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Re-expression of *miR-200* by novel approaches regulates the expression of PTEN and MT1-MMP in pancreatic cancer

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Membrane type-1 matrix metalloproteinase (MT1-MMP) is often activated and expressed in tumor cells with significant invasive properties, and is associated with poor prognosis of patients. This could partly be due to deregulated expression of microRNAs (miRNAs) which regulates the expression of MT1-MMP and PTEN (phosphatase and tensin homolog) contributing to tumor invasion and metastasis. We initially compared the expression profile of *miR-200* family, PTEN and MT1-MMP expression in six pancreatic cancer (PC) cell lines by qRT-PCR and western blot analysis. We found loss of expression of *miR-200a*, *b* and *c* in chemo-resistant PC cell lines, which was correlated with loss of PTEN and over-expression of MT1-MMP. Based on our initial findings, we chose BxPC-3, MIAPaCa-2 and MIAPaCa-2-GR cells for further mechanistic studies. We assessed the effect of two separate novel agents CDF (a synthetic analog of curcumin) and BR-DIM (a natural agent) on PC cells. The expression of *miR-200* family and PTEN was significantly re-expressed whereas the expression of MT1-MMP was down-regulated by CDF and BR-DIM treatment. Forced over-expression or silencing of *miR-200c*, followed by either CDF or BR-DIM treatment of MIAPaCa-2 cells, altered the morphology of cells, wound-healing capacity, colony formation and the expression of MT1-MMP and PTEN. These results provide strong experimental evidence showing that the loss of *miR-200* family and PTEN expression and increased level of MT1-MMP leads to aggressive behavior of PC cells, which could be attenuated through re-expression of *miR-200c* by CDF and/or BR-DIM treatment, suggesting that these agents could be useful for PC treatment.

Introduction

It has been estimated that 43 920 people will be newly diagnosed with pancreatic cancer (PC) in 2012, and it is the fourth leading cause of cancer-related deaths in the USA (1). Although research effort has advanced toward targeted therapies, late diagnosis and/or diagnosis with metastatic therapy-resistant disease has made PC the leading cause of high mortality. Hence, better understanding of the underlying mechanisms involved in therapeutic resistance and findings ways to overcome drug resistance is critical for improving the dismal survival statistics and therapeutic efficacies in PC patients.

Amongst the multiple matrix metalloproteinases activated and expressed in a wide range of tumors, matrix metalloproteinase 14 (MMP-14), also known as membrane type-1 matrix metalloproteinase (MT1-MMP), is believed to play a crucial role in facilitating the tumor cells' penetration through extracellular matrix with significant potential for angiogenesis and invasive characteristics (2,3). MT1-MMP has also been associated to promote tumor invasion by the activation of proMMP-2, and is also directly associated with increased potential for angiogenesis and metastasis (4–6). According to another report, binding of endothelial cells to extracellular matrix shows the existence of two phases of MMP regulation, one through rapid inhibition of pro-MMP-2 activation, through inhibition of MT1-MMP, and the other by slower response of cell spreading and changes in the cytoskeleton to suppress the levels of MT1-MMP mRNA and protein (7). The over-expression of MT1-MMP has also been associated with metastatic behavior of virtually all types of cancers (8,9), including PC in K-Ras transgenic mouse model (10) and in biopsies from triple-negative breast cancer (11). However, little is known about the expression of MT1-MMP and the underlying mechanisms involved in human PC, suggesting that understanding the regulation of MT1-MMP and finding ways to inhibit its expression would be important for designing novel therapies for PC.

Besides the deregulation of MT1-MMP, the loss of expression of phosphatase and tensin homolog (PTEN), which is a ubiquitous tumor suppressor gene, has been shown to correlate with tumor aggressiveness and was also associated with up-regulation of miRNA expression such as *miR-21* (12,13). Additionally, over-expression of MT1-MMP was found to be associated with loss of PTEN expression in prostate cancer cells derived from mice through the activation of the PI3K/Akt pathway (14); whereas in renal cell carcinoma, loss of PTEN induces HIF-2α transcriptional activity through antagonism of PI3K signaling (15). Moreover, in H-59 cells of lung carcinoma, IGF-1 receptor controls tumor cell invasion through MT1-MMP activation mediated by activation of PI3K/Akt/mTOR signaling (16). Thus, the loss of PTEN expression and increased MT1-MMP expression could have a significant impact on the regulation of cell growth, invasion, migration and aggressiveness of PC cells. Therefore, it is vital to find novel agents that could mechanistically regulate MT1-MMP and PTEN expression in PC, which would likely advance our knowledge in designing novel and improved therapies for the treatment of PC.

Emerging evidence suggest that microRNAs (miRNAs), highly conserved and small non-coding regulatory RNAs, play a major role in the regulation of gene expression through post-transcriptional repression, and appear to be important in PC. The expression of *miR-200* family has been established by us and others both *in vitro* and *in vivo* as one of the most intensively studied epithelial to mesenchymal transition (EMT)-related miRNAs that target multiple genes (12,17–20). Recent evidence has also shown down-regulation of *miR-200* family by ZEB1 due to suppression of stemness-inhibiting miRNAs in the 38 different carcinoma cell lines of the NCI-60 cell line panel (20). In lung cancer cell lines derived from mice, *miR-200* altered the tumor environment, inhibiting the processes of EMT and metastasis (21). These findings suggest that the expression of *miR-200* in PC is closely correlated with stemness, metastasis and EMT, which is due to targeting multiple genes. Hence, re-expression of *miR-200* family either by transfection with its precursors or treatment by novel agents (natural agents) could have the potential for the inhibition of EMT and stemness markers, suggesting that such a strategy could become a novel therapeutic approach for the treatment of PC.

Although the loss of PTEN has been shown to regulate *miR-21* expression, the extent to which it is affected through modulation of *miR-200* and its role in the deregulation of MT1-MMP and PTEN has not been previously examined. Therefore, the aim of the current study was to investigate the interplay between the expression of MT1-MMP and PTEN deregulation mediated through the expression of *miR-200* in PC cell lines. We further mechanistically investigated the putative role of *miR-200c* and its effects on the expression of MT1-MMP and PTEN by transfecting *pre-miR-200c* (precursor) or *ASO-miR-200c* (inhibitor) in human PC MIAPaCa-2 cell line. We found that the re-expression of *pre-miR-200c* led to decreased cell migration and clonogenicity, which was associated

Abbreviations: EMT, epithelial to mesenchymal transition; miRNAs, microRNAs; MT1-MMP, membrane type-1 matrix metalloproteinase; PC, pancreatic cancer; PTEN, phosphatase and tensin homolog.

with down-regulation of MT1-MMP and re-expression of PTEN. Moreover, instead of transfection, our novel agents, BR-DIM and CDF, were able to cause re-expression of *miR-200c* and down-regulated the expression of MT1-MMP, which was consistent with the up-regulation of PTEN expression.

Materials and methods

Cells culture, drugs and reagents

Human PC cell lines AsPC-1, BxPC-3, COLO-357, MIAPaCa-2, MIAPaCa-GR (gemcitabine resistant) and PANC-1 were chosen for this study. The cell lines have been tested and authenticated using the core facility–Applied Genomics Technology Center at Wayne State University, on 13 March 2009. The method used for testing was short tandem repeat profiling using the PowerPlex® 16 System from Promega (Madison, WI, USA). These cells were stored in multiple vials in liquid nitrogen for our use. CDF was synthesized as described in our earlier publications (22,23), and BR-DIM, a formulated DIM with higher bioavailabilty (24), was obtained from Dr. Michael Zeligs (BioResponse, LLC, Boulder, CO, USA). Both of these novel agents have been extensively used in our laboratory for the treatment of most cancer cells *in vitro* and *in vivo* including PC, as shown by many of our published papers (12,24–31).

Protein extraction and western blot analysis

We initially tested a range of concentrations for BR-DIM (10–50 µM) and CDF (0.5–2 µM), and found that 25 µM of BR-DIM and 0.5–1 µM of CDF was optimal for further studies. Based on these initial results, MIAPaCa-2, MIAPaCa-2-GR and BxPC-3 cells were treated with BR-DIM (25 µM) or CDF (0.5–1 µM) for all subsequent assays for 24, 48 and 72 h. Light micrographic pictures were taken at every time point with both BR-DIM and CDF treatment. Total protein was extracted from untreated and both BR-DIM- and CDF-treated cells at 24, 48 and 72 h and were loaded with 50 µg of protein and subjected to western blot analysis as described previously (32) to evaluate the expression of PTEN and MT1-MMP. The data were adjusted against loading control using β-actin expression.

Real-time reverse transcriptase-polymerase chain reaction (RT-PCR)

To determine the basal level of *miR-200* family (*miR-200*a, *miR-200b, miR-200c*) in all six PC cell lines, and also cells treated with BR-DIM (25 µM) or CDF (0.5–1 µM) for 24 h, we used TaqMan MicroRNA (miRNA) Assay kit (Applied Biosystems) and the available primer and probe for miR-200 family from Applied Biosystems, following manufacturer's protocol. Total RNA was extracted and 10 ng from each sample were reverse transcribed as described earlier (33). The expression of *miR-200a*, *200b* and *200c* of untreated and treated with BR-DIM and CDF were then carried out in a total volume of 10 µl reaction mixture by qRT-PCR as described earlier (33). All reactions, including controls and the experiments, were performed in triplicate, using StepOnePlus Real-Time PCR (Applied Biosystems). Relative expression of miRNAs was analyzed using C_t method and was normalized by *RNU48* expression.

Pre-miR-200c and antisense miR-200c oligonucleotide transfection

MIAPaCa-2 cells were plated in 6-well plates and incubated overnight. Cells were transfected with either control miRNA or *pre-miR-200c* or *ASO-miR-200c* (Ambion, Austin, TX, USA) at a final concentration of 20 nM, using DharmaFECT transfection reagent (Dharmacon), followed by BR-DIM or CDF treatment for 48–72 h. After 24 h of transfection, the medium was changed to avoid cell death during transfection. Transfected cells were then tested for wound healing and clonogenic assay, and also harvested for the extraction of total RNA and protein using standard methods.

Wound healing assay of transfected cells

Wound healing assay was performed to examine the capacity of cell migration and invasion, as described previously (13). Briefly, after the cells grew in about 80–90% confluence in 6-well plates, the wound was generated by scratching the surface of the plates with a 200 µL pipette tip. The cells were then transfected with either control miRNA, *pre-miR-200c* or *ASO-miR-200c*, followed by either BR-DIM or CDF treatment for 18 h. Photographic images were taken at 0 h and 18 h using a microscope (Nikon ECLIPSE TS100).

Clonogenic assay of transfected cells

Transfected cells as described above were trypsinized and 1000 viable cells were plated in 100-mm petri dishes. The cells were then incubated for about 10–12 days at 37°C in a 5% CO_2/5% O_2/90% N_2 incubator. Colonies were stained with 2% crystal violet and scanned for images.

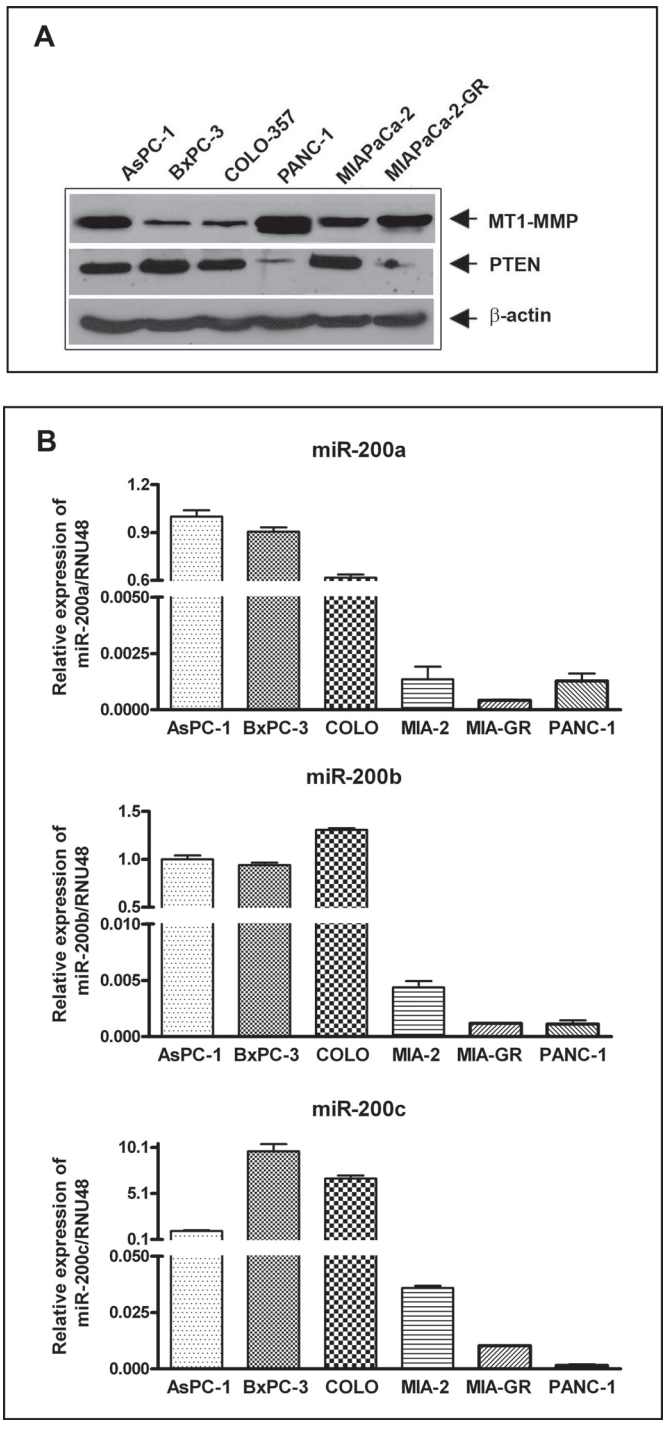

Fig. 1. Basal levels of MT1-MMP and PTEN expression in human PC cell lines AsPC-1, BxPC-3, COLO-357, PANC-1, MIAPaCa-2 and MIAPaCa-2-GR cells. MT1-MMP expression was significantly higher in AsPC-1, PANC-1 and MIAPaCa-2-GR cells while PTEN expression was significantly lower in PANC-1 and MIAPaCa-2-GR cells (**A**). Relative expressions of *miR-200a, miR-200b and miR-200c* (**B**), as assessed by qRT-PCR in the above six PC cell lines. The expression of *miR-200a*, *miR-200b* and *miR-200c* was significantly lower in MIAPaCa-2, MIAPaCa-2-GR and PANC-1 cells. Each experiment was repeated, at least, three times independently, and the bar represents standard deviation from all three experiments.

Statistical methods

Comparisons of treatment outcome were tested for statistical difference by the paired t-test. Statistical significance was assumed at a P value of <0.05.

Results

MT1-MMP and PTEN expression in PC cells was associated with deregulated expression of miRNAs

MT1-MMP is often expressed in tumor cells with significant invasive properties, and is associated with poor prognosis of patients. In contrast, PTEN, a well-known tumor suppressor gene, has been reported to be lost in tumors (12). We examined the basal level of MT1-MMP and PTEN expression in six human PC cell lines. MT1-MMP expression was highly elevated in AsPC-1, PANC-1 and MIAPaCa-2-GR cells, and moderately elevated in MIAPaCa-2 cells compared with BxPC-3 and COLO-357 cells (Figure 1A). In contrast, the expression of PTEN was significantly lower in PANC-1 and MIAPaCa-2-GR cells. Similarly, *miRNA-200* family were differentially expressed in all six cell lines of which MIAPaCa-2, MIAPaCa-2-GR and PANC-1 cells showed significantly lower expression of *miR-200a*, *miR-200b* and *miR-200c*, compared with AsPC-1, BxPC-3 and COLO-357 cells (Figure 1B). The loss of expression of *miR-200* family was correlated with the level of PTEN expression. For our subsequent studies, three cell lines (BxPC-3, MIAPaCa-2 and MIAPaCa-2-GR) were chosen as documented below.

Light micrographic pictures

Figure 2A and 2B demonstrate the morphological differences in MIAPaCa-2 and MIAPaCa-2-GR cells. The MIAPaCa-2 cells were exposed to gemcitabine every other week for a period of 6 months which led to this mesenchymal phenotype as reported earlier (12,34). MIAPaCa-2 and MIAPaCa2-GR cells treated with either BR-DIM or CDF for 24, 48 and 72 h (Figure 2A and 2B) were photographed and were subsequently used for all our experiments. BxPC-3 cells were also treated similarly (images not shown) and were used for all our subsequent experiments as shown below.

Re-expression of miR-200 family was achieved by BR-DIM treatment

We determined the expression levels of *miR-200* family (*miR-200a*, *miR-200b* and *miR-200c*) after treatment with 25 µM of BR-DIM for 24 h in MIAPaCa-2, MIAPaCa-2-GR and BxPC-3 cells. The expression level was determined by real-time RT-PCR. We found a significant up-regulation in the expression of *miR-200a*, *miR-200b* and *miR-200c* in all three cell lines treated with BR-DIM (Figure 3A).

Re-expression of miR-200 family was achieved by CDF treatment

We also determined the expression levels of *miR-200a*, *miR-200b* and *miR-200c* after treatment of PC cells with 0.5–1 µM of CDF for 24 h and assessed by real-time RT-PCR. We found a significant up-regulation in the expression of *miR-200a*, *miR-200b* and *miR-200c* in all three cell lines treated with CDF (Figure 3B). To further validate whether the protein expression of MT1-MMP and PTEN could be altered by either BR-DIM or CDF treatment, we investigated the effect of treatment in all three cell lines by western blot analysis as presented below.

Modulation of MT1-MMP and PTEN expression by BR-DIM and CDF

BxPC-3, MiaPaCa-2 and MiaPaCa-2-GR cells were used to evaluate the effects of BR-DIM and CDF treatment on the expression of MT1-MMP

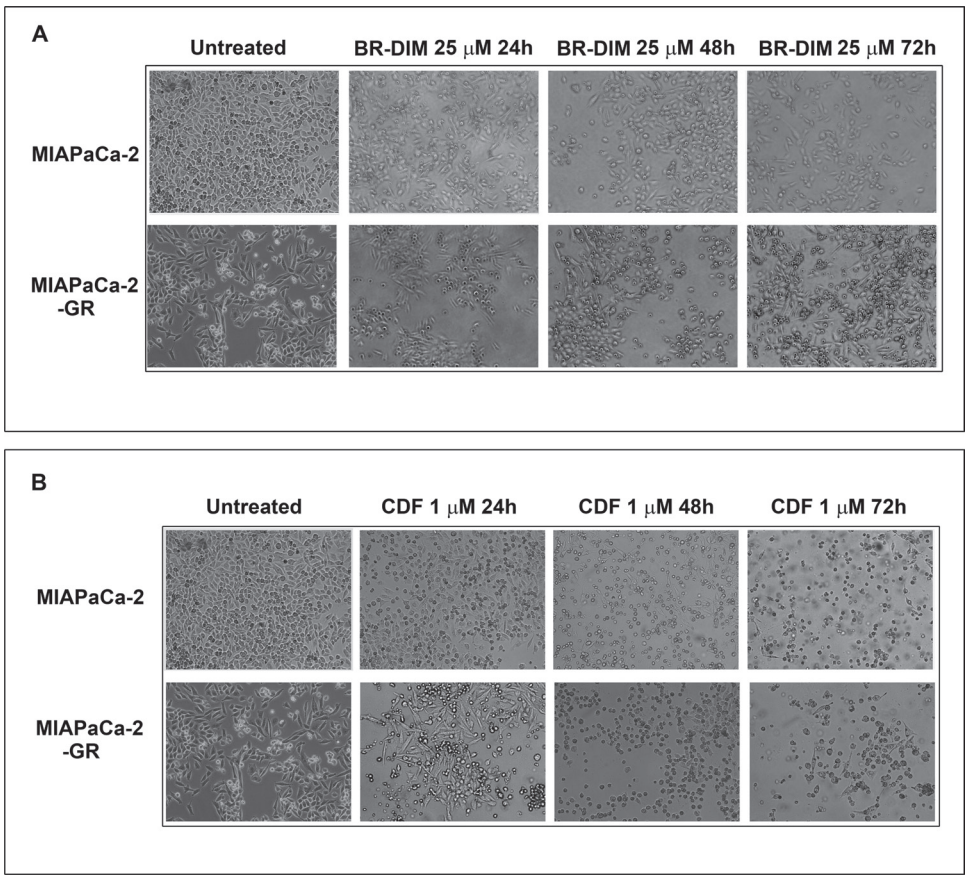

Fig. 2. The time-dependent effect of treatment with both BR-DIM (**A**) and CDF (**B**). Light photomicrographs of MIAPaCa-2 and MIAPaCa-2-GR cell lines untreated and treated with BR-DIM (25 µM) for 24, 48 and 72 h and untreated and treated with CDF 1 µM for 24, 48 and 72 h. MIAPaCa-2 cells were exposed to gemcitabine and the paired cell line was called MIAPaCa-2 and MIAPaCa-2-GR based on their changes in morphology from epithelial-like to mesenchymal-like phenotype. Each experiment was repeated, at least, three times independently.

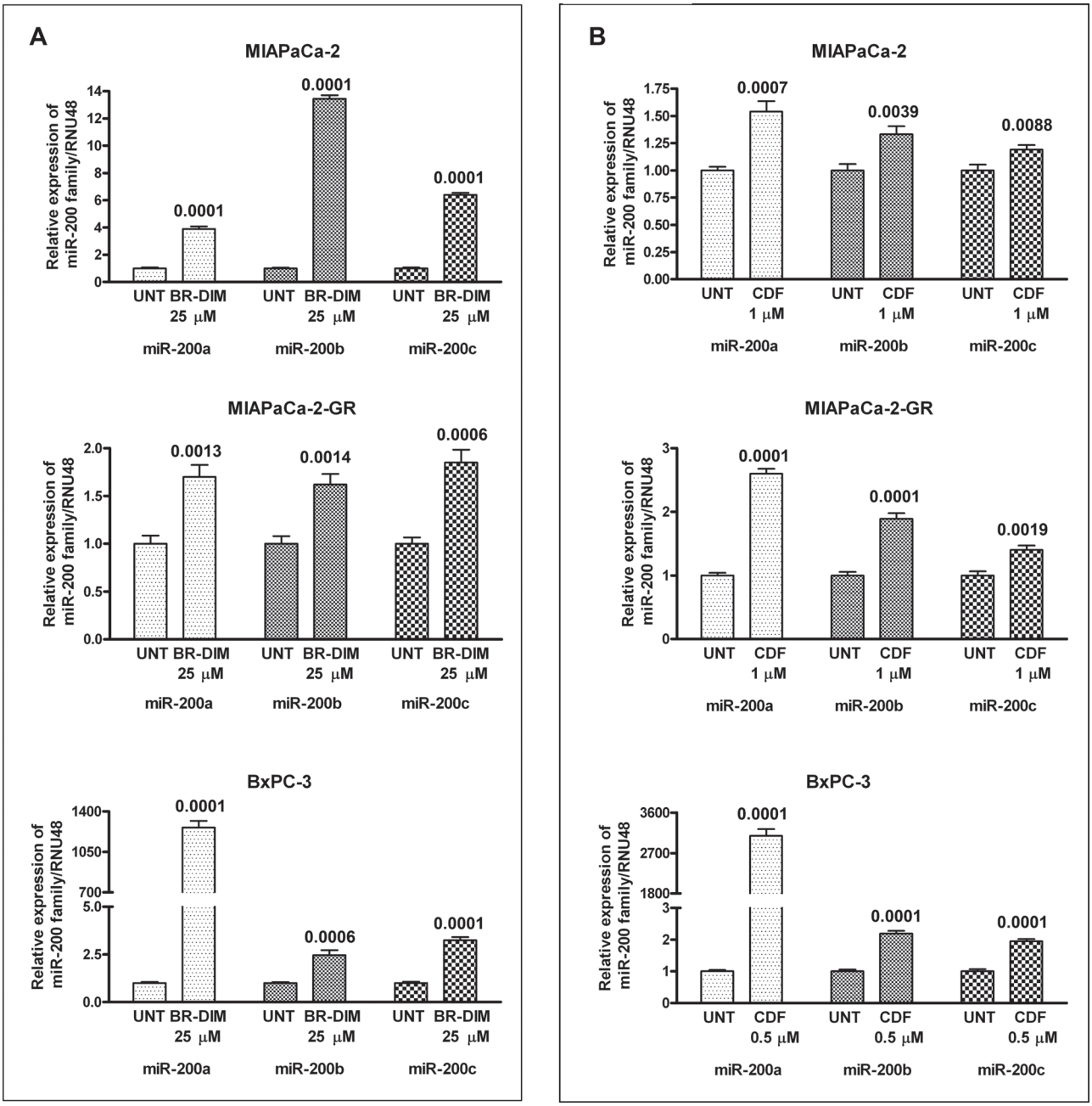

Fig. 3. Effect of treatment on the expression level of *miR-200* family (*miR-200a*, *miR-200b* and *miR-200c*) as determined by real-time RT-PCR on MIAPaCa-2, MIAPaCa-2-GR and BxPC-3 cells with 25 μM of BR-DIM (**A**), and 1 μM of CDF (**B**) for 24 h. We found significant up-regulation in the expression of *miR-200a*, *miR-200b* and *miR-200c* in all three cell lines treated with both BR-DIM and CDF. *P* values represent comparison between untreated and BR-DIM or untreated and CDF-treated cells as calculated by the paired *t* test. Each experiment was repeated, at least, three times independently, and the bar represents standard deviation from all three experiments.

and PTEN. Cells were treated with 25 μM BR-DIM or 0.5–1 μM CDF in a time-dependent manner for 24, 48 and 72 h. Expression of MT1-MMP proteins was significantly reduced in all three cell lines treated with either BR-DIM or CDF when compared with untreated control (Figure 4). In contrast, the expression of PTEN, a tumor suppressor gene, was found to be decreased in MIAPaCa-2-GR cells compared with BxPC-3 or MIAPaCa-2 cells and was significantly enhanced with both BR-DIM and CDF treatment in all three cell lines. These results suggest that BR-DIM and CDF could be effective for re-expression of PTEN. To further validate whether *miR-200* indeed could target the MT1-MMP, or PTEN expression, we chose to investigate the effect of transfection of *miR-200c* with both precursor and antisense oligonucleotide in MIAPaCa-2 cells, and also treated the cells with BR-DIM and CDF as presented below.

MT1-MMP and PTEN expression are regulated by miR-200c and affecting wound-healing capacity and colony formation of MIAPaCa-2 cells

In order to test whether *miR-200c* expression could regulate MT1-MMP and PTEN expression, we over-expressed *miR-200c* with *pre-miR-200c* in MIAPaCa-2 cells, which express relatively low basal levels of *miR-200c* compared with BxPC-3 cells. MIAPaCa-2 cells were seeded in

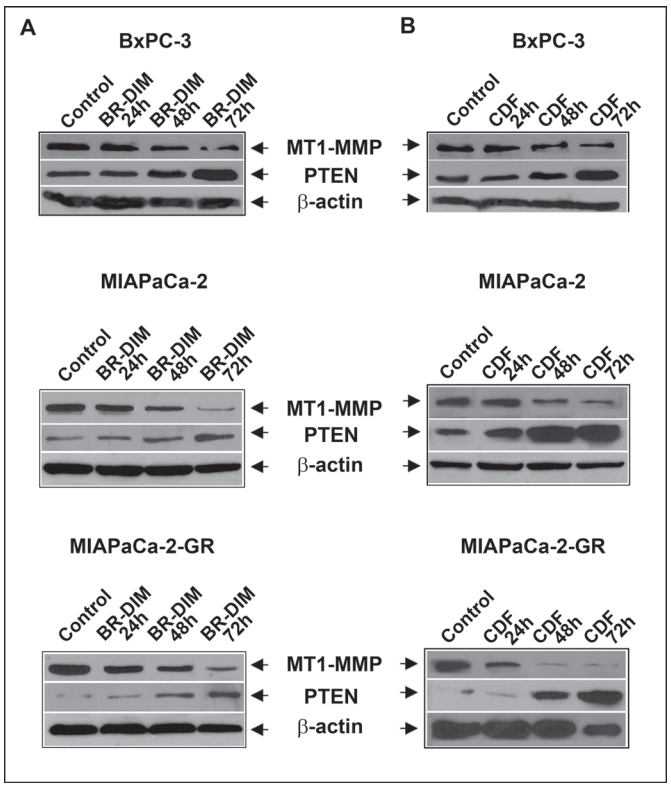

Fig. 4. Effect of treatment on the expression level of MT1-MMP and PTEN as determined by western blot analysis of BxPC-3, MIAPaCa-2 and MIAPaCa-2-GR cells with 25 μM of BR-DIM for 72 h (**A**) and 1 μM of CDF for 72 h (**B**). We found significant down-regulation of MT1-MMP and up-regulation of PTEN in all three cell lines treated with either BR-DIM or CDF compared with untreated cells in a time-dependent manner.

6-well plates and were transfected with *pre-miR-200c* precursor for 48 h followed by treatment with BR-DIM/CDF. Ectopic expression of *miR-200c* with *miR-200c* precursor led to changes in morphology of cells (Figure 5A), increased expression of *miR-200c* compared with control by qRT-PCR (Figure 5B) and significantly inhibited wound-healing capacity (Figure 5C). Interestingly, the re-expression of *miR-200c* led to decreased expression of MT1-MMP with simultaneous increase in PTEN expression (Figure 5D), and led to a significant decrease in colony formation (Figure 5E). These effects were further enhanced after BR-DIM/CDF treatment compared with control, suggesting the mechanistic role of *miR-200c* in the regulation of MT1-MMP and PTEN and that these effects could be easily achieved by treating the cells with CDF, which showed better effects than BR-DIM. These results further prompted us to conduct a reverse experiment by further inactivation in the expression of *miR-200c* using *ASO-miR-200c* transfection and assessed the expression of MT1-MMP and PTEN and correlated our findings with wound-healing capacity and clonogenic growth as presented below.

Transfection of antisense miR-200c in MIAPACa-2 cells increased cell migration and colony formation, and caused altered protein expression of MT1-MMP and PTEN

We investigated the consequence of inactivation of *miR-200c* expression by transfecting the cells with ASO-*miR-200c* on the expression of MT1-MMP and PTEN and also assessed cellular consequence on cell migration and colony formation. Interestingly, we found that the morphology of the cell changed to mesenchymal phenotype from epithelial phenotype after ASO-*miR-200c* transfection as shown in Figure 6A, which was correlated with decreased expression of *miR-200c* compared with control as assessed by qRT-PCR (Figure 6B). The inactivation of *miR-200c* using *ASO-miR-200c* transfection also led to an increased wound-healing capacity and clonogenicity in the transfected cells compared with control cells (Figure 6C and 6E), and these cellular characteristics were correlated with decreased expression of PTEN and increased expression of MT1-MMP (Figure 6D). Moreover, these changes were attenuated by treating the cells with CDF, which was superior than BR-DIM, as shown in Figure 6.

Discussion

MT1-MMP have been shown to be over-expressed in many human malignant tumors, including PC, and it is a crucial enzyme necessary during normal development but its activation as well as over-expression is also important in malignant processes (35,36). MT1-MMP is the only MMP shown to play a significant role in renal development both *in vivo* and *in vitro* (37). Messaritou *et al.* demonstrated that the collagen internalization receptor Endo180 is a novel regulator of MT1-MMP and uPA activity (38). In the present study, we have demonstrated that the over-expression of MT1-MMP was associated with reduced expression of PTEN in aggressive PC cell lines, which is consistent with previous studies in prostate, renal and lung cancer (14–16); however, to the best of our knowledge this is the first study to demonstrate the interplay between MT1-MMP and PTEN expression in PC. Since the majority of PC contains mutant K-Ras, the association between K-Ras mutation and PTEN expression has been reported especially in genetically engineered mouse model showing interactions of K-Ras^{G12D} and the loss of PTEN, resulting in increased metastasis through activation of NF-κB and its downstream cytokine pathway (39).

In the current study, we investigated the role of miRNAs especially because there is considerable interest in the mechanistic understanding of the role of miRNAs involved in tumor growth and metastasis, and their deregulation either by over-expression or knockdown by precursors and inhibitors are an emerging area of research. Computational algorithms have been the major methods in predicting miRNA targets based on the base pairing of miRNA and target gene 3'-UTR (40). Based on our experimental evidence and TargetScanHuman 5.2, we found *miR-200b* and *miR-200c* to possess a match for base pairing with the 3'-UTR of PTEN. Based on our initial finding, we chose *miR-200c* for further mechanistic studies in MIAPaCa-2 cells. We found that the loss of PTEN was directly correlated with low expression of *miR-200*, and that the forced over-expression of *miR-200c* with precursor or treatment of cells with our novel agents especially CDF resulted in increased PTEN expression, suggesting that *miR-200c* could regulate the expression of PTEN by translational regulation, and thus we believe that deregulation of miRNAs could become a newer strategy for the treatment of PC.

Previous studies have shown that PC cell lines exhibit significantly lower levels of expression of *miR-200* family, which was associated with increased EMT, suggesting that *miR-200* play an important role in several key aspects of tumor initiation and progression (12,41). Others have reported down-regulation of *miR-200* family expression through Smad signaling-dependent manner during the progression of renal fibrosis (42). Moreover, the loss of p53 was correlated with decrease in *miR-200c* expression and an increase in EMT and stemness markers in a cohort of breast tumors (43). These evidences clearly suggest the role of *miR-200* expression in tumor aggressiveness. In our current study, we found that *miR-200* expression was drastically down-regulated in aggressive PC cell lines, and that the re-expression of *miR-200c* using *pre-miR-200c* transfection of MIAPaCa-2 cells led to decreased expression of MT1-MMP with concomitantly increased expression of PTEN. Interestingly, these effects were further pronounced by treatment of cells with both BR-DIM and/or CDF treatment, suggesting that these agents especially CDF could be useful for deregulation of important molecular events that are associated with tumor aggressiveness. In contrast, following further knockdown of *miR-200c* by *ASO-miR-200c*, we found a marked increase in MT1-MMP expression, which resulted in the down-regulation

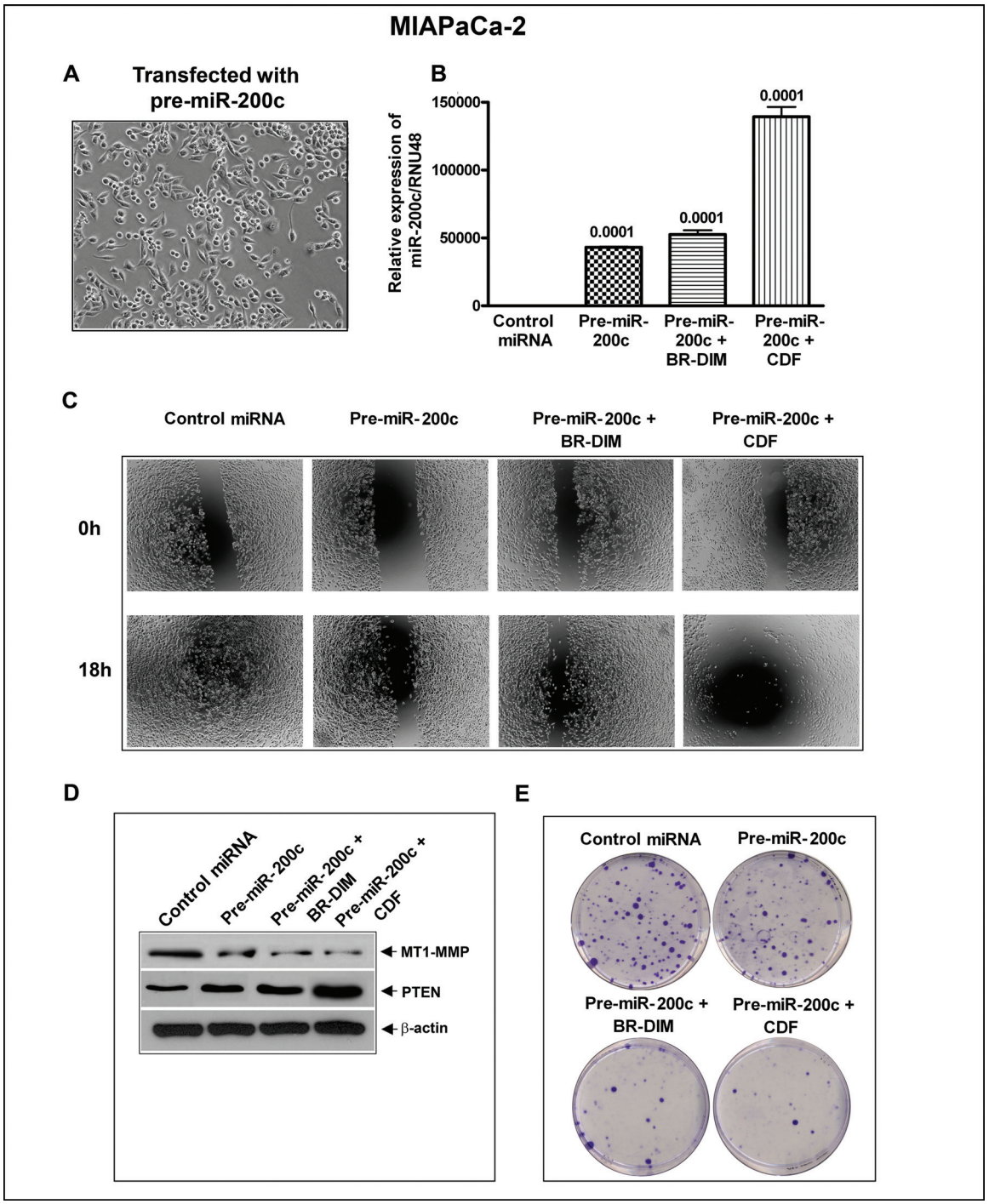

Fig. 5. Re-expression of *miR-200c* by *pre-miR-200c* precursor transfection in MIAPaCa-2 cells led to change in morphology of cells (**A**). Transfection of *miR-200c* or treatment of MIAPaCa-2 cells by BR-DIM or CDF increased expression of *miR-200c* as assessed by qRT-PCR (**B**), decreased in cell migration (**C**), decreased levels of MT1-MMP, and up-regulation of PTEN by western blot analysis (**D**) decreased clonogenicity as determined by colony formation assay (**E**) as compared with control cells. Each experiment was repeated, at least, three times independently.

of tumor suppressor gene PTEN in PC cell lines. Moreover, the inactivation in the expression of *miR-200c* resulted in the acquisition of EMT phenotype and tumor cell aggressiveness, which is consistent with our previous findings (12). Based on our observations, we conclude the importance of the *miR-200c* miRNA because it may serve as the key regulators of MT1-MMP and PTEN expression, and that the restoration of PTEN expression and down-regulation of MT1-MMP could be easily achieved with both CDF and BR-DIM treatment, which was mediated through re-expression of *miR-200c* although CDF was found to be superior than BR-DIM.

We have previously demonstrated an increase in MT1-MMP expression in K-ras Cre-mediated activation of a mutant K-ras allele ($Kras^{G12D}$) and deletion of a conditional Ink4a/Arf tumor suppressor allele in transgenic mouse model (10), which led to tumor initiation and progression. Another recent study reported that dimerization of MT1-MMP is required to promote cell

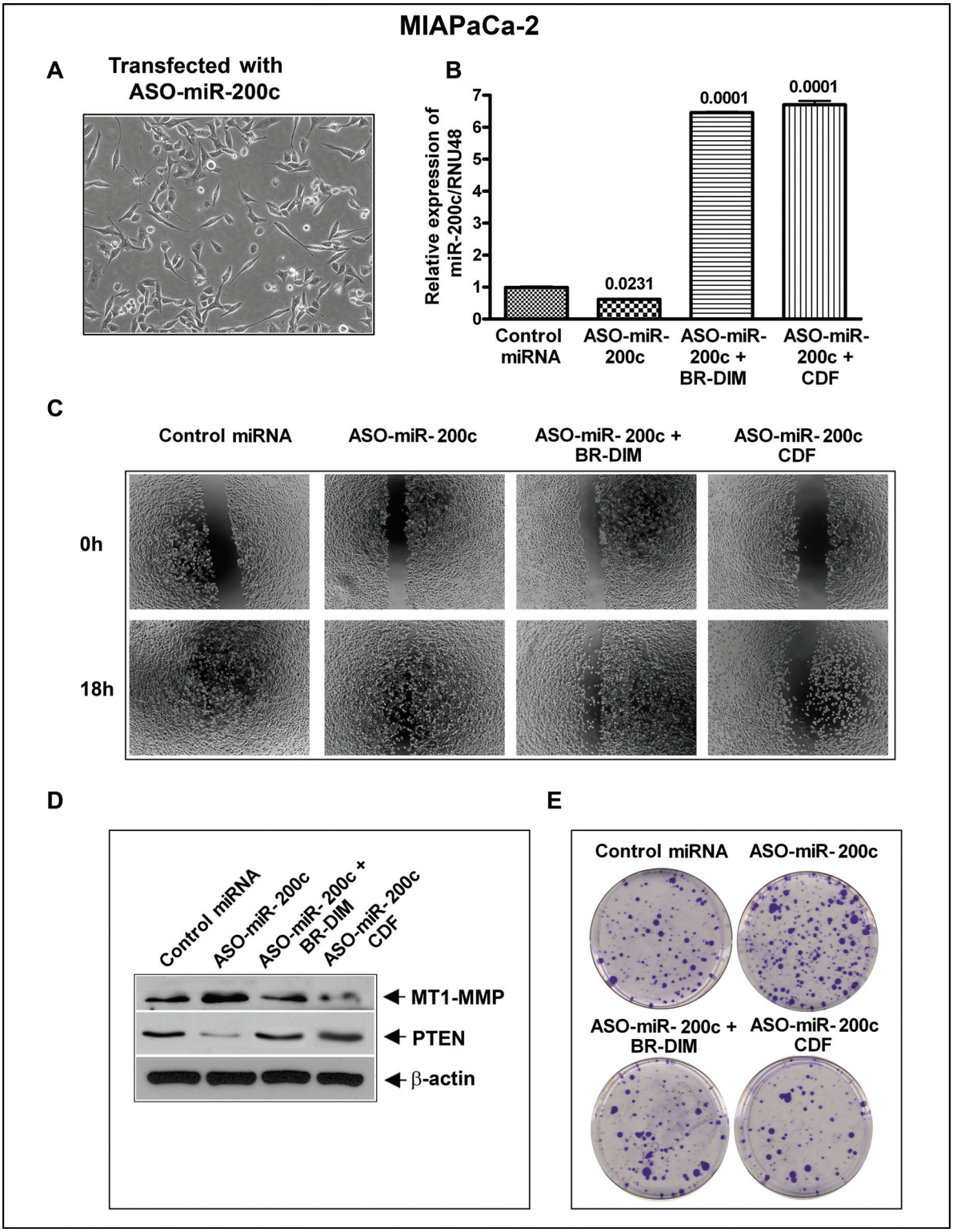

Fig. 6. Inhibition of *miR-200c* by *ASO-miR-200c* transfection led to change in morphology of cells (**A**), decreased expression of *miR-200c* as assessed by qRT-PCR (**B**), increased in cell migration (**C**), increased levels of MT1-MMP, and inhibition of PTEN by western blot analysis (**D**) increased clonogenicity as determined by colony formation assay (**E**) as compared with control cells. All the above changes were rescued by BR-DIM or CDF treatment. Each experiment was repeated, at least, three times independently.

invasion in a collagen-enriched environment (44). RNA silencing of endogenous MT1-MMP expression in fibrosarcoma and gastric carcinoma cell lines down-regulated only MT1-MMP expression, but not other MMPs which caused significant inhibition in the migration and invasion of tumor cells (45). Our observation in this study showed that over-expression of MT1-MMP increased cell migration in aggressive PC cell lines, which was significantly reduced by the treatment of cells with our novel agents BR-DIM

and CDF. In conclusion, our current findings clearly suggest that increased expression of MT1-MMP and decreased expression of PTEN is in part due to loss of expression of *miR-200c* in PC. Moreover, we have provided experimental evidence, supporting that targeted re-expression of *miR-200c* by BR-DIM and CDF led to decreased expression of MT1-MMP and causing re-expression of PTEN, resulting in reducing tumor cell aggressiveness. Hence these agents, especially CDF, could serve as a novel approach for the treatment of PC.

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Conflict of Interest Statement: None declared.

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