the invasion capacity of MDA-MB-231 human breast cancer cells, which suggests that this protein plays an important role in cell invasion. However, the silencing of ADAM9 had no effect on MDA-MB-231 cell adhesion, migration, proliferation, or MMP-2 and 9 activities. Our results showed that ADAM9 silencing had no impact on MMP-2 and MMP-9 expression or gelatinase activity indicating that reduced invasion in cells
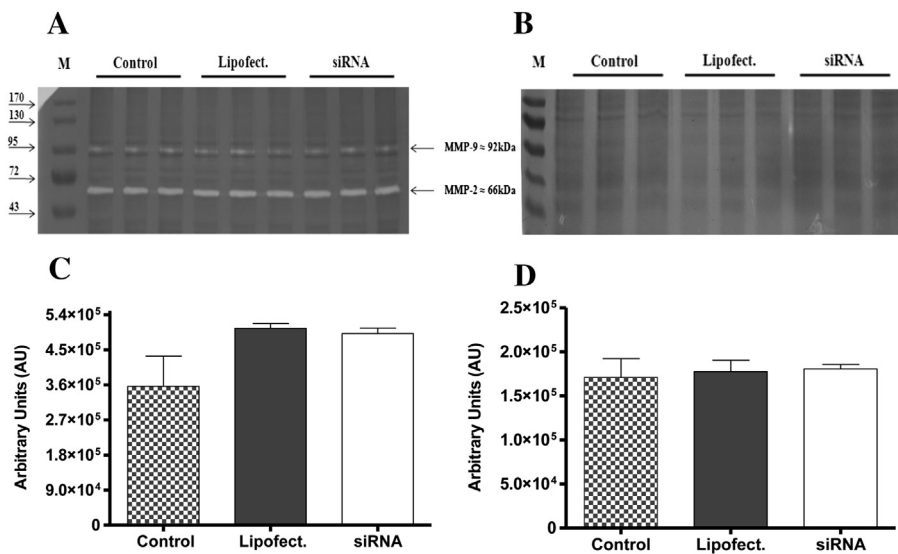

Fig. 7. Analysis of concentration and activity of MMP-2 and MMP-9 in the MDA-MB-231 breast tumor cells. (A) Zymography in 1% gelatin-SDS-PAGE or (B) EDTA-treated samples. Lane 1: molecular mass marker; lanes 2–4: control cells; lanes 5–7: cells treated with lipofectamine; and lanes 8–10: cells treated with ADAM9 siRNAs ($n = 3$; 20 μg of total protein was loaded in each lane). (C) MMP-2 and (D) MMP-9 concentrations were determined by the sum of integrated optical density (IOD) obtained for the intermediate bands. Gels were analyzed by densitometry, and activity was expressed as arbitrary units.

expression ADAM9 siRNA is unlikely to be due to indirect inhibition of MMP-2/9. We propose that ADAM9 proteolytic activity may directly contribute to matrigel invasion by MDA-MB-231 cells since ADAM9 has been reported to cleave laminin [31], a major constituent of matrigel.

Shintani et al. [40] showed that the overexpression of ADAM9 enhances adhesion and cell invasion in lung cancers, via modulation of other adhesion molecules and changes in sensitivity to growth factors. According to this study, ADAM9 may either directly degrade the ECM or induce the activation of other proteases in the ECM, such as matrix MMPs, thereby allowing tumor cell penetration into the brain matrix.

Our results are in agreement with Mazzocca et al. [31] who showed that ADAM9-S, an alternatively spliced variant secreted by activated hepatic stellate cells, induces colon carcinoma cell invasion in vitro and that this process requires both protease activity and binding to the $\alpha_6\beta_4$ and $\alpha_2\beta_1$ integrins.

Contradictorily to our results, Fry and Toker [41] demonstrated that the silencing of both soluble (ADAM9-S) and transmembrane (ADAM9-L) isoforms, increased the migration of BT549 breast cancer cells. In this work, they also showed that the overexpression of ADAM9-S is responsible for increasing cell migration in BT549 cells through its metallopeptidase domain. Moreover, they also showed that ADAM9-L is responsible for inhibiting cell migration through its disintegrin domain. Thus, both isoforms have different and opposite responses during cancer progression. Whether MDA-MB-231 cells have ADAM9-S and L isoforms and the effects of isoform silencing in this cell line will be further investigated.

Some ADAMs may induce proliferation by catalyzing the cleavage of growth factors, such as HB-EGF, and its membrane anchored form (proHB-EGF) can act as a negative regulator of proliferation [42,43]. Izumi et al. [44] showed that after induction with TPA (an activator of protein kinase C), ADAM9 interacted with PKCδ and cleaved proHB-EGF; however, we have demonstrated here that ADAM9 is not involved in the proliferation of MDA-MB-231 cells. In another work, RNAi-mediated ADAM9 silencing was responsible by a reduction in adenoid cystic carcinoma metastasis both in vitro and in vivo [19]. In this work, the authors also demonstrated that ADAM9 is essential for cancer cell proliferation and invasion and that its expression could be used as a prognostic of metastatic risk, since it was elevated in a high metastatic potential cell line (SACC-LM) when compared to a low metastatic potential cell line (SACC-83) [19].

Klessner et al. [39] demonstrated that ADAM9 participates in the shedding of desmoglein 2 (Dsg2), resulting in stronger cell–cell adhesion, which could, in turn, reduce the rate of migration and cell invasion. The ADAMs can also interact with β_1 integrins, and this association facilitates the recognition and location of their substrates for proteolytic shedding [45,46], as reported by Mahimkar et al. [47].

In a recent work, Hamada et al. [48] reported that miR-126 was found to target ADAM9 and that siRNA-based knockdown of ADAM9 in pancreatic cancer cells resulted in reduced cellular migration, invasion, and induction of epithelial marker E-cadherin.

Taken together, the literature results and the data found in the present study suggest that ADAM9 participates in the invasion of tumor cells by either directly degrading the ECM, by inducing activation of other proteases, such as MMPs, by co-localizing with other molecules, such as β_1 integrin, present on the surface of MDA-MB-231 cells (data not shown) or by interacting with other regulators such as miRNAs. A more conclusive demonstration that ADAM9 is a suitable target for metastatic breast cancer will require the use of a stable expression vector in vivo and/or inhibitors of this protein alone or in combination with conventional clinical therapies.

5. Conclusions

The results presented in this study reinforce the importance of the ADAM9 role in the invasion of breast tumor cells. Considering the significance of cell invasion in metastatic progression, ADAM9 can be pointed as an interesting target for the design of drugs involved in the treatment or prevention of breast cancers. We conclude that ADAM9 has an essential role in cell invasion and may be involved in metastatic spread. Therefore, it may be an interesting target for anti-metastatic therapy.

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