

ETS1 promotes chemoresistance and invasion of paclitaxel-resistant, hormone-refractory PC3 prostate cancer cells by up-regulating MDR1 and MMP9 expression

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Abstract

ETS1, which belongs to the ETS transcription factor family, plays important roles in diverse aspects of cancer such as drug resistance and metastasis. In the present study, we examined the functional roles of ETS1 in paclitaxel resistance and invasion using human prostate cancer PC3 cells and paclitaxel-resistant PC3PR cells established from PC3 cells. Our results showed that ETS1 mRNA and protein expression was markedly up-regulated in paclitaxel-resistant PC3PR cells compared with paclitaxel-sensitive PC3 cells. The mRNA levels of MDR1 as well as MMP1, MMP3, MMP9 and uPA were positively correlated with that of ETS1. In PC3PR cells, silencing of ETS1 expression by siRNAs inhibited the activity of the MDR1 promoter containing ETS binding sites, reduced the mRNA and protein levels of MDR1 and suppressed paclitaxel resistance. Furthermore, ETS1 knockdown decreased secretion of MMP9 as well as its intracellular mRNA level, and dramatically inhibited invasion of PC3PR cells. Our results suggest that ETS1 promotes paclitaxel resistance and invasion in part by up-regulating MDR1 and MMP9 expression. Taken together, a novel therapeutic strategy targeting the ETS1 gene could be designed to overcome chemoresistance and metastasis of taxane-resistant, hormone-refractory prostate cancer.

Key words: ETS1, MDR1, MMP9, prostate cancer, paclitaxel resistance, metastasis

Abbreviations: ETS1; E26 transformation specific sequence 1, MDR1; multi drug resistance 1, uPA; urinary type plasminogen activator, MMP; matrix metalloprotease, ECM; extra cellular matrix, TBP; TATA-binding protein, GAPDH; glyceraldehydes 3 phosphate dehydrogenase, ADT; androgen deprivation therapy, PSA; prostate specific antigen, CRPC; castration-refractory prostate cancer, ABCB1; ATP-binding cassette, sub-family B (MDR/TAP), member 1, P-gp1; P-glycoprotein 1

1. Introduction

The *ets* oncogene (*v-ets*) was discovered as part of a fusion protein with *gag* and *myb* expressed by the E26 avian erythroblastosis virus. The vEts protein contains an 84 amino acid sequence, the 'ets domain', that has been found in a variety of species ranging from humans to *Drosophila*. ETS1 is the prototypic member of the ETS transcription factor family [1]. ETS1 plays important roles in diverse aspects such as drug resistance and metastasis in several types of cancer [2-6].

MDR1 describes a complex phenotype whose predominant feature is resistance to wide range of structurally unrelated anticancer agents, resulting in a serious limitation to the effective chemotherapeutic treatment [7]. Matrix metalloproteases (MMPs) family consists of more than 26 endopeptidases that share homologous protein sequences, with conserved and specific domains that are related to substrate specificity and recognition of other proteins [8]. Urinary-type plasminogen activator (uPA, PLAU) is a serine proteinase that functions in conversion of the circulating zymogen plasminogen to the active, broad-spectrum serine proteinase plasmin [9]. MMPs and uPA are up-regulated in various types of metastatic cancer, and play crucial roles in invasion, migration and metastasis of cancer through destruction of extra cellular matrix (ECM) [8-10].

It has been demonstrated that ETS1 transcriptionally controls expression of MDR1, uPA and MMPs, and promotes chemoresistance and invasion in several types of cancer such as pancreatic, ovarian and breast cancers [2-4,11-15]. In terms of taxane-resistance, DNA microarray analysis suggested that ETS1 may regulate MDR1 in breast cancer cells [2]. However, there have been no reports describing the functional roles of ETS1 and its target genes in paclitaxel resistance and invasion. We previously established paclitaxel-resistant PC3PR cells from paclitaxel-sensitive PC3 prostate cancer cells [16]. In the present study, we demonstrated increased expression of ETS1

as well as MDR1, uPA, and MMPs in PC3PR cells compared with PC3 cells. We then evaluated the effects of ETS1 knockdown on expression of these genes, paclitaxel resistance and invasion in PC3PR cells, providing evidence that ETS1 promotes chemoresistance and invasion by up-regulating MDR1 and MMP9 expression.

2. Material and methods

2.1. Reagents and antibodies

Paclitaxel was purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-ETS1 and -MDR1 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and anti-MMP9 and -GAPDH antibodies were from Cell Signaling Technology (Beverly, MA, USA).

2.2. Cell culture

Human prostate cancer PC3 cells were obtained from American Type Cell Collection (Manassas, VA, USA) and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum in humidified atmosphere containing 5% CO₂. Paclitaxel-resistant PC3PR cells were generated from parental PC3 cells by a stepwise increase of paclitaxel concentrations in the culture medium as described previously [16]. In the present study, PC3PR cells viable at 20 nM of paclitaxel were used.

2.3. Total RNA extraction and quantitative reverse transcription-PCR

Total RNA was extracted using the RNeasy mini kit (Qiagen, Valencia, CA, USA) with on-column DNase I treatment according to the manufacturer's protocol. To determine the mRNA levels of ETS1, MDR1, uPA and MMPs 1, 3 and 9, quantitative RT-PCR was performed using the Primescript RT reagent kit and SYBR premix EX Taq II (Takara, Otsu, Japan). The Ct values for each gene were normalized to TBP (TATA-binding protein) levels, and the relative fold change values were calculated using the $\Delta\Delta C_t$ method as described previously [17]. Primer sequences used in the present study were as follows: ETS1 (sense: 5'-CAAGCCTGTCATTCCTGCTG-3', antisense: 5'-ATTCCCAGCCATCTCCTGTC), MDR1 (sense: 5'-GAAGCTAACCCCTTGATTTTGG-3', antisense:

5'-TCAGCTACTGCTCCAGCTTTTG-3'),	uPA	(sense:
5'-AGTGTCTCAGCAGCCCCACTACTAC-3',		antisense:
5'-CCTTGGAGGGAACAGACGAC-3'),	MMP1	(sense:
5'-ACGAATTTGCCGACAGAGATG-3',		antisense:
5'-CAGTTCTAGGGAAGCCAAAGGAG-3'),	MMP3	(sense:
5'-CAGGCTTTCCCAAGCAAATAG-3',		antisense:
5'-CTCCAAGTGTGAAGATCCAGTAAG),	MMP9	(sense:
5'-AAGTGGCACCACCACAACATC-3', antisense: 5'-CAAAGGCGTCGTCAATCACC-3'),		
TBP (sense: 5'-GACCTAAAGACCATTGCACTTCG-3',		antisense:
5'-TTCTTCACTCTTGGCTCCTGTG-3').		

2.4. Small interfering RNA transfection

Two small interfering RNAs (siRNA) for human ETS1 were purchased from Thermo Scientific Dharmacon (Lafayette, CO, USA). Sequences of the siRNAs were as follows: (ETS1 siRNA1, sense: 5'-GAUAAUCCUGUCAGUCUU-3', antisense: 5'-AAGACUGACAGGAUUUAUC-3', ETS1 siRNA2, sense: 5'-GCAUAGAGAGCUACGAUAG-3', antisense: 5'-CUACGUAGCUCUCUAUGC-3'). Negative Control Medium GC Duplex #2 obtained from Invitrogen (Carlsbad, CA, USA) was used as a negative control siRNA.

2.5. Western blot analysis

To prepare whole cell lysates, cells were resuspended in ice-cold lysis buffer (1% Nonidet P-40, 0.1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 150 mM NaCl, 10 mM Tris-HCl [pH 7.4]). To harvest secreted proteins, the supernatant of the conditioned medium was precipitated overnight with 4 volumes of cold acetone and the precipitate was pelleted by centrifugation. Cell lysates and pellets were subjected to

electrophoresis on 10% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). After blocking in 5% skim milk, membranes were probed with a primary antibody followed by a horseradish peroxidase-linked secondary antibody. After washing, bound proteins were detected using the ECL Western blotting detection system (Amersham GE Healthcare, Piscataway, NJ, USA).

2.6. Promoter reporter assay

We constructed an MDR1 promoter reporter gene according to a previous report [11]. The MDR1 promoter region (-137 to +30) containing ETS binding sites was amplified by PCR, which was subcloned into the pGL4.12 firefly luciferase reporter vector (Promega, Madison, WI, USA). The DNA sequence of the inserted DNA was confirmed. The firefly luciferase reporter gene was co-transfected with Renilla luciferase expressing plasmid, phRL-TK (Promega) using Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, firefly and Renilla luciferase activities were determined by Pikkagene dual luciferase assay system (Toyo B-Net, Tokyo, Japan). Firefly luciferase activity was normalized to Renilla luciferase activity.

2.7. Cell invasion assay

The ability of cells to invade across Matrigel coated inserts was assayed using the BioCoat Matrigel invasion chambers (Becton Dickinson, San Jose, CA, USA) according to manufacturer's instructions. Briefly, serum-free RPMI 1640 medium (0.5 ml) containing 2.0×10^5 cells was added to the upper chamber, and 0.75 ml of RPMI 1640 medium containing 10% fetal bovine serum was added to the lower chamber as a chemo-attractant. After overnight incubation at 37°C under 5% CO₂, cells on the upper surface of the filter were removed and cells that migrated to the lower surface of the filter

were fixed in 100% methanol and stained with 0.005% crystal violet. For each filter, the number of migrated cells in 5 high-power fields was counted using bright field microscopy and the cell image was photographed (x100, BZ-8000, Keyence, Woodcliff, NJ, USA).

2.8. Cell viability determination

The viable cell number was determined by trypan blue exclusion assay.

2.9. Statistical analysis

The data analysis was performed by Student's t-test and Fisher's LSD test following one-way ANOVA using the SPSS software version 11 (Armonk, NY, USA). $p < 0.05$ was considered statistically significant.

3. Results

3.1. Up-regulation of ETS1 and its potential target genes in PC3PR cells

We first examined if ETS1 expression was increased in PC3PR cells compared with PC3 cells. As shown in Fig. 1, quantitative RT-PCR revealed that the ETS1 mRNA level in PC3PR cells was significantly higher than that in PC3 cells (x 5.6 fold, $p < 0.05$). We then studied if expression of ETS1 potential target genes was also elevated in PC3PR cells. Indeed, the mRNA expression levels of MDR1, uPA and MMPs 1, 3 and 9 were 69.2-, 4.3-, 5.4-, 16.7- and 25.8-fold higher in PC3PR cells than in PC3 cells ($p < 0.05$). These results indicated that ETS1 and its potential target genes are up-regulated in PC3PR cells compared with parental PC3 cells.

3.2. Inhibition of MDR1 and MMP9 expression by ETS1 knockdown in PC3PR cells

In order to determine which of potential target genes are regulated by ETS1 in PC3PR cells, we knocked down ETS1 expression by two different siRNAs and examined their effects on their mRNA expression. Both siRNAs effectively diminished ETS1 protein expression in PC3PR cells (Fig. 2A). Under the experimental condition in which ETS1 expression was eliminated, mRNA expression of both MDR1 and MMP9 was decreased (Fig. 2B), but that of uPA and MMPs 1 and 3 was not affected (Suppl. Fig. 1). The knockdown effect was much stronger on MMP9 than on MDR1, indicating that MMP9 gene is more dependent on transcriptional regulation by ETS1 than MDR1 gene. These findings suggest that MDR1 and MMP9 genes are targets of ETS1 in PC3PR cells. Consistent with qRT-PCR results, MDR1 protein expression was significantly decreased (ETS1 siRNA1: 0.66 fold, siRNA2: 0.46 fold) by ETS1 knockdown, whereas MMP9 secretion into the medium was almost totally abolished (Fig. 2A). In an attempt to demonstrate inhibition of MDR1 transcription by ETS1 silencing, we generated an MDR1 promoter reporter gene (-137 to +30) containing ETS

binding sites. The firefly luciferase assay showed that the MDR1 promoter activity was up-regulated in PC3PR cells than in PC3 cells (data not shown). Both siRNAs 1 and 2 decreased the MDR promoter activity, but the inhibitory effect was more profound with siRNA2 (Fig. 2C). Taken together, these results indicated that ETS1 silencing inhibits MDR1 and MMP9 expression in PC3PR cells.

3.3. Inhibition of paclitaxel resistance by ETS1 knockdown in PC3PR cells

Since ETS1 silencing reduced the expression level of MDR1 that is crucial for chemoresistance, we studied if ETS1 knockdown could attenuate paclitaxel resistance of PC3PR cells. The results showed that both siRNAs significantly suppressed paclitaxel resistance, but to a lesser extent with siRNA1 (Fig. 3).

3.4. Inhibition of invasion by ETS1 knockdown in PC3PR cells

Expression and secretion of MMP9 that enhances ECM degradation during cancer cell invasion were diminished by ETS1 knockdown. We therefore performed cell invasion assay to examine if ETS1 silencing could inhibit invasion of PC3PR cells. We found that cell invasion was remarkably suppressed by knockdown of ETS1 expression (Fig. 4).

4. Discussion

Prostate cancer is one of the most common malignancy and cause of cancer related death in industrialized countries [18,19]. Androgen deprivation therapy (ADT) is very effective for patients initially diagnosed as prostate cancer and also suppresses PSA levels in 80-90% of patients with metastatic prostate cancer. ADT in metastatic disease, however, is considered palliative, as disease progression occurs at a median of 2-3 years, with a subsequent expected survival of 16-18 months from the time of progression [20]. At the current situation, taxane-based chemotherapy (paclitaxel and docetaxel) is the standard therapeutic arm to castration-refractory prostate cancer (CRPC). Although the established treatment plan offers an overall survival benefit in patients with metastatic CRPC, there is typically a finite amount of time before the prostate cancer cells develop resistance to the taxane-based chemotherapy [18]. Taxane resistance poses a major obstacle to the success of advanced prostate cancer chemotherapy. Identifying novel therapeutic target is essential to overcome taxane-resistant CRPC.

In order to study the mechanisms of taxane resistance, we previously established paclitaxel-resistant cell lines (PC3PR) from androgen-independent PC3 prostate cancer cells. PC3PR cells are not only paclitaxel-resistant but also docetaxel-resistant [16]. Taxane resistance is thought to be due in large part to the adenosine triphosphate-dependent drug efflux pump MDR1 (ABCB1, P-gp1) [19]. Indeed, our previous studies demonstrated that MDR1 expression was up-regulated in paclitaxel-resistant PC3PR cells than in paclitaxel-sensitive PC3 cells [16,21]. DNA microarray data from another group also showed that MDR1 was overexpressed in paclitaxel-resistant PC3 cells compared to parental PC3 cells [22]. These data suggest that MDR1 plays a pivotal role to acquire paclitaxel resistance in prostate cancer.

ETS1 has been shown to regulate various aspects such as chemoresistance and

invasion by regulating expression of genes such as MDR1 as well as uPA and MMPs in several types of cancer [2-4,11,12]. In prostate cancer, there is a positive correlation between ETS1 expression and Gleason score and ETS1 is overexpressed in overt prostate cancer than in latent cancer, suggesting that ETS1 plays an important role in prostate cancer aggressiveness, progression and prognosis [6]. Our results showed that ETS1 expression is markedly elevated in PC3PR cells than in PC3 cells (Figs. 1 and 2A), indicating that ETS1 overexpression is associated with the development of paclitaxel resistance. The mechanism by which ETS1 expression is up-regulated in PC3PR cells remains to be studied.

To understand the functional roles of ETS1 in PC3PR cells, we knocked down ETS1 by using two different siRNAs. Both siRNAs effectively diminished ETS1 expression (Fig. 2A). Silencing of ETS1 expression decreased the activity of the MDR1 gene promoter containing ETS binding sites as well as its mRNA and protein levels (Fig. 2). A previous study reported that ETS1 interacts with mutant but not wild-type p53 and selectively up-regulates MDR1 transcription [11]. p53 is a tumor suppressor in which mutations are often found in cancer and associated with clinically aggressive features [23], but androgen-independent PC3 cells are p53 null [24]. Our results showed that ETS1 alone increases MDR1 transcription in the absence of p53, which was consistent with a previous report [11]. Since ETS1 positively regulated MDR1 expression, we examined if ETS1 knockdown could improve paclitaxel sensitivity of PC3PR cells. As expected, silencing of ETS1 expression suppressed paclitaxel resistance (Fig. 3). Together, we demonstrated that ETS1 contributes to the development of paclitaxel resistance by up-regulating MDR1 expression.

In addition to MDR1, quantitative RT-PCR showed increased expression of genes that are involved in metastasis such as uPA and MMPs 1, 3 and 9 in PC3PR cells compared with PC3 cells (Fig. 1). These results suggest that PC3PR cells may have

acquired aggressive metastatic potential during the development of paclitaxel resistance. Several reports also demonstrated that uPA and MMPs are overexpressed in chemoresistant cancer cells [2,3,25]. Silencing of ETS1 expression decreased secretion of MMP9 as well as its intracellular mRNA level (Fig. 2), and dramatically inhibited cancer cell invasion (Fig. 4). Since ETS1 has been shown to positively regulate MMP9 transcription [26], ETS1 may transcriptionally up-regulate MMP9 expression and increase its secretion, thereby enhancing invasion ability of PC3PR cells. Although previous studies demonstrated that ETS1 directly binds to the promoter regions of uPA and MMPs 1 and 3 genes, and controls their transcription [12-14], its knockdown did not affect their expression (Suppl. Fig. 1), suggesting that ETS1 may not target these genes in PC3PR cells. It is noteworthy that ETS1 regulated both MDR1 and MMP9 expression in PC3PR cells, whereas DNA microarray analysis suggested that ETS1 may regulate MDR1 but not MMPs 1 and 9 in taxane-resistant breast cancer MCF-7 cells [2]. The functional roles of ETS1 in regulation of gene expression may vary depending on cancer cell types.

In the present study, we demonstrated that ETS1 promotes paclitaxel resistance and invasion by up-regulating MDR1 and MMP9 expression in paclitaxel-resistant PC3 prostate cancer cells. To our knowledge, this is the first report that described the functional roles of ETS1 and its target genes in paclitaxel-resistant cancer cells. Although confirmatory studies using animal models are needed, our results suggest that a novel therapeutic strategy targeting the ETS1 gene could be designed to overcome taxane-resistance of CRPC.

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Figure legends

Fig. 1 Expression of ETS1 and its potential target genes in PC3 and PC3PR cells.

Total RNA was isolated from PC3 and PC3PR cells and subjected to quantitative RT-PCR for ETS1, MDR1, uPA and MMPs 1, 3 and 9. Data are expressed as mean \pm SD from three independent experiments in duplicate. * $p < 0.05$, compared with PC3 cells, as determined by Student's t-test.

Fig. 2 Effects of ETS1 knockdown on MDR1 and MMP9 expression in PC3PR cells.

(A) Whole cell lysates and secreted proteins were harvested from PC3 and PC3PR cells as well as from PC3PR cells transfected with a control siRNA, ETS1 siRNA1 or ETS1 siRNA2. Conditioned medium was collected after 24 h serum-deprivation and secreted proteins were isolated from conditioned medium by acetone precipitation. Samples were subjected to Western blot analysis using anti-ETS1, -MDR1, -MMP9 and -GAPDH antibodies. A representative blot from three independent experiments is shown.

(B) Total RNA was harvested from PC3PR cells transfected with a control siRNA, ETS1 siRNA1 or ETS1 siRNA2 and subjected to quantitative RT-PCR for MDR1 (upper column) and MMP9 (lower column). Data are expressed as mean \pm SD from three independent experiments in duplicate. * $p < 0.05$, compared with PC3 cells, as determined by Fisher's LSD test following one-way ANOVA.

(C) The MDR1 promoter reporter gene and phRL-TK control vector were transfected into PC3PR cells along with a control siRNA, ETS1 siRNA1 or ETS1 siRNA2. Twenty-four hours later, firefly and Renilla luciferase activities were measured. The MDR1 promoter activity was calculated as a ratio of firefly to Renilla luciferase activity. Data are expressed as mean \pm SD from three independent experiments in duplicate. * $p < 0.05$, versus cells transfected with control siRNA, as determined by Fisher's LSD test following one-way ANOVA.

Fig. 3 Effects of ETS1 knockdown on paclitaxel resistance in PC3PR cells.

(A) PC3PR cells were transfected with a control siRNA, ETS1 siRNA1 or ETS1 siRNA2. After incubation in fresh medium for 48 h, cells were reseeded onto six-well plates and cultured for additional 24 h. Cells were then treated with 300 nM paclitaxel for 48 h. Viable cell number was counted at the beginning and end of the paclitaxel treatment, and photographs were taken at the end. Data are expressed as mean \pm SD of cell number from 5 high-power fields. * $p < 0.05$, compared with cells transfected with a control siRNA, as determined by Fisher's LSD test following one-way ANOVA. (B) Photographs of PC3PR cells at the end of the treatment (x400).

Fig. 4 Effects of ETS1 knockdown on invasion in PC3PR cells.

PC3PR cells were transfected with a control siRNA, ETS1 siRNA1 or ETS1 siRNA2. After incubation in fresh medium for 48 h, cells were reseeded onto the Matrigel invasion chambers. Twenty-four hours later, invaded cells were fixed, stained and counted, and photographs were taken. (A) Invaded cell number. Data are expressed as mean \pm SD of cell number from 5 high-power fields. * $p < 0.05$, compared with cells transfected with a control siRNA, as determined by Fisher's LSD test following one-way ANOVA. (B) Photographs of invaded cells (x100).

Figure 1

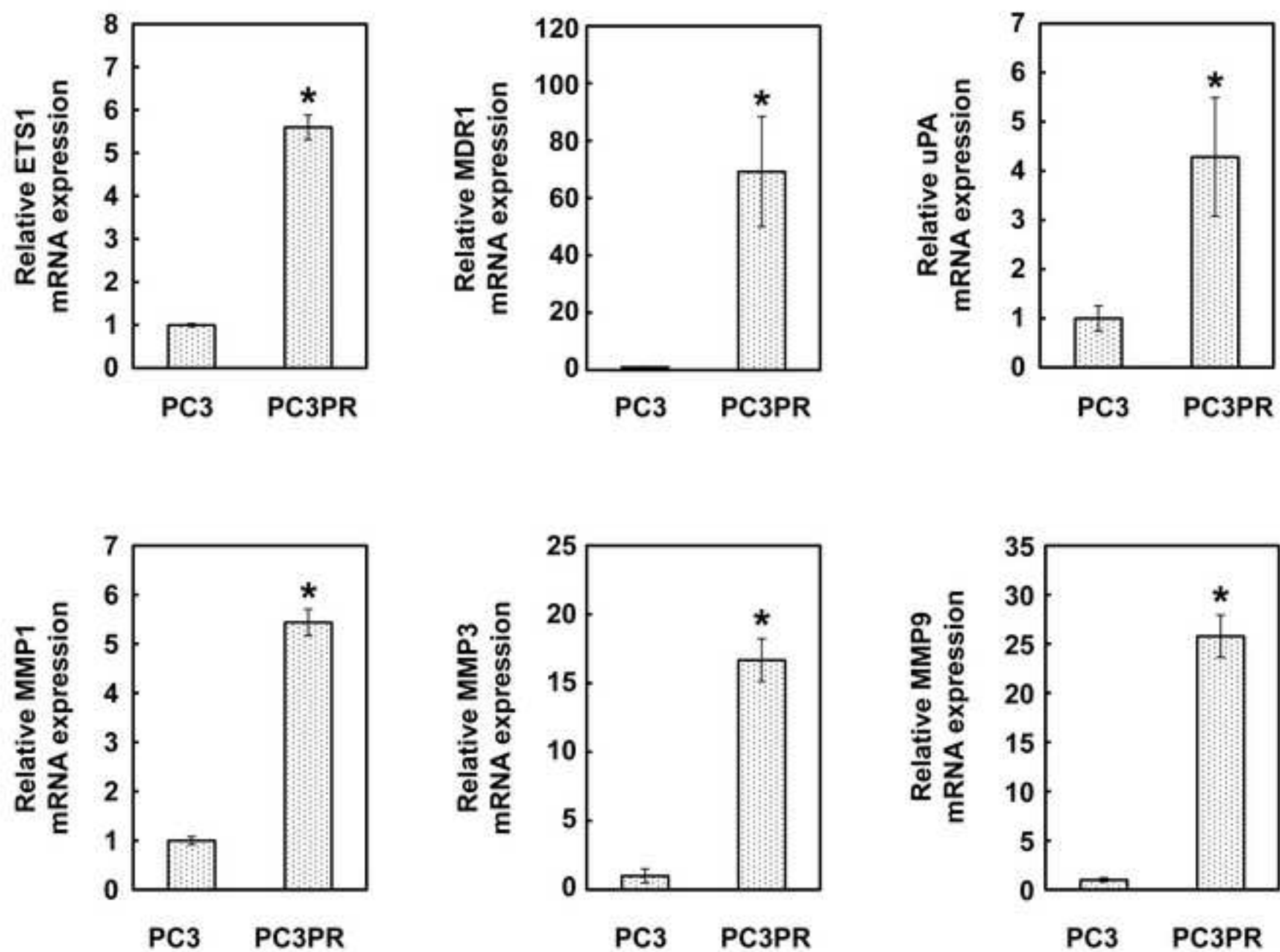


Figure 2

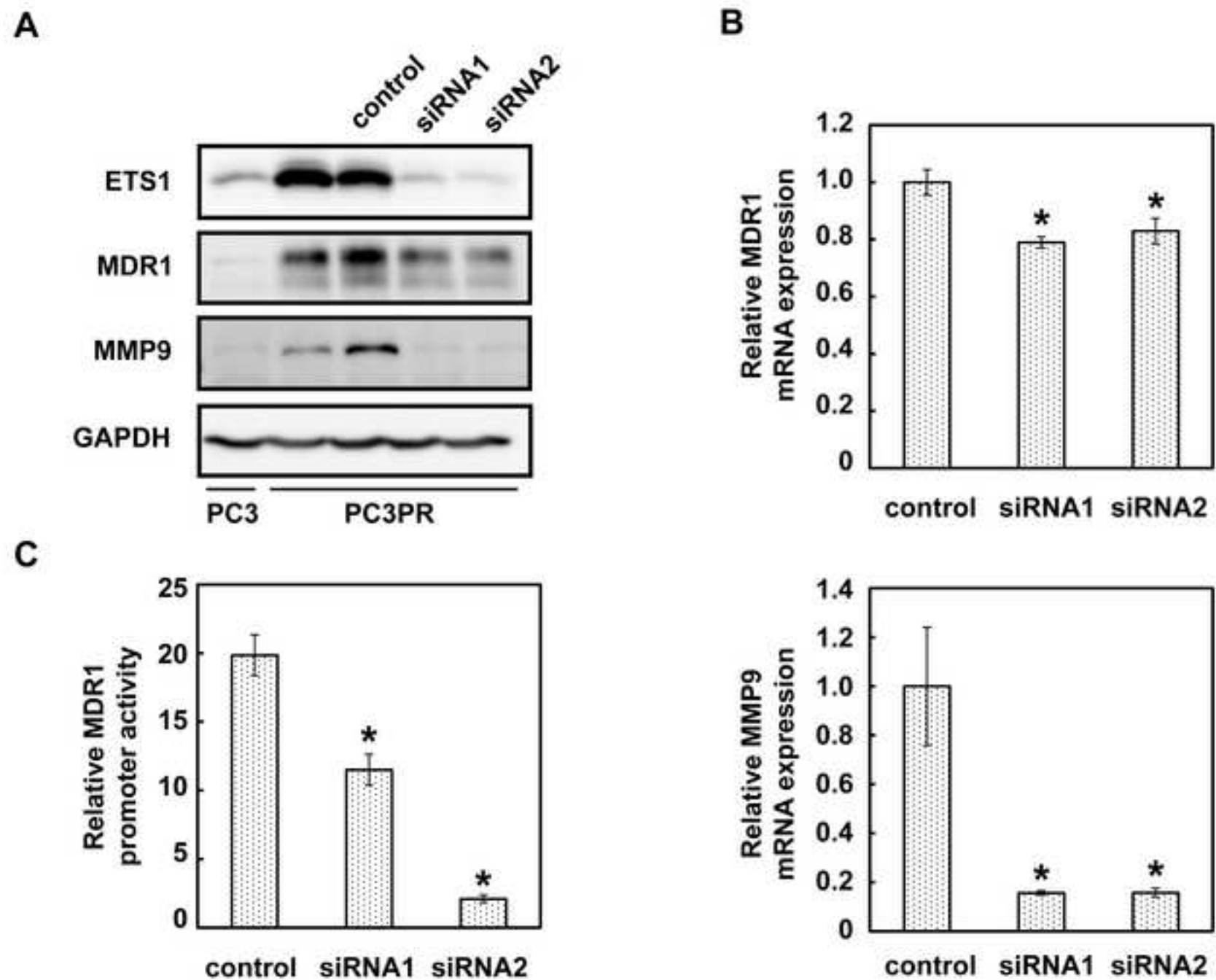
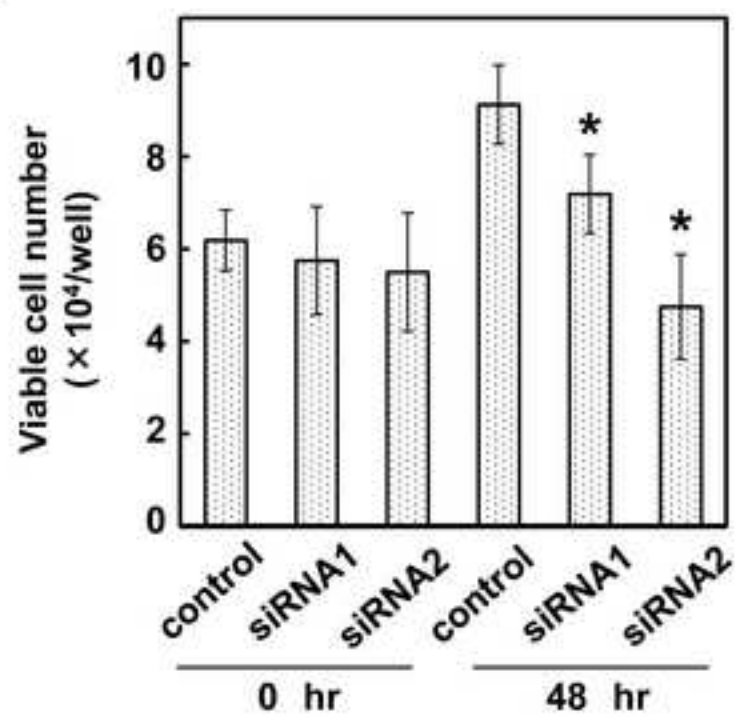
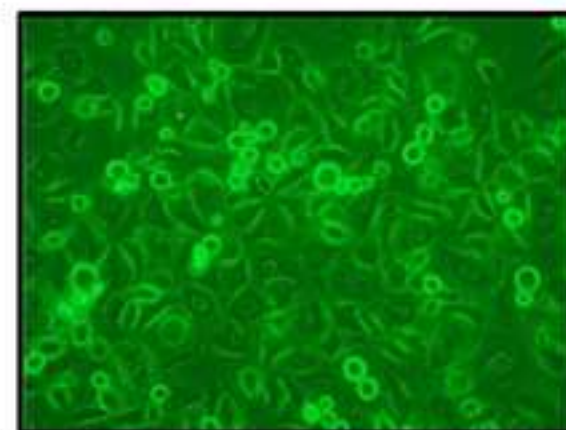


Figure 3

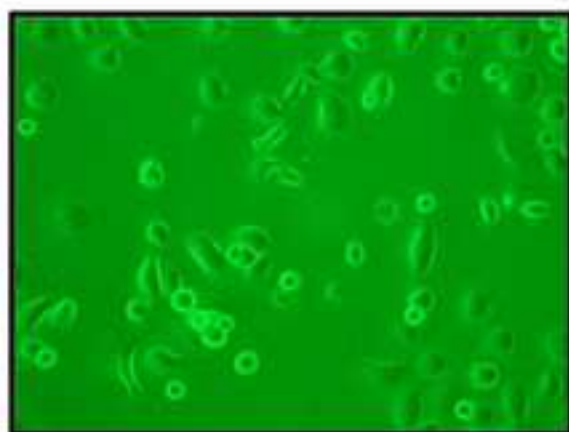
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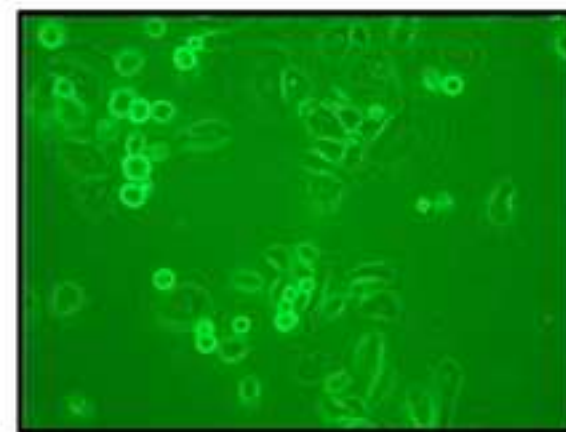
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control



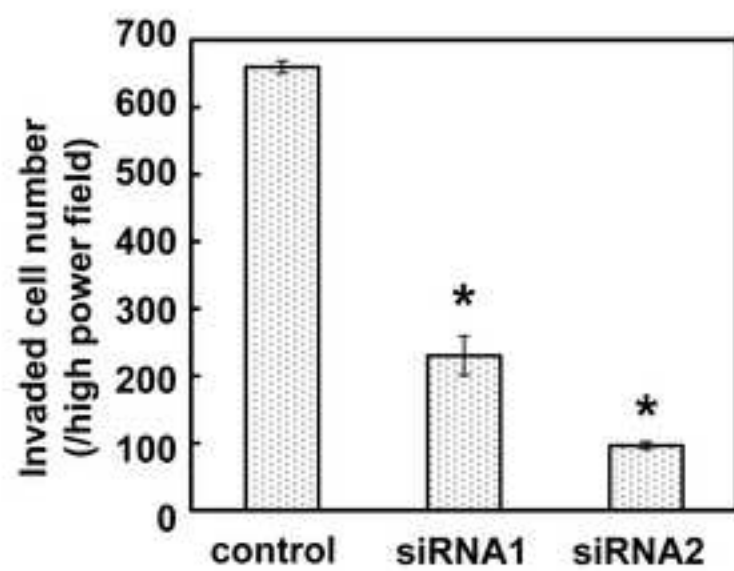
siRNA1



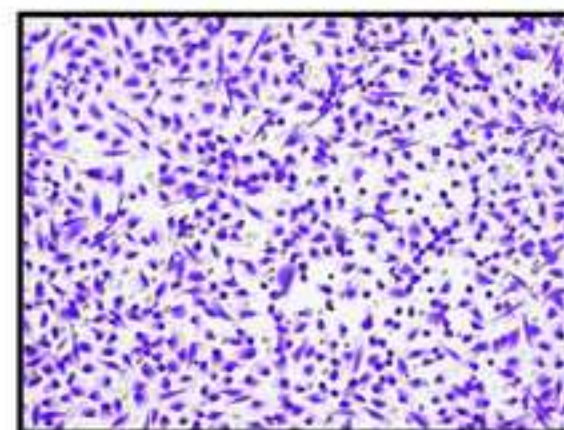
siRNA2

Figure 4

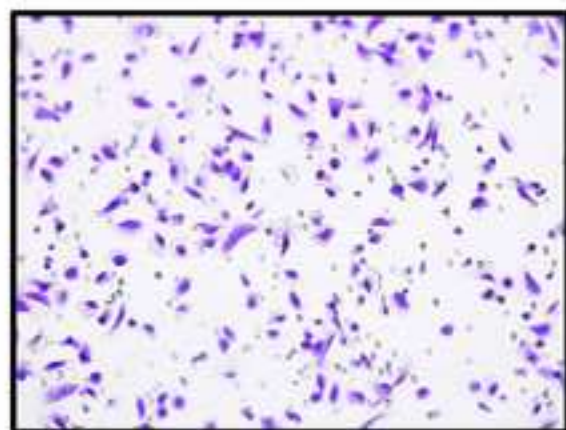
A



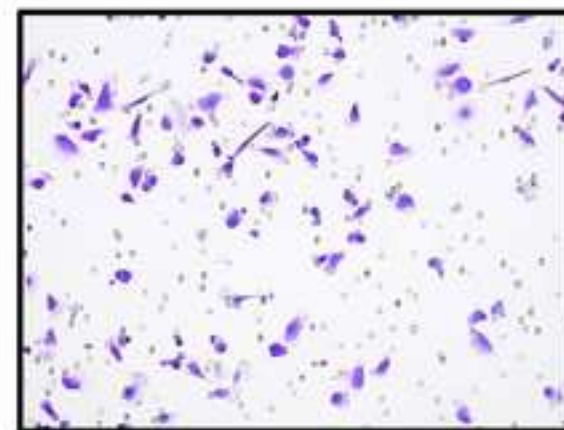
B



control



siRNA1



siRNA2