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Additive effect of metastamiR-193b and breast cancer metastasis suppressor 1 as an anti-metastatic strategy

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Abstract

Background It has been reported that enhancing the cellular levels of miR-193b as well as breast cancer-metastasis-suppressor-1 (BRMS1) protein is associated with diminished metastatic characteristics in breast cancer. In view of these facts, as a new therapeutic intervention, we employed a restoration-based strategy using both miR-193b-3p mimic and optimized BRMS1 in the context of a chimeric construct.

Methods miR-193b-3p and BRMS1 genes were cloned and the resulting plasmids were transfected into the MDA-MB231, MCF-7 and MCF-10A cell lines. microRNA expression levels were assessed by rea time PCR using LNA-primer and protein expression was confirmed by western blot method. Then, apoptosis, MTT, colony formation and invasion assays were carried out.

Results The expression levels of miR-146a, miR-146b and miR-373 were up-regulated, while the miR-520c, miR-335 and miR-10b were down-regulated following the exogenous BRMS1 expression. The exogenous over-expression of BRMS1 was associated with higher amounts of endogenous miR-193b-3p expression and enabled more efficient targeting of the 3′UTR of uPA. Although, miR-193b-3p and BRMS1 are individually capable of suppressing breast cancer cell growth, migration and invasion abilities, their cistronic expression was capable of enhancing the ability to repress the breast cancer cells invasion.

Conclusions Our results collectively indicated the existence of an additive anti-metastatic effect between miR-193b-3p and BRMS1. Moreover, it has been hypothesized that the exogenous expression of a protein can effect endogenous expression of non-relevant microRNA. Our findings provide new grounds for miR-restoration therapy applications as an amenable anti-metastatic strategy.

Keywords Breast cancer metastasis · BRMS1 · miR-193b · uPA

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Introduction

Recent advent of microRNAs attracted increasing attention in the field of metastasis investigations [1, 2]. microRNAs are highly conserved small non-coding RNAs, involved in post-translational gene expression control [3–5]. Using miRNAs to suppress the genes involved in different stages of the metastasis provides a new horizon in this field. Two classes of the most important regulators of metastasis are: metastasis suppressors and metastamiRs [6, 7]. metastamiRs are defined as the members of miRNA family which are involved in metastasis regulation. "metastasis-regulatory microRNAs" or "metastamiRs" was first coined by Hurst et al. [8]. Essentially, metastamiRs are categorized into two functionally distinguished subgroups: (1) the group with pro-metastatic activity which are involved in metastasis

activation and called metastasis-promoting miRNAs or oncomiRs, (2) the group with anti-metastatic activity which are involved in metastasis inhibition and called metastasis-suppressing miRNAs or anti-metastamiRs. Due to unique nature of oncomiRs and anti-metastamiRs, they have opened a new direction in the field of metastasis miR-therapy. Two main approaches could be considered to use metastamiRs in miRNA restoration based therapies. One approach could be the augmentation of the anti-metastamiRs expression using miRNA mimic technology (miR mimic technology is based on double stranded RNA molecules designed and chemically synthesized to mimic endogenous mature miRNA molecules upon transfection into cells), while the other approach could be the decrease of the oncomiRs expression using inhibitory technologies. Contemporary, miR restoration therapy has garnered a lot of attention in the field of cancer and metastasis inhibition.

miR-193b is a member of metastamiR family capable of inhibiting metastatic genes by post-transcriptional silencing and changing the expression of several proteins related to metastasis and cell signaling [9–12]. The sequence analyses of miR-193b target genes have confirmed the existence of miR binding sites. Amongst, the ERα and urokinase-type plasminogen activator (uPA) were the genes involved in breast cancer pathology which are targeted by the miR-193b-3p strand [13, 14]. uPA can play a pivotal role as a trigger of epithelial–mesenchymal transition (EMT) [15].

BRMS1 has garnered a lot of attention owing to its various anti-metastatic functions. The BRMS1 gene is located at 11q13. Seraj et al. were the first to delineate the functional mechanism of BRMS1 protein [16]. The mechanism underlying BRMS1 action was further analyzed by Nikolaev and Hurst in 2004 and 2006, respectively [17, 18]. This molecule exerts its function mainly by altering SIN3/histone deacetylase (HDAC) chromatin remodeling complexes. SIN3 is a co-repressor which contains several protein–protein interaction domains. It interacts with sequence specific transcription factors via direct DNA binding ability [17]. On the other hand, SIN3 interacts with the HDAC complex, which contains histone binding proteins [19]. The alteration in SIN3/HDAC could lead to changes in migration, invasion, apoptosis, angiogenesis, adhesion, cytoskeleton rearrangement, gap junctional communication, and enhancing immune recognition [20, 21]. Moreover, BRMS1 bridges between metastasis suppression and metastamiR regulation by up regulation of metastasis-suppressing miRNAs (miR-146a, miR-146b and miR-335) and down regulation of metastasis-promoting miRNAs (miR-10b, miR-373 and miR-520c). BRMS1 is a metastasis suppressor capable of transcriptome, post-transcriptome and proteome regulation at molecular level [22].

Restoring metastasis suppressor levels (including miRNAs or genes) in invasive cancer cells, especially when they act pleiotropically, represents a powerful therapeutic option for metastatic cancers. Re-establishing the expression of a single gene or miRNA (to that of the non-diseased tissue) will generate a more extensive therapeutic effect compared to drugs that follow the one-drug-one-target pattern. This effect is due to the ability of pleiotropic genes or miRNAs to target multiple genes/pathways [23]. In the present study, we have analyzed three different cell lines including non-tumorigenic MCF-10A (non-transformed mammary epithelial cells), tumorigenic but non-metastatic MCF-7 and metastatic MDA-MB231 breast cancer cell. We have demonstrated that restoring the combined expression of anti-metastatic miR-193b and BRMS1 could reduce in vitro invasion/migration in breast cancer cell lines. Besides, we have showed that exogenous expression of BRMS1 could directly or indirectly lead to up-regulation of endogenous miR-193b. In turn, the higher amount of miR-193b-3p enabled more efficient targeting of the 3′UTR region from uPA mRNA and consequently down-regulates uPA expression. Hence, the combination of these two metastasis suppressors may inhibit metastasis processes more effectively. Exogenous expression refers to the expression of genes reside on the exogenous constructs transfected into the cells, while endogenous expression refers to the expression of genomic genes.

Materials and methods

Generation of DNA constructs

All fragments cloned into pcDNA6.2-GW/EmGFP/miR-negative plasmid (Invitrogen, Carlsbad, CA, USA) according to the kits' instruction (pc/GFP/miR-negative abbreviated: pc/GFP/neg). miR-negative is predicted not to target any known vertebrate genes (Supplementary 1). Mature miR-193b-3p sequence was retrieved from miRBase, then the top and bottom oligo sequences of miR-193b-3p were designed and chemically synthesized. By 10X oligo annealing buffer, they were annealed and cloned into pcDNA (pc/GFP/miR-193b abbreviated: pc/GFP/193) (Supplementary 1). With the intention of reaching the highest possible level of expression, the BRMS1 gene sequence has been optimized by GenScript (Genscript Corporation Piscataway, NJ, USA). The optimized gene then was cloned into pcDNA 6.2-GW/EmGFPmiRneg and pc/GFP/miR-193b by *Sal*I and *Dra*I restriction enzymes (Roche Applied Science, Castle Hill, NSW, Australia) to make pc/BRMS1/miR-negative (abbreviated: pc/B/neg) and pc/BRMS1/ miR-193b (abbreviated: pc/B/193) constructs respectively.

This chimeric vector allows for (co-cistronic) co-expression of BRMS1 in the place of EmGFP and miRNA-193b embedded in a murine miR-155 context under the human cytomegalovirus promoter. Since the GFP fragments

replaced with BRMS1 by these enzymatic digestion, we also eliminated GFP fragment in control plasmid (pc/miR-193b abbreviated: pc/193) by self ligation of *Dra*I blunt ends. Finally we had 5 constructs and the fidelity of all chimeric vectors was confirmed by sequencing.

Cell culture

MDA-MB231 cell line (high invasiveness triple negative breast cancer cells, basal form and claudin low cell line) was used as the metastatic breast cancer cells; MCF-10A cell line was used as non-tumorigenic cell; and MCF-7 (luminal breast cell line) cells were used as the tumorigenic but non-metastatic cells. The medium for MCF-7 cells is DMEM/F12, 10% Fetal Bovine Serum (FBS), 1% penicillin/streptomycin (Gibco/ Invitrogen, Carlsbad, CA, USA). MCF-10A cell line was propagated in DMEM/F12 supplemented with 5% donor horse serum, 20 ng/ml EGF, 10 μg/ml insulin and 0.5 μg/ml hydrocortisone. MDA-MB231 cell was grown in Dulbecco's modified Eagle's Minimal Essential Medium (DMEM; Life Technologies, Inc., Grand Island, NY, USA) supplemented with 10% FBS (Hyclone Laboratories, Logan, UT, USA) and 1% penicillin–streptomycin solution (Life Technologies, Inc.) in a humidified atmosphere of 5% CO_2 in air.

Optimization of transfection

Our study was performed in 3 different 24-well plates for three cell lines (MCF-10A, MCF7, MDA-MB231), for each plate we had 6 groups in triplicate forms: untreated group, treated by pc/GFP/neg, pc/GFP/193, pc/B/neg, pc/B/193, pc/193. According to the manufacturer's recommendations, 2 μl of Lipofectamine 2000 (Invitrogen) was used with 1 μg of each construct. 72 h post-transfection, the efficiency was determined by green fluorescent protein (GFP) and flow cytometry. By Blasticidin antibiotic (Invitrogen, Carlsbad, CA, USA) selection (2.5 μg/ml), Transfected cells were isolated from un-transfected cells.

RNA extraction and Real time PCR

Total RNA was extracted from three cell lines by RNeasy mini kit (Qiagen, Hilden, Germany). cDNA was randomly primed from total RNA using the RevertAid First Strand cDNA Synthesis Kit (Fermentas, Thermo Fisher Scientific, Waltham, MA). To identify potential target genes of miR-193b-3p, the TargetScan (http://www.targetscan.org), miRanda (http://www.microrna.org), miRDB (http://www.mirdb.org/miRDB/) and PicTar (http://pictar.mdc-berlin.de) databases were used. Real time PCR assays were performed in triplicate by FastSrart SYBR Green MasterMix (Roche) on a Corbett RotorGene real-time PCR

using primers in Supplementary 1. Relative expression levels were determined using the comparative quantification characteristic of the Rotorgene software. All mRNA quantification data were normalized to β-actin and the comparative Ct (Pfaffl) method was used to determine the expression fold change.

miRNA extraction and real time PCR

Using High pure miRNA isolation kit (Roche), miRNAs were extracted 48 h after transfection from all samples according to manufacturer's instruction. Then, cDNA was synthesized by Universal cDNA Synthesis kit (Exiqon). Real time PCR for mature miRs (miR-191 as endogenous control) was done with miRCURY LNA Universal RT microRNA PCR kit, while the primers of each miR was LNA microRNA from Exiqon Company. RotorGene PCR machine (Corbett Research, San Francisco, USA) was used to evaluate gene expression levels. All reactions were performed in triplicate and used the average values for relative quantification. We used the Pfaffl method to determine the relative quantity of expression. Briefly, we normalized the cycle of the threshold (Ct) values of the target gene to the endogenous control and compared it with a calibrator.

MTT assay

Cell density was adjusted to 5×10^3 cells/well and seeded into 96-well plates, after cells reached 70% confluency, transfections were performed as described before. All transfections were done in triplicates. 48 h after transfection, each well was incubated with 10 μl methyl thiazolyltetrazolium (MTT, 5 mg/ml in Phosphate-buffered saline (PBS); Sigma, St Louis, Missouri) for 4 h at 37 °C in 5% CO_2 incubator. Formazan crystals were dissolved using 100 ml dimethyl sulfoxide (Sigma, St Louis, Missouri) per well. The optical density was measured using an enzyme-linked immunosorbent assay plate reader at 570 nm wavelength (reference wavelength 630 nm).

Apoptosis assay

Apoptosis were assessed by Annexin-V-FLUOS Staining Kit (Roche) According to the manufacturer's recommendations. As described before, Cells were transfected by pc/B/193 construct, 48 h after transfection cells were harvested by trypsinization. The cell pellet re-suspended in 100 μl incubation buffer followed by 2 μl Annexin-V-Fluorescein and Propidium iodide. After 10–15 min incubation at 15–25 °C, they were analyzed by a flow cytometer.

Cell proliferation and colony formation assay

In the clonogenic assay, MDA-MB231 and MCF-7 were seeded in a 6-well plate at 2×10^3 and transfected with constructs. The cells were incubated in a CO_2 incubator at 37 °C for 2 weeks until the formation of colonies with substantial sizes. The medium was removed, cells were fixed in 1 ml of 4% formaldehyde solution, and the plates were incubated at 4 °C for 30 min. After removal of the fixation solution, 1 ml of 2% crystal violet solution was added, and the plates were incubated at room temperature (RT) for 30 min. Subsequently, crystal violet solution was removed, and the plates were rinsed. The plates were air-dried at RT. Subsequently, 1 ml of 10% acetic acid was added to each well to dissolve the crystal violet. The absorbance of individual wells was determined at 595 nm using ELISA reader. The maximum absorbance (OD) was considered as the base line and was assumed to be 100.

Wound healing assay

MDA-MB231 and MCF-7 were plated in six-well plates and cultured in medium containing 10% FBS to near confluence of the cell monolayer. Then a linear wound was created in the monolayer by scratching using a plastic pipette tip. The monolayer was washed twice with PBS to remove debris or detached cells. The wounded monolayer was incubated for 24 h. Cell migration into the wound area was observed by an inverted microscope at time zero and 24 h after the scratch.

Transwell migration assay

Transwell migration assay was performed using transwell cell culture chamber units (Millipore, Billerica, MA, USA) with 8-μm pore size polycarbonate membranes. Briefly, cells (2.5×10^4) which were transfected by constructs and serum starved were plated in the upper chamber non-coated wells, while serum-containing medium was added to the lower chamber wells as chemoattractants. After incubation for 24 h at 37 °C in a 5% CO_2 humidified incubator, cells in upper wells were completely removed from the upper surface of the filters with cotton swabs. Cells that had migrated to the lower surface of the filters were fixed with 4% formaldehyde for 30 min and stained with crystal violet. The migratory phenotypes were determined by counting the cells that migrated to the lower side of the filters in different fields under a phase-contrast microscope at $200 \times$. The cell counting was done using the ImageJ software. Percentage of these counted migrated cells relative to the primary seeded cells, were taken. Assays were repeated in triplicate.

Tranwell invasion assay

The cell invasion assay was performed in a same way as migration assay, except that transwell cell culture chambers were coated by matrigel. After trypsinization and seeding in serum-free medium, the cells were placed on matrigel-coated transwell chambers precoated with medium containing 10% FBS. FBS (10%) in the lower chamber served as a chemoattractant. Then, the 24-well plate was incubated at 37 °C in 5% CO_2. After 24-h transfection, the non-invaded cells were removed with a cotton swab. The migrated cells were fixed in 4% formaldehyde for 30 min. Subsequently, cells were stained with crystal violet for 5 min, followed by PBS wash for 15 min. An inverted microscope was used to count the migrated cells. The same procedure was essentially followed to measure transwell cell migration toward a chemoattractant by replacing matrigel-coated transwell chambers with uncoated transwell chambers.

Western blotting

Primarily, to investigate the protein expression level of a functionally optimized BRMS1 protein, western blot was done. Since BRMS1 was placed instead of GFP under CMV promoter, there was no fluorescent marker to trace the transfected cells from not-transfected cells. As a solution, two groups of MDA-MB231 cells were transfected by pc/B/neg and pc/GFP/neg in parallel. For both the same transfection condition and antibiotic selection were done. Finally in pc/GFP/neg transfected group, only GFP expression cells and in pc/B/neg transfected group, only BRMS1 expression cells were retained. Cells were lysed in RIPA (radio-immunoprecipitation assay) buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% sodium deoxycholate, 1% NP-40) enriched with protease inhibitors cocktail (PMSF). The cells were scraped by a cell scraper and transferred to a tube on ice and sonicated briefly. By centrifuge extract for 10 min at $14,000 \times g$ in a cold microfuge, total proteins were obtained. Millipore ultrafiltration column for concentration protein were used. The protein concentration was measured by Bicinchoninic acid assay (Thermo Fisher Scientific). Protein (40 μg) were separated on a 12.5% SDS-polyacrylamide gel and transferred electrophoretically onto Nitrocellulose Transfer membranes (PROTRAN, Schleicher & Schuell BioScience, Germany). The membranes were blocked with 3% skimmed milk in Tris-buffered saline/0.05% Tween − 20 for 1 h, subsequently blotted with horseradish peroxidase-conjugated antibody (Abcam Company) for 1 h. The protein bands were visualized with enhanced chemiluminescence detection kit (Thermo Fisher Scientific).

Statistical analysis

All experiments were performed at least three times in triplicates for each group. The results are presented as the mean SD. Statistical significance was determined using Student's *t* test, and an asterisk means significant ($P < 0.05$).

Results

Quantitative real-time PCR (qRT-PCR)

The level of endogenous BRMS1 was also evaluated before any treatments. The results demonstrated that the level of BRMS1 expression in MCF-7 and MCF-10A was ten and sixfold upper than MDA-MB231 (Fig. 1a). After transfection by pc/B/neg and pc/B/193, the exogenous BRMS1 expression increased. This increasing in
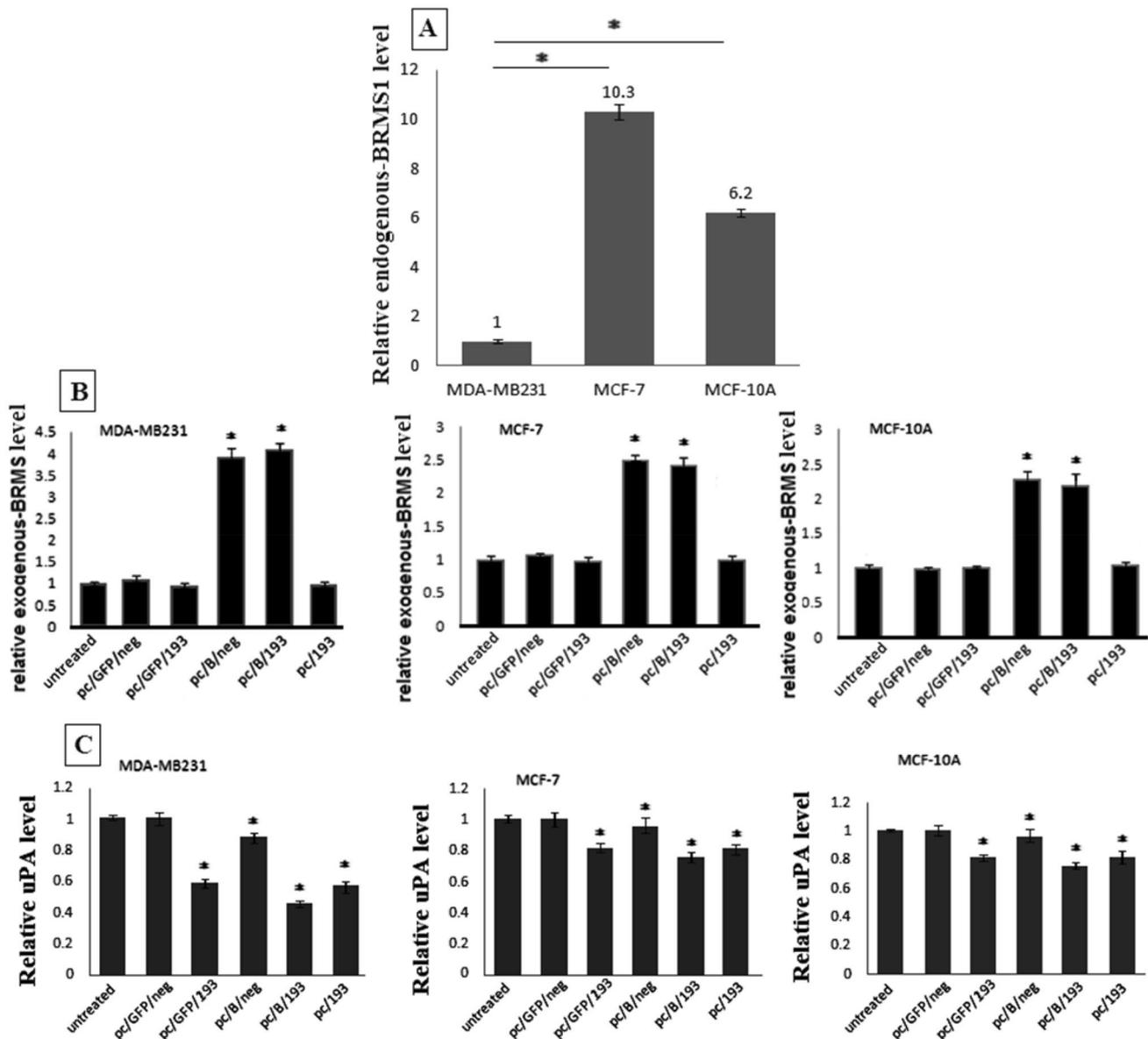
Fig. 1 Gene expression levels in MCF-7, MCF-10A and MDA-MB231. **a** The expression levels of endogenous BRMS1 were examined in untreated breast cancer cells using real-time PCR (Pfaffl ratio). All values were calibrated to basal expression in MDA-MB231 cells. **b** Relative exogenous BRMS1 expression compare to the untreated cells. **c** Relative uPA expression compare to the untreated cells. Data are presented as means ± SD of three separate experiments, $n = 3$, *$P < 0.05$

MDA-MB231 was more significant (Fig. 1b). Obviously qRT-PCR showed less uPA expression in miR-193b transfection (Fig. 1c) but surprisingly the exogenous BRMS1 expression (pc/B/neg) also causes a reduction in uPA expression (Fig. 1c). This indicated that most uPA down-regulation occurred after cistronic co-expression of BRMS1 and miR-193b-3p. The reduction in transfected MDA-MB231 was more than two other cell lines. Relative expression of the genes compared to β-actin as the house-keeping gene was obtained according to pfaffl relevance.

Functional exogenous BRMS1

MDA-MB231 cells were transfected by pc/GFP/neg and were survived by the antibiotic selection (Fig. 2a). The same result was obtained after pc/B/neg transfection. Figure 2b shows the MDA-MB231 cells which expressed exogenous BRMS1. The BRMS1 proteins encoded by pc/B/neg construct were examined after transfection by western blot. Total protein was extract and western blot approved more BRMS1 protein expression level in transfected cells (Fig. 2c). The structured of optimized BRMS1 could be equal with endogenous BRMS1 and both of them were detected on western blot.

Real time PCR for metastamiRs

As shown in Fig. 3a, the expression level of miR-193b is significantly increased in all cells transfected with the miR mimic constructs containing miR-193b (pc/GFP/193, pc/B/193 and pc/193). However, the enhancement in miR-193b expression levels is more significant in MDA-MB231 cell line compared to two other cell lines. Not only ectopic expression of miR-193b increased expression levels of miR-193b, but also ectopic expression of exogenous BRMS1 increased expression levels of miR-193b. Also BRMS1 downstream metastamiRs were investigated: Anti-metastamic miR-146a, 146b and 335 were increased (Fig. 3b–d) and on the other hands oncomiRs 10b, 520c and 373 were decreased (Fig. 4a–c).

The effect of constructs on cell death/growth

To obtain evidence for which, the level of constructs toxicity, we performed a MTT assay on transfected breast cell line. The data showed that BRMS1 construct have much higher level of toxicity in comparison to miR-193b construct (Fig. 5a). For apoptosis assay, Cells were transfected by all constructs, but it should be noted that the pc/GFP/neg and the constructs that contained the miR-193b-3p fragment (pc/
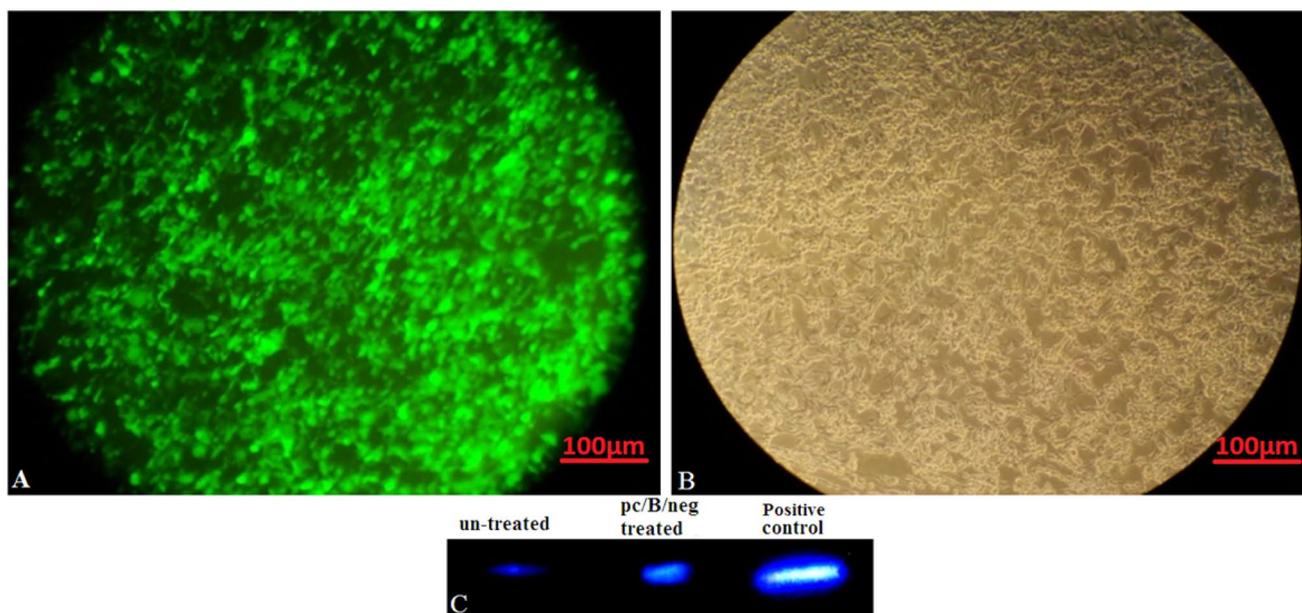

Fig. 2 Protein expression levels. **a** MDA-MB231 cells were transfected by pc/GFP/neg. By the vector antibiotic selection (Blasticidin), all transfected cells with GFP expression were retained. **b** MDA-MB231 cells were transfected by pc/B/neg. By the vector antibiotic selection (Blasticidin), all transfected cells with BRMS1expression were retained. **c** Western blot with BRMS1 antibody [horseradish peroxidase-conjugated antibody (Abcam Company)] on: untreated MDA-MB231 cells, pc/B/neg transfected MDA-MB231, and a positive control (pure commercial BRMS1 protein). transfected MDA-MB231 cells by pc/B/neg showed more BRMS1 protein expression than untreated MDA-MB231 cells
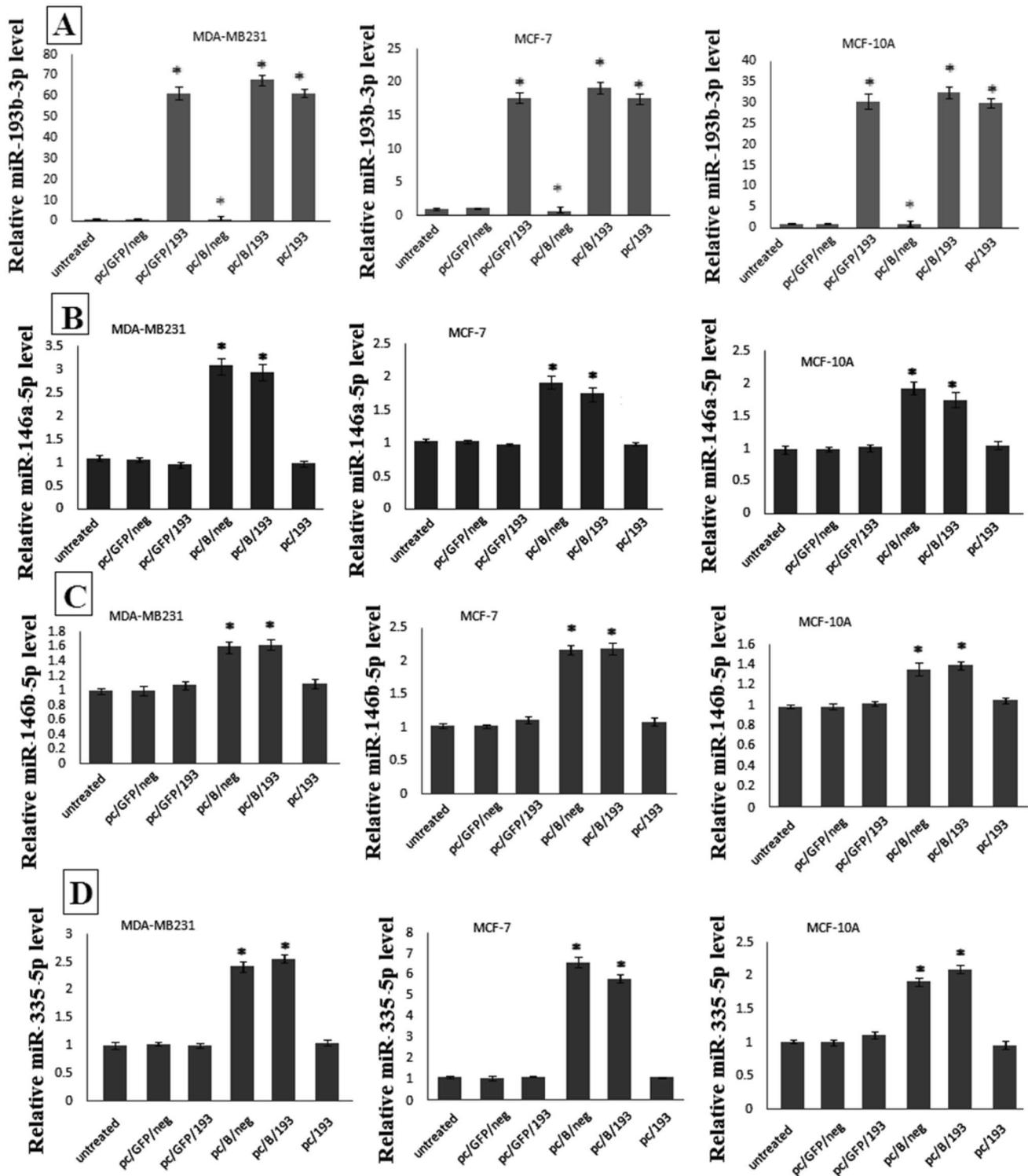
Fig. 3 Up-regulated miR expression levels in MCF-7, MCF-10A and MDA-MB231. **a** miR-193b, **b** miR-146a, **c** miR-146b, **d** miR-335. These kinds of metastamiRs were tumor suppressive miRs and after transfection, they have been increased. Data are presented as means $\pm$ SD of three separate experiments, $n = 3$, $*P < 0.05$ vs untreated group
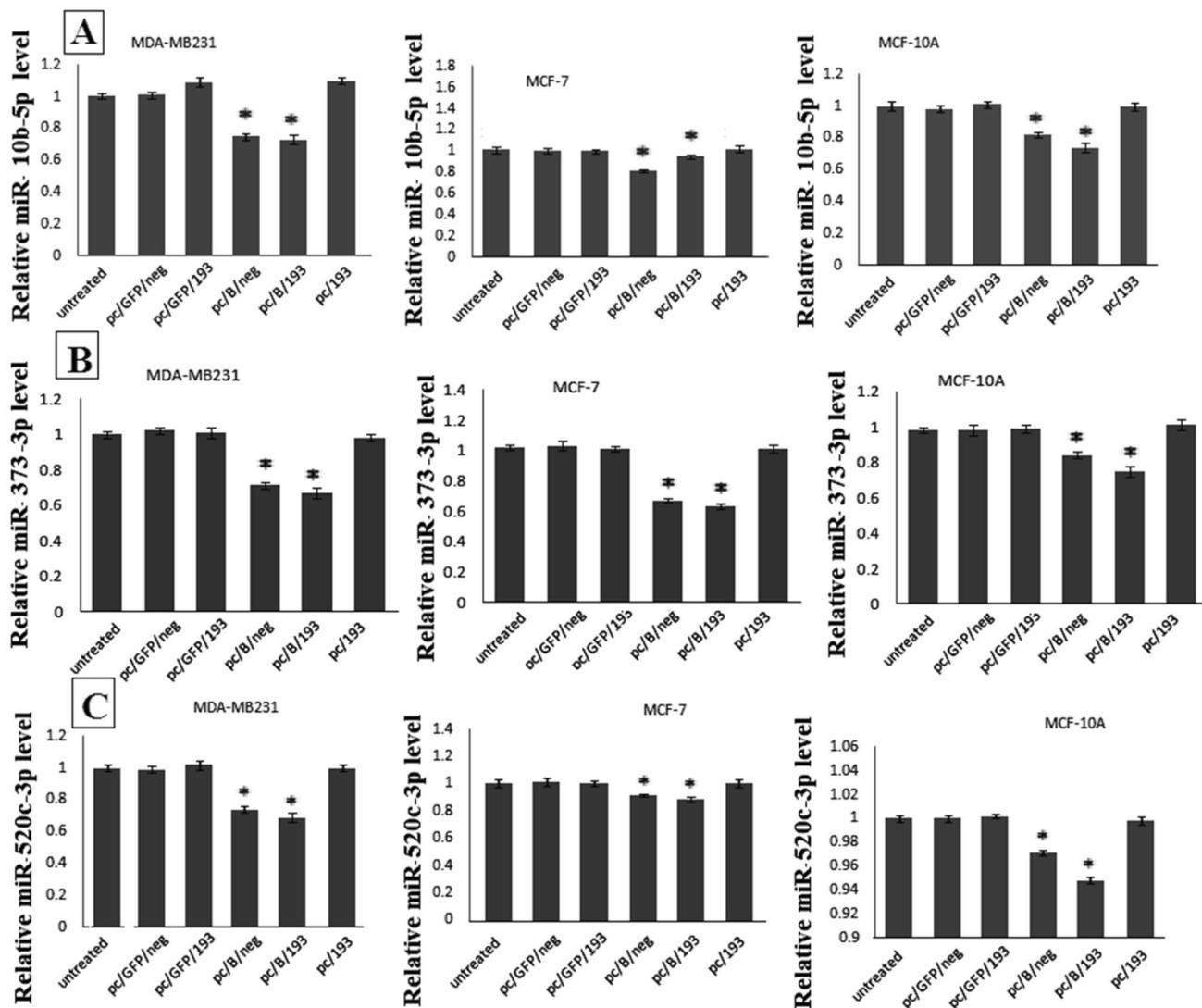
Fig. 4 Down-regulated miR expression levels in MCF-7, MCF-10A and MDA-MB231. **a** miR-10b, **b** miR-373, **c** miR-520c. These kinds of metastamiRs were oncomiRs and af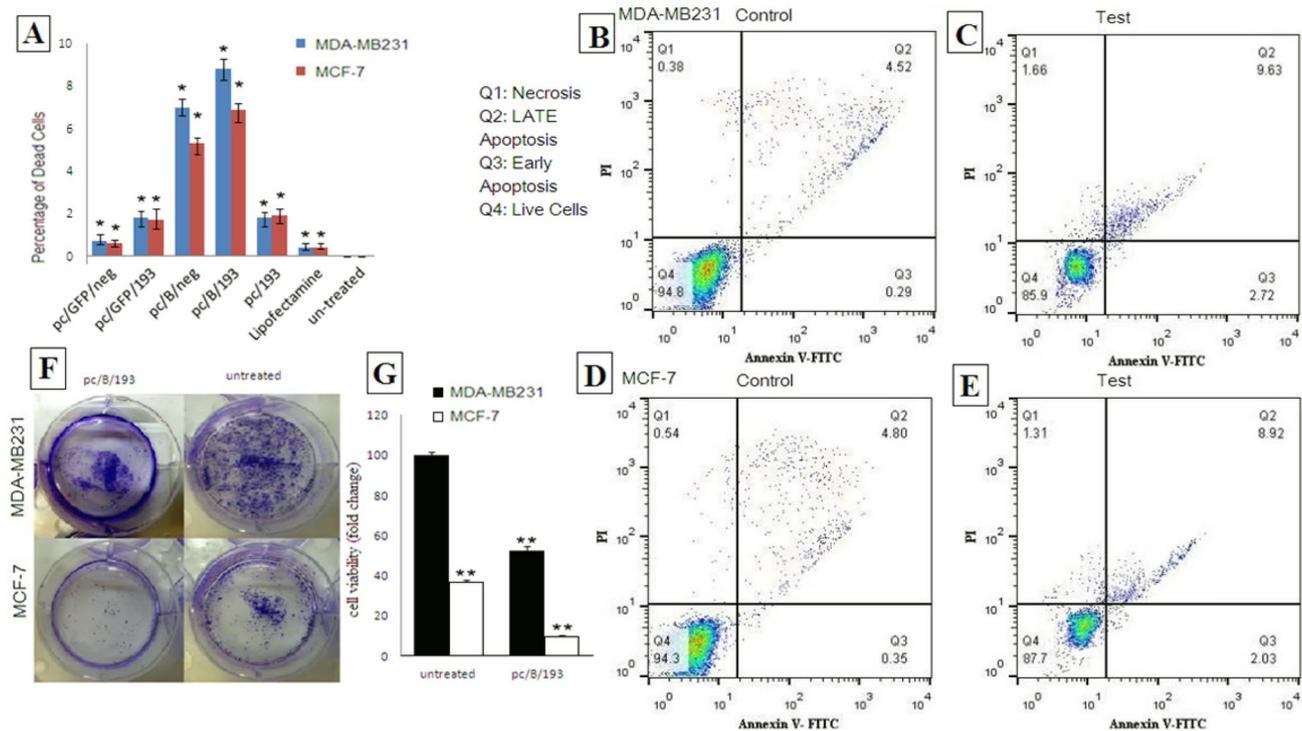ter transfection, they have been decreased. Data are presented as means ± SD of three separate experiments, $n = 3$, *$P < 0.05$ vs untreated group

GFP/193, pc/193), did not show a remarkable difference in induction of apoptosis. However, the constructs that contained the BRMS1 fragment (pc/B/neg or pc/B/193) induced apoptosis (Fig. 5c, e). The difference in the Q2 area (late apoptosis) is clear. Therefore, apoptosis analysis indicated that exogenous expression of BRMS1, but not miR-193b, leads to apoptosis. This means "the functional optimized BRMS1" promoted apoptosis. The ectopic expression of BRMS1 and miR-193b-3p significantly suppressed breast cancer cell colony formation (Fig. 5f, g). The motility of human breast cancer cells lines MCF-7 and MDA-MB231 were examined by wound healing assay when treated with miR mimic, BRMS1 and both of them. Confluent monolayer

of cells was scratched to be wounded and cultured for 24 h (Fig. 6a, b). The treatment with both miR mimic and exogenous expression of BRMS1 (pc/B/193 construct) led to significant increase of scratch width (distance between two edges of the wound) and decrease of cell proliferation compared to the un-treated (Fig. 6c, d).

Ectopic miR-193b-3p and BRMS1 contribute to breast cancer migration and invasion

To determine whether miR-193b-3p and BRMS1 regulates breast cancer cell invasion and metastasis, we performed in vitro gain-of-function analyses by ectopic expression

Fig. 5 Ectopic expression of miR-193b-3p and BRMS1 affected cell death and growth. **a** Results for MTT assay which indicates significant toxicity of BRMS1 constructs in comparison to the miR-193b. Data are presented as means ± SD of three separate experiments, $n = 3$, *$P < 0.05$ vs untreated group. **b, c** Results for apoptosis analysis of MDA-MB231 and MCF-7 cells respectively without any treatment as control groups. **d, e** Results for apoptosis analysis of MDA-MB231 and MCF-7 treated by pc/B/193 as test groups. **f** Colony forma-tion assay results which was performed after transfection of MDA-MB231 and MCF7 cells with both miR-193b-3p mimics and optimized BRMS1 (pc/B/193 construct) for 2 week. The cell images of a representative experiment are shown. **g** Quantification results using absorbance of colored colonies with crystal violet. A graph shows quantified values relative to the untreated MDA-MB231 (maximum absorbance) as 100. Data are presented as means ± SD of three separate experiments, $n = 3$, **$P < 0.05$ vs untreated MDA-MB231

of miR-193b and BRMS1 in MCF-7 and MDA-MB231. Transwell migration and invasion assays were performed on the transfected cells by all constructs. As illustrated in Fig. 7c—migration of MDA-MB231 cell line—the construct which only contains the miR-193b-3p fragment (pc/193), was capable of decreasing the migration rate down to 11%, while the BRMS1 containing construct (pc/B/neg) managed to decrease the migration rate down to 30%. Therefore, the suppressive effect of miR-193 was much higher than BRMS1. The same effects were observed analyzing the changes in invasion rate following the treatments. It was found that ectopic simultaneous expression of miR-193b and BRMS1 significantly (almost 68% reduction) suppressed the invasion of MDA-MB231 in Transwell assays with Matrigel and also reduced the migration of these cells in Transwell assays without Matrigel (Fig. 7).

Discussion

The investigation about the anti-metastatic proteins like BRMS1 could bring about important clues about the mechanism underlying the cellular processes of metastasis initiation [24, 25]. In this regard, our real time PCR results indicated that the lowest BRMS1 expression belongs to the metastatic breast cancer cell line (MDA-MB231). The rationale behind this observation could lay in the fact that BRMS1 is a metastasis suppressor and mostly influence the metastatic process rather than the proliferation of cancer cells. It has been demonstrated that BRMS1 is additionally involved in inhibition of melanoma and bladder cancers [26, 27]. Moreover, in 2014, Zhang et al. have showed the effects of BRMS1 expression in breast cancer [25]. Given these facts, it seems quite normal for the metastatic MDA-MB231 cells to have low amounts of BRMS1 expression.
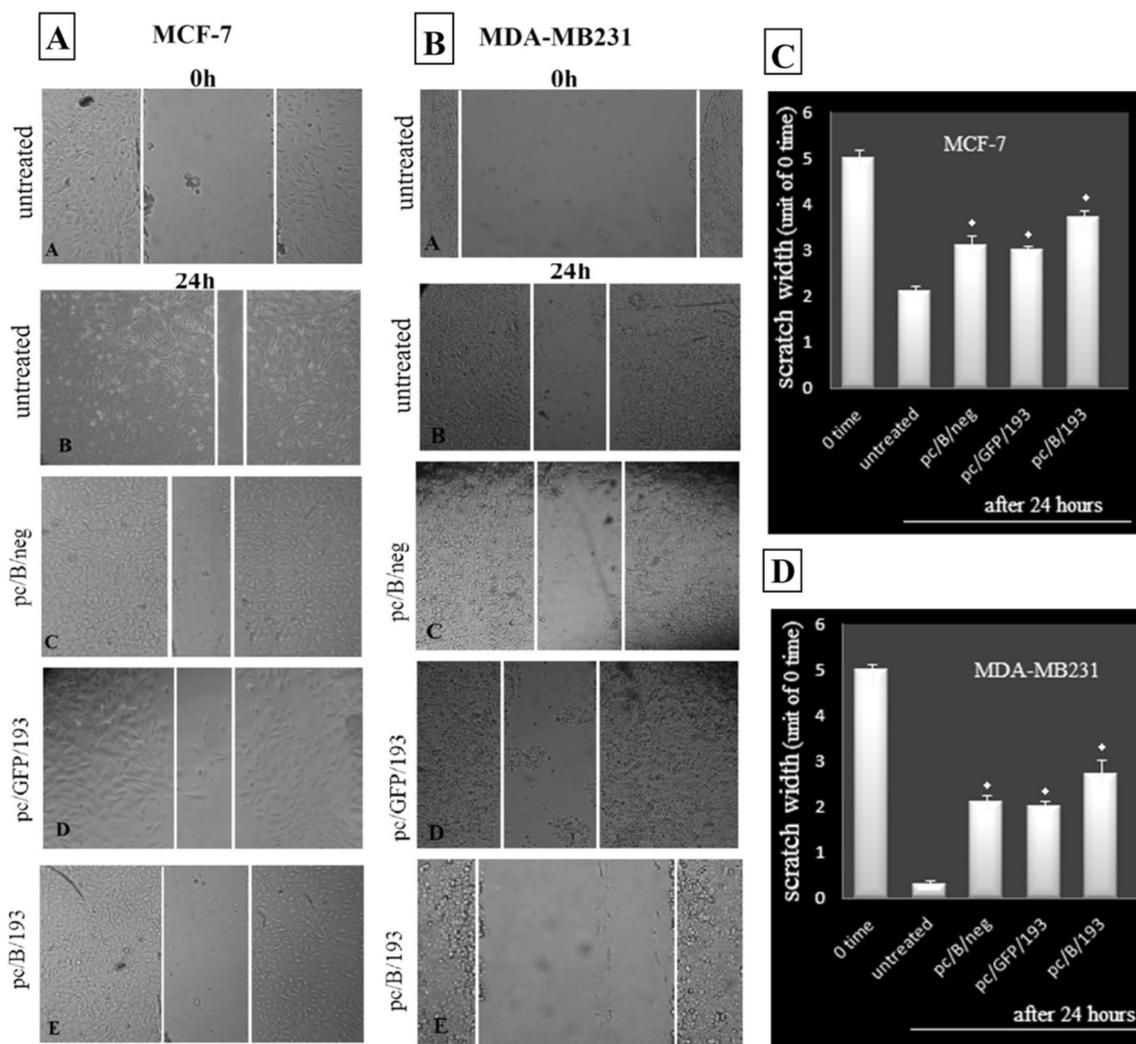
Fig. 6 The effect of constructs on proliferation and motility. **a** Movement of MCF-7 and **b** MDA-MB231 cells into the wound was shown for miR mimic, BRMS1, both of them, transfected and untransfected cells. **c, d** Quantification of the wound healing assay. Data are presented as means ± SD of three separate experiments, $n = 3$, *$P < 0.05$ vs untreated group after 24 h

On the other hands, the observed increase in the BRMS1 expression was highest for the transfected MDA-MB231 cells. Exerted exogenous expression of BRMS1 has led to a fourfold increase in BRMS1 expression. Since the western blot analyses indicated that the exogenously expressed BRMS1 protein could be structurally similar to endogenous BRMS1, equal functional effects are expected to be inflicted by both proteins.

Our data showed that the ectopically expressed miR-193b-3p and BRMS1 contribute to the observed decrease in breast cancer cell growth. Lombardi et al. have shown that the decrease in metastasis and migration behavior of assessed cells could be the consequence of exogenous

BRMS1 expression [28]. Moreover, Liu et al. have shown that BRMS1 is involved in apoptosis initiation [29]. In line with these studies, the performed apoptosis test confirmed the apoptosis-inducing effect of BRMS1 containing construct. The MTT-assay results indicated that the pc/B/neg construct is endowed with higher cell mortality in comparison to pc/GFP/193 which in turn confirms the BRMS1 effects on apoptosis pathway. Negative regulation for the nuclear translocation of NF-kB and urokinase-type plasminogen activator transcripts is described to be among the other roles played by BRMS1. Hurst and Edmonds have indicated that aside from affecting several anti-metastatic genes, BRMS1 exerts its functions through changes in

the expression of some members of metastamiR families. BRMS1 regulates the expression of metastamiRs to inhibit metastasis [18, 22, 30]. It have been shown that ectopic expression of BRMS1 as a pleiotropic gene could up-regulate anti-metastamiRs such as miR-146a, miR-146b [31] and miR-335 [32], while oncomiRs such as miR-10b [33], miR-373 and miR-520c are down-regulate [30, 34]. Accordingly BRMS1 represses metastasis by inhibiting multiple steps in the cascade through regulation of various metastasis associated genes as well as metastasis-regulatory microRNAs [35]. In this regard, the functionality of optimized BRMS1 gene was assessed by checking the expression of its downstream metastamiRs. Our results confirmed the fluctuations observed in metastamiRs expression proposed by Hurst and Edmonds et al. it should be taken into account that the construct merely containing BRMS1 (pc/B/neg) gene did not lead to increased miR-193b expression. This means that unlike the other miRs (miR-146a, miR-146b, miR-373, miR-520c, miR-335 and miR-10b) the expression of miR-193b is not under the direct control of BRMS1. More interestingly, none of the constructs led to increased expression of "endogenous" BRMS1 (in all transfected cells, the expression pattern of endogenous BRMS1 were similar to Fig. 1a).

Intriguingly unlike the BRMS1 gene, the expression of miR-193b was higher in the context of cells transfected by pc/B/193 (simultaneous expression of miR-193b and BRMS1). Although the LNA primers do not distinguish between the endogenious and exogenious miR-193b, but we hypothesized that the observed difference in miR-193b expression is the consequence of "endogenous" miR-193b expression.

The pc/GFP/193 and pc/193 constructs (produce different mRNA length) did not show significant differences regarding the expression of miR-193b, besides the pc/GFP/193 and pc/B/193 contain inserted sequences of approximately similar size (the sizes of BRMS1 and GFP gene are similar) and these two constructs produce single mRNA molecules which eliminates the possible expression variations arises from uneven mRNA lengths. Therefore, in spite of what we observed in our results, similar ectopic expression of miR-193b was anticipated to be observed from both constructs. This variation in miR-193b expression could be construed as the existence of an additive effect between BRMS1 and miR-193b to "genomic" miR-193b expression.

In this regard, Cicek et al., suggested that there is a reverse correlation between BRMS1 and uPA expressions [36]. Recent studies have shown that the NF-kB transcription factor positively regulates uPA expression. uPA promoter contains an NF-kB binding site [37]. On the other hands, BRMS1 diminishes NF-kB activity by catalyzing the deacetylation of NF-kB subunit [36]. Thus, the increase in BRMS1 expression leads to decrease in uPA protein expression. Various theories could be hypothesized to explain the logic behind the decreased uPA expression observed in transcriptional level. The involvement of upstream microRNAs could be deemed as a fair theory to delineate this issue. The upstream microRNAs of a target gene or protein could be increased to exert its inhibitory effects on the downstream gene or protein. The existence of a correlation between the miR-193b and the uPA expression has been reported in previous studies [14]. Similar results have already been obtained that BRMS1 expression leads to over expression of microRNA-146a/b, beside miRN-146 family suppresses NF-kB activity in breast cancer [31]. Our bioinformatics analyses revealed a miR-193b targeting site on 3′UTR region of uPA mRNA. The seed match region is 7mer-m8 in length and is exactly matching to positions 2–8 of the mature miRNA (the seed + position 8) (Supplementary 2A). Although the exact mechanism of BRMS1 effects on microRNAs has not been determined [30], given our data, it could be deduced that the increased BRMS1 expression down-regulates uPA expression even directly by interaction with NF-kB or indirectly by increasing "genomic" miR-193b expression (Supplementary 2B).

The potential target genes of miR-193b-3p which are correlated with BRMS1 were obtained. This network was illustrated in STRING database (Supplementary 2C). Prior studies conducted separately on miR-193b and BRMS1 reported that restoring the expression of these molecules could return the normal phenotype in replacement therapy. It involves the re-introduction of a molecule (for example, gene or miRNA molecules) to restore a loss-of-function, and therefore, represents a new opportunity to explore the remedial potential of metastasis suppressors [38, 39]. As such, our findings provided strong evidences that restoration of miR-193b and BRMS1 expression inhibits cell migration and invasion in breast cancer cells via regulation of various molecules involved in metastatic cascade. Thus, the concept of exploiting chimeric replacement therapy constructs could serve as a potential therapeutic remedy for breast cancer metastasis.
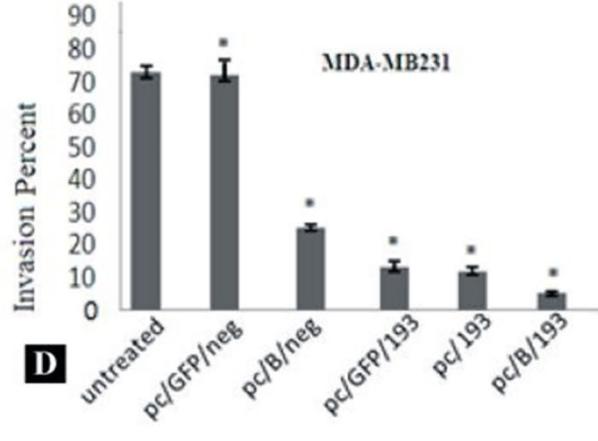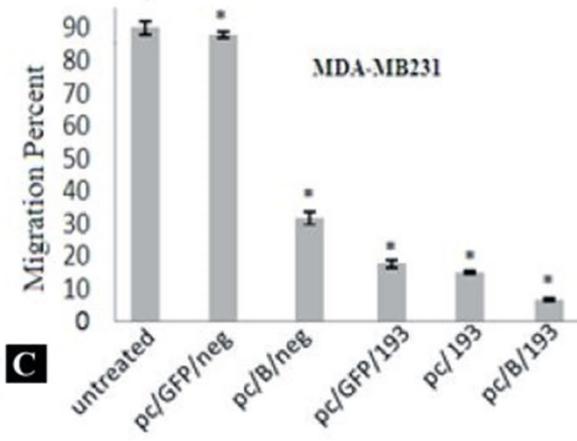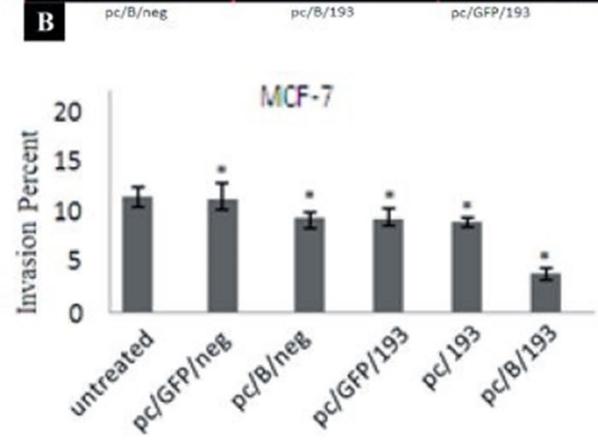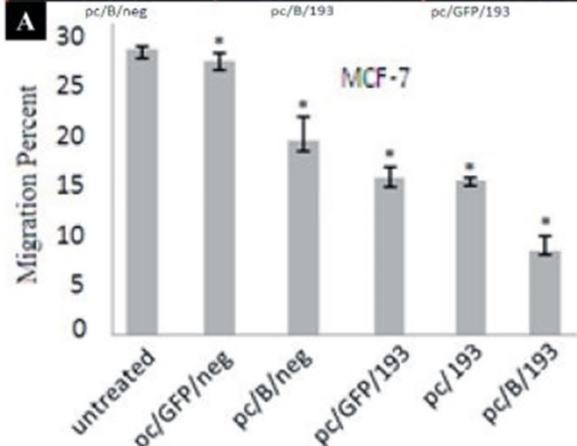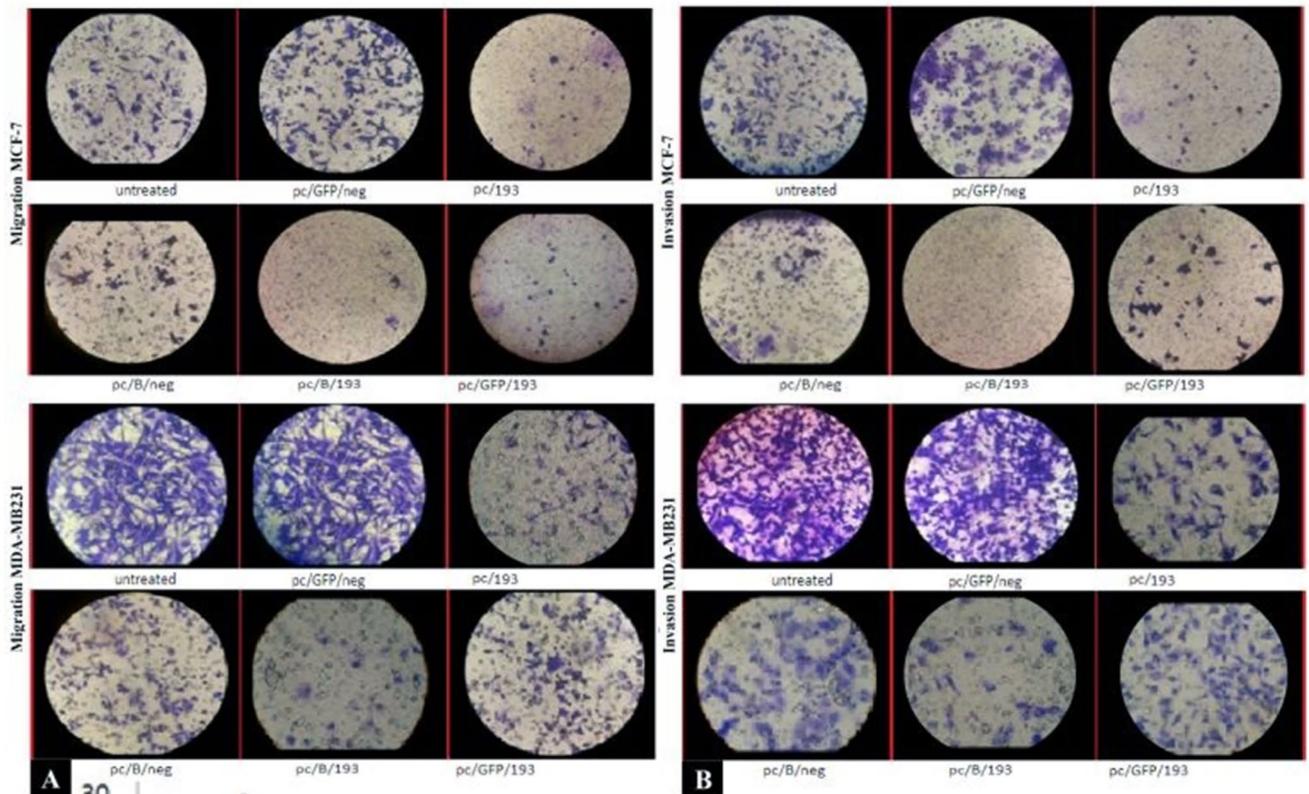
◄**Fig. 7** Effects of miR-193b-3p and BRMS1 on cell migration/invasion in vitro. The migration and invasion abilities of MDA-MB231 and MCF-7 cells transfected with all constructs for 24 h were assessed using the Transwell assay. Subsequently, migrated cells were stained with crystal violet solution. The cell images of a representative experiment are shown in **a** for the migration assay and **b** for the invasion assay. The colored migrated cells were counted using ImageJ software and the migration percentage (**c**) and the invasion percentage (**d**) was calculated. These graphs show quantified values. Data are presented as means ± SD of three separate experiments, $n = 3$, *$P < 0.05$ vs untreated group

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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