MDM2 and PSMA Play Inhibitory Roles in Metastatic Breast Cancer Cells Through Regulation of Matrix Metalloproteinases

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Abstract. *Background/Aim: Mouse double minute 2 (MDM2) and prostate-specific membrane antigen (PSMA) are currently under investigation as individual therapeutic targets due to their overexpression in many cancer types, as well as their pro-tumorigenic effect on cells. Recently, knockdown of PSMA was linked to a decrease in MDM2 and matrix metalloproteinase 2 (MMP2) and an increase in MMP3 and MMP13 expression. We aimed to assess the link between PSMA, MDM2 and the MMPs in metastatic breast cancer cell lines. Materials and Methods: Real-time quantitative polymerase chain reaction (PCR) and western blotting were used to assess siRNA-mediated knockdown of MDM2 and PSMA in MDA-MB-231 and ZR-75.1 breast cancer cells. Assays to assess the growth, adhesion, migration and invasion of the cells following siRNA treatment were undertaken. MMP and tissue inhibitor of matrix metalloproteinases (TIMP) levels were assessed via quantitative PCR. Results: Knockdown of MDM2 resulted in a decrease in PSMA expression levels and vice versa; although this trend was not replicated at the protein level. Knockdown of each of the molecules resulted in a decrease in growth, adhesion, migration and invasive ability of breast cancer cells. Both knockdowns led to a decrease in MMP2 and an increase in MMP3, -10 and -13 gene expression. Conclusion: MDM2 and PSMA may co-regulate the expression of certain MMPs and, thus, the functionality of cells in metastatic breast cancer.*

The breast is the most common cancer site in women in all European countries, with extremely high incidence and mortality rates in Northern Europe (1). However, these mortality rates are falling due to mammographic screening, leading to earlier diagnosis, as well as improved treatment for those affected by the disease (2). Despite these encouraging improvements, metastasised breast cancer is incurable in many patients and is not well understood in terms of molecular drivers.

Mouse double minute (MDM2) overexpression occurs in numerous human tumours including breast, ovarian, prostate, cervical and brain (3). MDM2 is best known for its negative regulation of p53 tumour suppressor (4), however, more recently, it has been reported to undertake p53-independent roles in the cell, which also contribute to tumourigenesis (5).

Expression of prostate-specific membrane antigen (PSMA) increases directly in cancer of high grade, castration-resistant prostate cancer and metastases (6, 7). Although originally thought to be 'specific' to the prostate, PSMA was recently found to play a role in other types of cancer, including breast (8, 9).

MDM2 and PSMA have each been linked to the growth, adhesive, migratory, invasive and angiogenic capacity of cell lines, with knockdown of both proteins being associated with a decrease in the aggression of cancer cells, and *vice versa* (10-13). Moreover, the expression of each of the proteins has been positively correlated with matrix metalloproteinase 9 (MMP9) in both cell lines and tissues (12-14) and PSMA has been linked to active MMP2 expression in endothelial cells (15). Recently, an article was published which linked MDM2, PSMA and the MMPs. Xu *et al*. showed that knockdown of *PSMA* in LNCaP prostate cancer cells resulted in a decrease in *MDM2* and *MMP2* and an increase in *MMP3* and *MMP13* expression (16).

The MMPs are molecules that are secreted by cells in response to their extracellular environment and can degrade both matrix and non-matrix proteins. In normal steady-state conditions, the action of most MMPs is negligible; however, the dysregulation of these molecules is known to play an important role in cancer progression (17, 18).

Thus, we aimed to investigate the hypothesis that MDM2 and PSMA work together to co-regulate expression of the MMP family and thus the progressive properties of cancer.

Materials and Methods

Cell culture. Human breast cancer cell lines MDA-MB-231 and ZR-75.1 were purchased from American Type Culture Collection (ATCC) (Middlesex, UK) and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS),

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penicillin and streptomycin. Human microvascular endothelial cells adult dermis (HMVECAD) were maintained in 131 Media with microvascular growth supplement (all MVGS; Gibco, Paisley, UK). All cells were maintained at 37˚C with 5% CO_2.

siRNA Transfection. A dose-dependence study was undertaken in order to optimise the concentration of siRNA to be used for individual cell lines (data not shown). MDA-MB-231 cells were seeded at 3×10^5 cells/well in a 24-well plate in serum-free medium (no antibiotics) and then allowed to attach overnight at 37˚C. Cells were then transfected with 100 nM ONTARGETplus MDM2-, FOLH1-, or Non-Targeting siRNA (GE Healthcare, Buckinghamshire, UK) using 1 μl/ml of DharmaFECT4 according to the manufacturer's instructions (GE Healthcare).

ZR-75.1 cells were seeded at 5×10^5 cells/well in a 24-well plate in serum-free media (no antibiotics) and then allowed to attach overnight at 37˚C. Cells were then transfected with 50 nM ONTARGET plus MDM2-, FOLH1-, or Non-Targeting siRNA using 2 μl/ml of DharmaFECT1 according to manufacturer's instructions (GE Healthcare).

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR). Following 72 h of siRNA treatment, RNA was extracted from cells using TRI reagent (Sigma-Aldrich, Poole, Dorset, UK) according to the manufacturer's instructions. RNA (500 ng) was used for reverse transcription with High Capacity cDNA Reverse Transcription Kits (Life Technologies, Paisley, UK). Conditions used for reverse transcription were 25˚C for 10 minutes, 37˚C for 120 min, and 85˚C for 5 minutes using Applied Biosystems 2720 Thermal Cycler (Life Technologies).

Quantitative PCR. cDNA was diluted 1:8 with ddH20. RT-qPCR was performed using TaqMan® Universal PCR Master Mix (Applied Biosystems) and Universal Primer, with forward and fluorescent tagged reverse primers designed for target gene amplification (MMP1, -2, -3, -7, -8, -9, -10, -11, -12, -12, TIMP1, -2) (Sigma Aldrich). Quantitative PCR amplification was performed using the StepOnePlus Real-Time PCR System (Applied Biosystems) with pre-denaturation for 10 min at 95˚C, followed by 85 cycles of denaturation at 95˚C for 10 sec, annealing at 55˚C for 30 sec and elongation at 72˚C for 10 seconds. Results were analysed using the $2^{-(\Delta\Delta CT)}$ method of normalisation to expression of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*).

Western blotting. Following 72 h of treatment with siRNA, cells were lysed using RIPA buffer then 30 μg of protein was mixed with an equal volume of 2x Laemlli buffer (Sigma Aldrich), heated to 95˚C for 3 min, then separated using SDS-polyacrylamide gel electrophoresis. The gel was run for 1.75 h at 100 V then transferred onto polyvinylidene fluoride (PVDF) membrane using wet transfer for 1 h at 100 V. The membrane was blocked in 5% Marvel/TBS-Tween for 1 h, then probed overnight with primary antibody (MDM2 SMP14 and GAPDH 6C5; Santa Cruz, Dallas, TX, USA; phospho-MDM2 ser166 and PSMA; Cell Signalling, Danvers, MA, USA). Membranes were washed three times in Tris-Buffered Saline (TBS)/Tween (0.01%) and then probed with appropriate secondary antibody. Protein bands were stained using EZ-ECL (Biological Industries, Cromwell, CT, USA) and visualised using G:BOX (Syngene, Cambridge, UK).

AlamarBlue® proliferation assay. A total of 5×10^3 cells were seeded in 100 μl of serum-free medium (no antibiotics) per well in a black 96-well plate, six replicates undertaken per condition, with four plates produced in total. Cells were allowed to attach overnight at 37˚C. Twelve hours later, cells were treated with siRNA and DhamaFECT solutions at the concentrations specified above. Six hours following transfection, all media were changed for 100 μl of normal DMEM (with antibiotics). At 24-h intervals from the time of transfection, 10 μl of AlamarBlue® Cell Viability Solution (ThermoFisher, Waltham, MA, USA) was added to each well. The plate was incubated for 4 h and then read on a GLOMAX® MULTI Detection System (Promega, Southampton, Hampshire, UK) at 570 nm in relative fluorescence units (RFU).

Tumour-endothelium adhesion assay. A total of 300,000 HMVECs/well were added to a 48-well plate and allowed to form a monolayer overnight. Following 72 h of siRNA treatment, cancer cells were diluted to 1×10^6 cells/ml and 5 μl of Vybrant DiO cell-labelling solution (Invitrogen) were added per millilitre of cell suspension. Cells were incubated for 20 min and then washed three times using phosphate buffer solution (PBS). Cells were resuspended at 2×10^5 cells/ml and 200 μl added to the HMVEC monolayer. Cells were allowed to attach for 30 min after which the monolayer was washed twice with PBS. 350 μl cell dissociation solution was then added to each well and the plates were further incubated for 1 h. The cell suspension was aliquoted into a black 96-well plate and read on a GLOMAX®\MULTI Detection System at 495 nm excitation and 519 nm emission.

Migration assay. Following 72 h of siRNA treatment, cells were harvested using HyQTase Cell Detachment Solution (GE Healthcare). Cells were re-suspended in serum-free medium, at a density of 1×10^5 cells/ml. DMEM (1 ml) containing 10% FCS (chemoattractant) was added to the receiver wells in triplicate, and 1 ml of serum-free DMEM (no chemoattractant) was added to the receiver well of control transwell. An 8-μm-pore ThinCert™ 24-well plate insert (Grenier, Kremunster, Austria) was placed in each of the receiver wells and 500 μl of cell suspension was added to each transwell insert. Following 4 h of incubation, transwells were washed with PBS and then incubated for 1 h in 350 μl Cell Dissociation Solution (CDS; Sigma Aldrich)/Calcein AM (eBioscience, Hatfield, UK) at a ratio of 1.2 μl Calcein AM in 1 ml CDS. Following this, the cell suspension was aliquoted into a black 96-well plate and read on a GLOMAX® MULTI Detection System, at 495 nm excitation and 519 nm emission. To analyse the total directed cell movement, the fluorescence of the well containing no chemoattractant was subtracted from that of the test wells.

Invasion assay. Invasion transwells were set up the day before the assay would be undertaken. Defrosted Matrigel (Corning, BD, Oxford, UK) was added to serum-free DMEM to gain a concentration of 500 μg/ml, then 100 μl of this solution was added to 8-μm-pore ThinCert™ 24-well plate inserts and allowed to dehydrate for 2 hours at 55˚C. Forty minutes prior to the experiment, the Matrigel was rehydrated with 100 μl of serum-free DMEM. The rest of the invasion assay was undertaken in the same way as the migration assay; except the running time was 24 hours.

Statistical analysis. Graphs were produced and statistically analysed using GraphPad Prism software version 6.01. *t*-Test, one- and two-way ANOVA were used to test for statistical significance where appropriate; *p*-values of 0.05 or less were considered significant.

Results

Knockdown of MDM2 in MDA-MB-231 and ZR-75.1 cells results in a decrease in PSMA transcript levels, and vice versa. Following treatment of MDA-MB-231 cells with MDM2-targeting siRNA (Figure 1 A and B), PSMA expression levels were significantly decreased (p=0.0051) (Figure 1C) compared to the non-targeting control-treated cells. Moreover, treatment of the cells with *PSMA*-targeting siRNA (Figure 1 D and E) led to a substantial decrease in *MDM2* expression levels (p=0.0001) (Figure 1F). Dual treatment with 50 nM of each siRNA showed a significant decrease in expression of each of the genes compared to the non-targeting control, independently of the siRNA used (Figure 1G and H).

Results were similar for ZR-75.1 cells, with knockdown of *MDM2* through siRNA treatment (Figure A and B) leading to a significant decrease in PSMA expression (p=0.002) (Figure 2C) and *vice versa* (p=0.0085) (Figure 2E and F). Dual treatment with siRNAs also led to significant decreases compared to the non-targeting control, regardless of which siRNAs were used for treatment (Figure 2G and H). However, in both cell lines, this decrease in gene expression of the protein targeted by the siRNA was not replicated at the protein level (Figure 1B and E, Figure 2B and E).

Knockdown of MDM2 and PSMA result in a decrease in growth and adhesion of breast cancer cells. Following siRNA treatment, 24-h time points were used to assess the growth of MDA-MB-231 and ZR-75.1 cells treated with *MDM2-, PSMA-, MDM2-PSMA-* and non-targeting siRNA. Both cell lines exhibited a highly significant decrease in growth of cells following knockdown of *MDM2, PSMA* or both molecules, compared to the non-targeting siRNA-treated cells. No cumulative effect on inhibition of growth was seen in cells treated with both targeting siRNAs (Figure 3 and B).

The adhesive capacity of both MDA-MB-231 cells (*MDM2*-targeted siRNA, p<0.0001; *PSMA*-targeted siRNA, p<0.0001) and ZR-75.1 cells (*MDM2*-targeted siRNA, p=0.002; *PSMA*-targeted, p=0.0201) decreased upon treatment with each of the siRNAs; with the cells treated with dual siRNA showing the biggest decrease in adhesion (MDA-MB-231, p<0.0001; ZR-75.1, p<0.0001) (Figure 3C and D). The siRNA treatment had a more profound effect on MDA-MB-231 cell adhesive ability compared to ZR-75.1 cells.

MDM2-targeted siRNA treatment led to a significant change in visible cell morphology, with MDA-MB-231 cells seeming unable to form their usual lengthened shape and ZR-75.1 cells unable to form the cluster of cells typical for this cell line. PSMA-targeted siRNA appeared to have no visible effect on cell morphology and when treated with both siRNAs, the cells exhibited an intermediate phenotype (Figure 3E and F).

Knockdown of MDM2 and PSMA leads to a decrease in the invasive and migratory capacity of breast cancer cells. Transwell migration assays were undertaken in order to assess the chemotactic migration of cells across an 8-μm-pore transwell. MDA-MB-231 cells exhibited a decrease in migration of more than half following *MDM2*-targeted siRNA treatment and a 70% decrease in migration when treated with *PSMA*-targeted siRNA (Figure 4A). Similarly, invasion was decreased following treatment with both siRNAs (MDM2-targeting 57%; PSMA-targeting 54%) (Figure 4b). The same trend was seen when ZR-75.1 cells were treated with the siRNAs, with a decrease in migration of almost 70% after MDM2-targeting siRNA treatment and a 90% decrease with PSMA-targeting siRNA (Figure 4c); along with a 70% decrease in invasion after MDM2-targeting siRNA treatment and a 75% decrease following PSMA-targeting siRNA treatment (Figure 4d).

Breast cancer cells with MDM2 and PSMA knockdown exhibit a similar pattern of MMP expression (Figure 5). Following 72 h of siRNA treatment, *MMP* and *TIMP* expression levels were assessed in the *MDM2*- and in *PSMA*-targeted siRNA-treated cells compared to those treated with the non-targeting siRNA control. *MMP1* expression was seen to be unchanged following *MDM2*-targeted siRNA treatment in MDA-MB-231 cells but significantly down-regulated following that with *PSMA*-targeted siRNA (p<0.0001). ZR-75.1 cells showed a significant decrease in *MMP1* expression following treatment with each of the siRNAs (p<0.0001). *MMP2* expression was seen to be down-regulated by each of the treatments in both cell lines (*MDM2*-targeting siRNA p=0.0003; all others p<0.0001). *MMP3* expression significantly increased following *MDM2*-targeted siRNA treatment in MDA-MB-231 (p=0.0004) and ZR-75.1 (p<0.0001) cells, and after *PSMA*-targeted siRNA treatment (p=0.0482 and p<0.0001, respectively). *MMP7* expression levels were unchanged in both cell lines following *MDM2*-targeted siRNA treatment, but significantly increased following *PSMA*-targeted siRNA treatment (MDA-MB-231, p<0.0001; ZR-75.1, p=0.0411). There were no significant changes in *MMP8* expression, following *MDM2*- and *PSMA*-targeted siRNA treatment in either cell line. An *MMP9* expression decrease was seen in both cell lines following *MDM2*-targeted siRNA treatment (MDA-MB-231 p<0.0001; ZR-75.1, p=0.0003); however, *PSMA*-targeted treatment did not lead to significant changes in expression levels. *MMP10* was seen to significantly increase in both cell lines after each treatment (MDA-MB-231: *MDM2*-targeting p=0.0115; MDA-MB-231, PSMA-targeting p=0.0014; ZR-75.1: MDM2-targeting p<0.0001, PSMA-targeting p=0.0084). *MMP11* expression only increased in MDA-MB-231 cells following *PSMA*-targeting siRNA treatment (p=0.0039), other treatments did not change expression levels significantly in either cell line. There were no significant changes to *MMP12* expression in

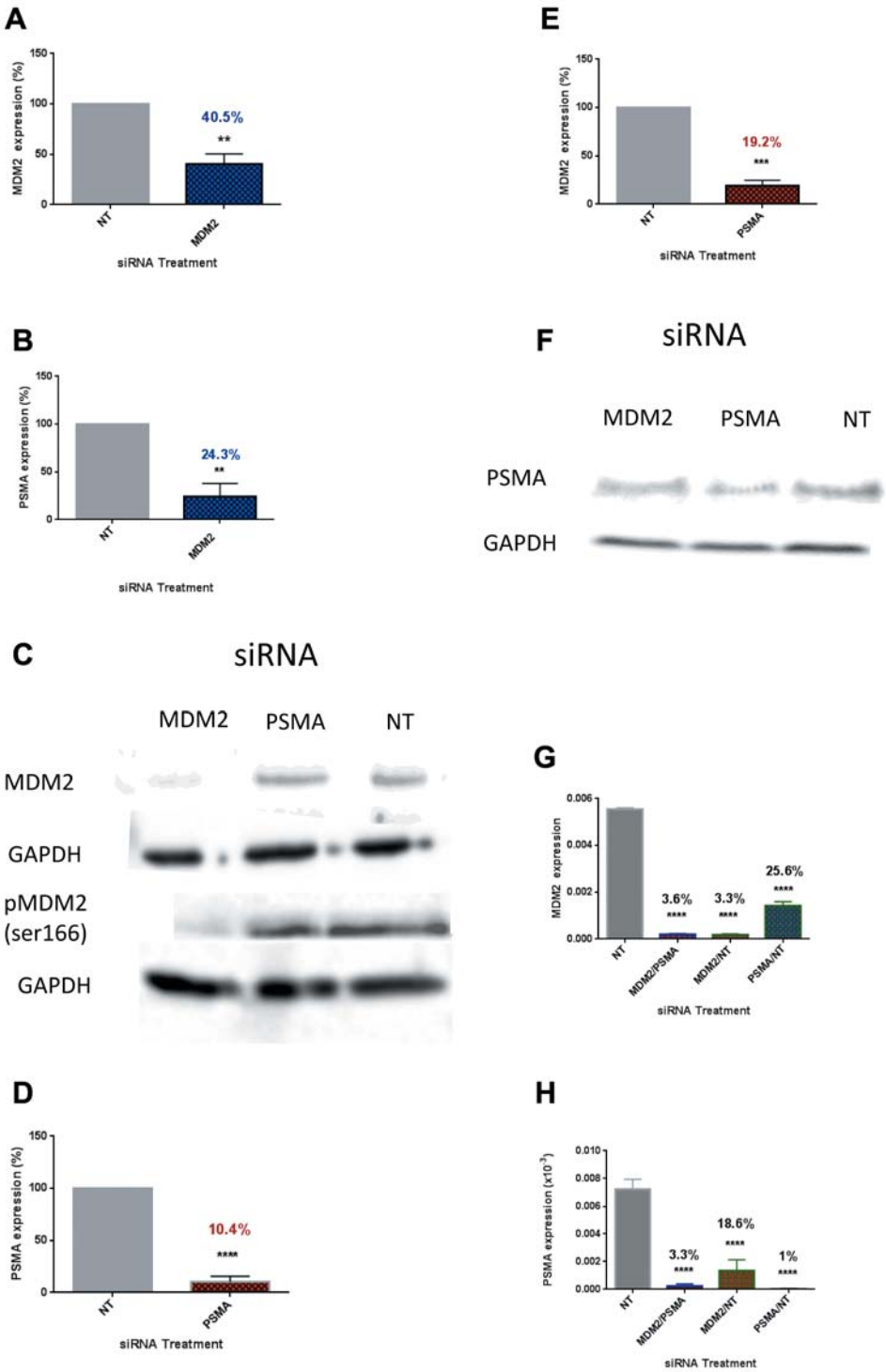

Figure 1. *Knockdown of mouse double minute (MDM2) and prostate-specific membrane antigen (PSMA) in MDA-MB-231 using targeted siRNA. A) MDM2 gene expression following treatment of cells with MDM2-targeted siRNA as a percentage of that of non-targeting (NT) control. B) MDM2 and phospho-MDM2 (ser166) protein levels following treatment of cells with MDM2- and PSMA-targeted siRNA. C) PSMA gene expression following treatment of cells with MDM2-targeted siRNA as a percentage of that of NT. D) PSMA gene expression following treatment of cells with PSMA-targeted siRNA as a percentage of that of NT control. E) PSMA protein levels following treatment of cells with MDM2- and PSMA-targeted siRNA. F) MDM2 gene expression following treatment of cells with PSMA-targeted siRNA as a percentage of NT control. G) MDM2 gene expression following dual treatment of cells with siRNA (mean+SD). H) PSMA gene expression following dual treatment of cells with siRNA (mean+SD). Data are mean+SEM, n=3, individual experiments were carried out in triplicate unless otherwise indicated. **p≤0.01, ***p≤0.001, ****p≤0.0001 compared to control.*

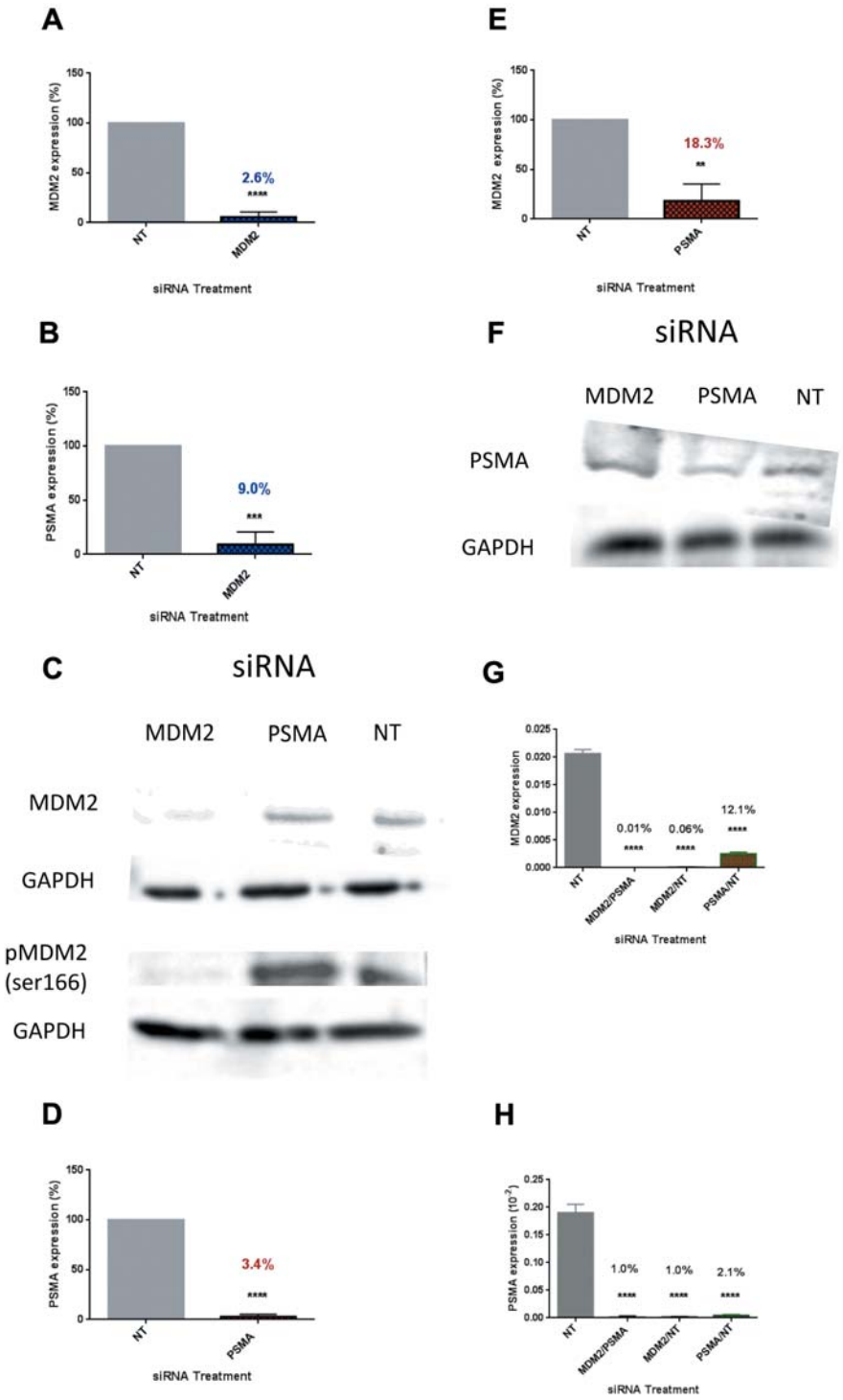

Figure 2. *Knockdown of mouse double minute (MDM2) and prostate-specific membrane antigen (PSMA) in ZR-75.1 using targeted siRNA. A) MDM2 gene expression following treatment of cells with MDM2-targeted siRNA as a percentage of that of non-targeting (NT) control. B) MDM2 and phospho-MDM2 (ser166) protein levels following treatment of cells with MDM2- and PSMA-targeted siRNA. C) PSMA gene expression following treatment of cells with MDM2-targeted siRNA as a percentage of that of NT. D) PSMA gene expression following treatment of cells with PSMA-targeted siRNA as a percentage of that of NT control. E) PSMA protein levels following treatment of cells with MDM2- and PSMA-targeted siRNA. F) MDM2 gene expression following treatment of cells with PSMA-targeted siRNA as a percentage of NT control. G) MDM2 gene expression following dual treatment of cells with siRNA (mean+SD). H) PSMA gene expression following dual treatment of cells with siRNA (mean+SD). Data are mean+SEM, n=3 individual experiments were carried out in triplicate unless otherwise indicated. **p≤0.01, ***p≤0.001, ****p≤0.0001 compared to control.*

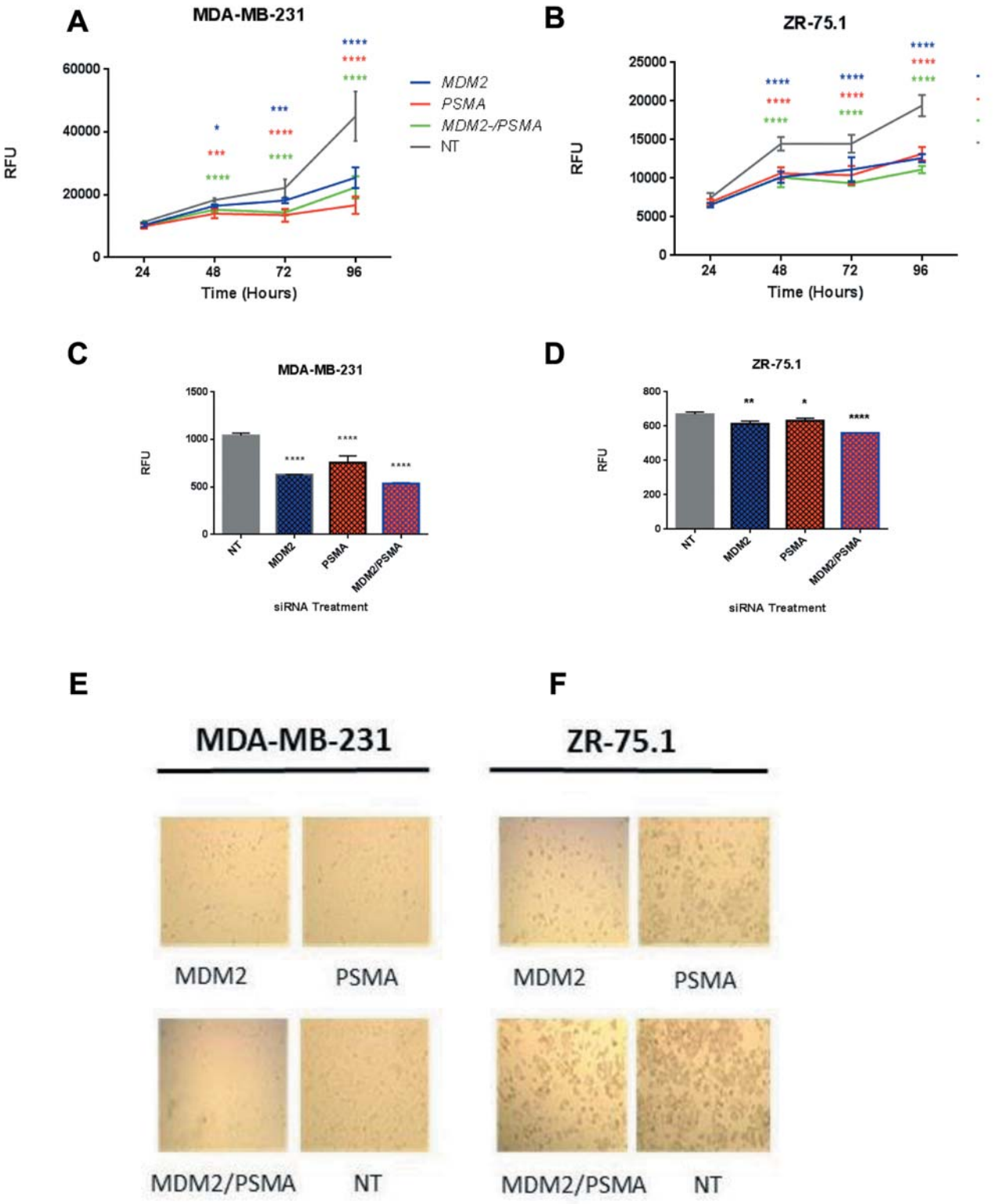

Figure 3. *Effects of mouse double mutant 2 (MDM2) and prostate-specific membrane antigen (PSMA) knockdown on the growth and adhesive properties of breast cancer cells. MDA-MB-231 (A) and ZR-75.1 (B) cell proliferation following treatment with MDM2-, PSMA-, MDM2-/PSMA- and non-targeting siRNA (NT) (mean±SD, representative data, n=3, six replicates were carried out in each experiment). Adhesive ability of MDA-MB-231 (C) and ZR-75.1 (D) following treatment with MDM2-, PSMA-, MDM2-/PSMA- and non-targeting siRNA (n=3, each experiment was carried out in triplicate). MDA-MB-231 (E) and ZR-75.1 (F) cell morphology following treatment with siRNAs.*

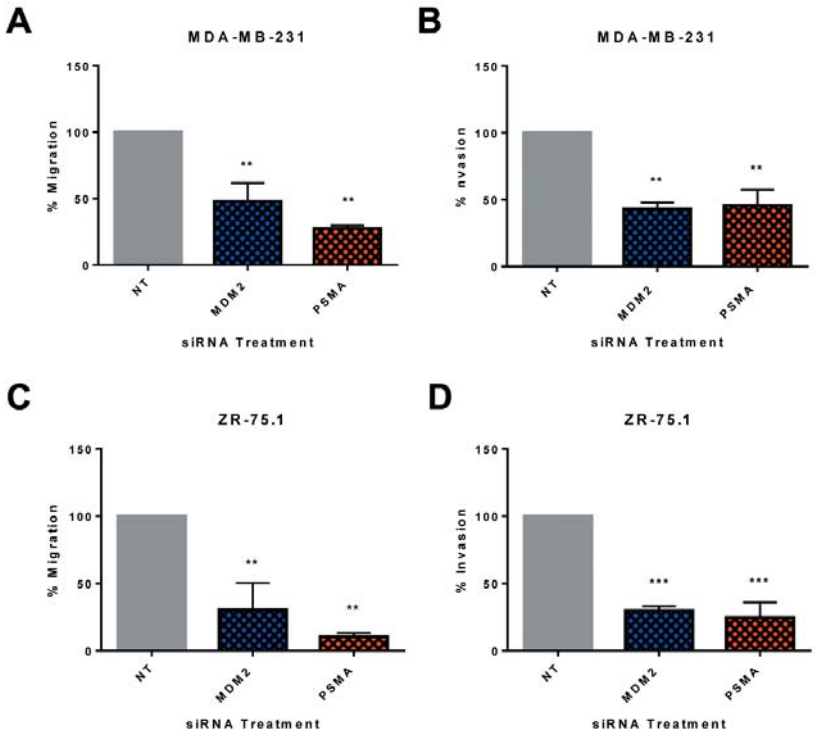

Figure 4. *Effect of mouse double minute 2 (MDM2) and prostate-specific membrane antigen (PSMA) knockdown on breast cancer cell migration and invasion. Migratory (A) and invasive (B) capacity of MDA-MB-231 cells following MDM2-/PSMA-targeting siRNA treatment as a percentage of non-targeting control (% mean+SEM, n=3, individual experiments carried out in triplicate). Migratory (C) and invasive (D) capacity of ZR-75.1 cells following MDM2-/PSMA-targeting siRNA treatment as a percentage of non-targeting control (% mean+SEM, n=3, individual experiments carried out in triplicate).*

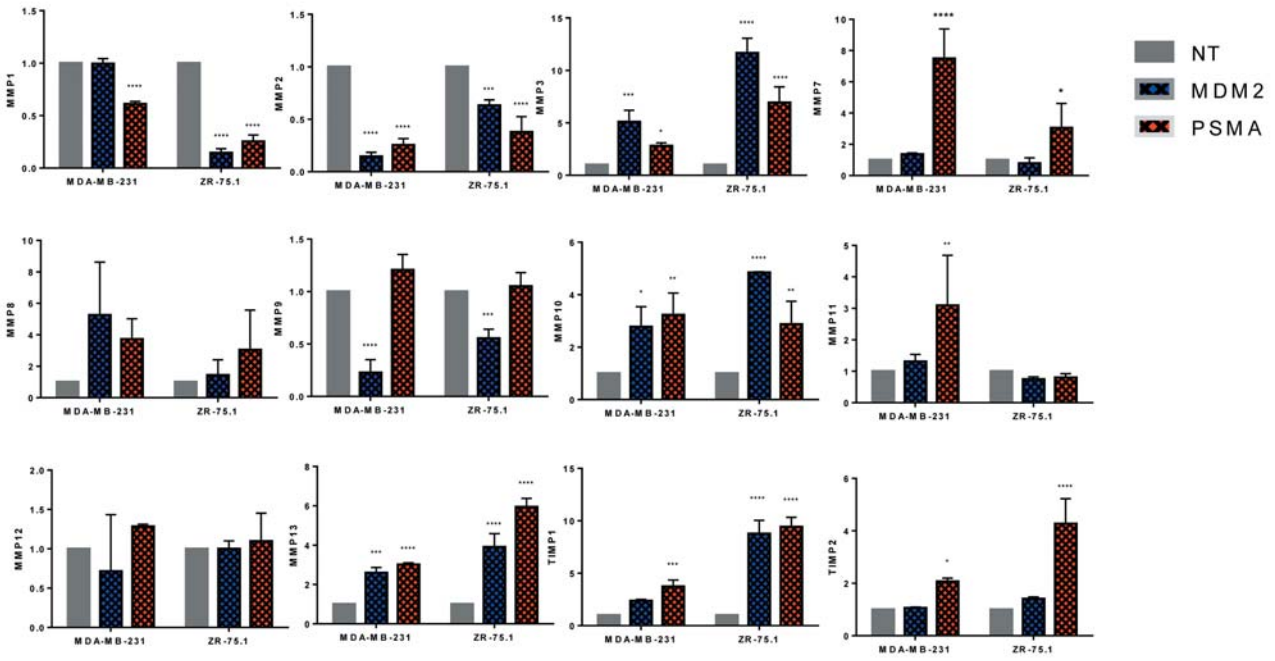

Figure 5. *Matrix metalloproteinase (MMP) and tissue inhibitor or matrix metalloproteinase (TIMP) gene expression levels in MDA-MB-231 and ZR-75.1 cells following siRNA treatment. Data are mean fold change + SD in expression of cells treated with mouse double minute (MDM2)-/prostate specific membrane antigen (PSMA)-targeting siRNA compared to non-targeting (NT) control (individual experiments were carried out in duplicate).*

either cell line after any treatment. *MMP13* expression was seen to significantly increase following both *MDM2*-targeted siRNA (MDA-MB-231 $p=0.0002$; ZR-75.1 $p<0.0001$) and *PSMA*-targeted siRNA (MDA-MB-231 $p<0.0001$; ZR-75.1 $p<0.0001$). *TIMP1* expression was not significantly changed in MDA-MB-231 cells following *MDM2*-targeted siRNA treatment; however, treatment with PSMA-targeted siRNA led to a significant increase in expression ($p=0.0008$). ZR-75.1 cells showed a significant increase in expression following treatment with both *MDM2*-targeting siRNA ($p<0.0001$) and *PSMA*-targeting siRNA ($p<0.0001$). Finally, *TIMP2* expression was unchanged through treatment with *MDM2*-targeting siRNA and significantly increased following treatment with *PSMA*-targeting siRNA in both cell lines (MDA-MB-231 $p=0.0111$; ZR-75.1 $p<0.0001$).

Discussion

MDM2 and PSMA are each considered a potential therapeutic target in their own right due to their overexpression in cancer cells and their knockdown in cancer cell lines resulting in a decrease in their tumour progressive properties. Recently, however, the expression of these two proteins and MMPs in the prostate cancer cell line LNCaP were linked (16). The report showed that knockdown of *PSMA* led to a decrease in *MDM2* transcript levels. We have shown that in two metastatic breast cancer cell lines, MDA-MB-231 and ZR-75.1, the same is true. We also revealed that the reverse is true: knockdown of *MDM2* leads to a decrease in *PSMA* transcript levels. However, we also showed that this decrease is not replicated at the protein level and the reason for this is still under investigation within our laboratory.

In our own knockdown breast cancer cell models, a highly significant decrease in MDM2 and pMDM2 was seen following *MDM2*-targeting siRNA treatment. However, unlike MDM2 which has a short half-life of 30 min (19), PSMA has a long half-life of 55 h (20). This means that at the 72-h time point, PSMA protein had only decreased to around half of its levels in the non-targeting siRNA-treated cells.

The functionality of the cells was assessed to gain an understanding of the molecules, which may be involved in the interplay of the proteins. Both *MDM2* and *PSMA* knockdown was shown to reduce the proliferative ability of cells, which is in agreement with other work on *PSMA* undertaken in prostate cancer cells (21, 22) and fits well with what is already known about the role of MDM2 in p53 suppression (4). When cells were treated with both siRNAs no cumulative effect was seen on cell growth, which could imply that MDM2 and PSMA are involved in the growth of cells *via* the same pathway. Interestingly, both proteins have been linked to the phosphatidylinositol 3-kinase/protein kinase B PI3K/AKT pathway (23-26), which is known to be involved in the growth and survival of cells (27).

The ability of cancer cells to adhere to HMVECs following knockdown was assessed in an attempt to mimic the metastatic environment encountered by cancer cells in the bloodstream before extravasation occurs. Knockdown of each protein in the cells led to a decrease in the adhesiveness of the cells, with knockdown of both proteins leading to a greater decrease in adhesion. Moreover, the morphology of targeted siRNA treated cells changed significantly, with MDA-MB-231 cells treated with MDM2-targeted siRNA losing their ability to lengthen, as is the usual phenotype for this cell line, and ZR-75.1 cells treated with *MDM2*-targeted siRNA losing their ability to clump, as this cell line usually does. However, *PSMA*-targeted siRNA treated cells look relatively normal, and cells treated with both siRNAs had an intermediate phenotype.

Knockdown of each molecule resulted in a decrease in migratory capacity, compared to cells treated with the control siRNA, which again fits with current literature (10, 12, 22, 23). In addition, lower individual expression of both proteins resulted in reduced invasion, which agrees with other reports on MDM2 (10, 12) and some on PSMA (22, 23), but not others (20). It is important to note that although ZR-75.1 cells exhibited a more significant decrease in migratory and invasive capacity in response to *MDM2*/*PSMA* knockdown, the cell line also showed a more significant reduction in gene-expression levels of both *MDM2* and *PSMA* compared to MDA-MB-231 cells, so at this moment it is unclear whether this difference in functionality is due to the response of the cell line to the knockdown or simply a result of reduced protein expression.

A family of molecules involved in all the above processes are the MMPs [for review see (25)] to which *PSMA* knockdown has already been linked (16). Thus, gene expression levels of the *MMPs* and their inhibitors, the *TIMPs*, were evaluated. Interestingly, knockdown of each of the proteins led to a similar expression profile, with a decrease in *MMP2* and an increase in *MMP3, MMP10* and *MMP13* being seen following both knockdowns, which reflected results seen previously (16). *TIMP1* expression also increased with each of the treatments in both cell lines, although the level following *MDM2*-targeted siRNA in MDA-MB-231 cells was not. Interestingly, *MMP9* was only seen to decrease following *MDM2* knockdown, while both MDM2 and PSMA have been positively correlated with its expression in the literature (12-14). This could imply that MDM2 regulates *MMP9* expression at the gene level, but PSMA regulates MMP9 protein levels.

In conclusion, it may be that the similarity of MMP2, -3 and -13 expression, following knockdown of *MDM2* and *PSMA*, individually, could indicate that the proteins may co-regulate the expression of certain MMPs, possibly through their effect on another molecule. This would explain the similarity in functionality of cells following knockdown of each of the proteins. Therefore, understanding the underlying mechanism of the interplay between MDM2 and PSMA may

lead to a new avenue in therapeutics, with dual inhibitor treatment becoming a feasible possibility.

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