Double transfection improves small interfering RNA-induced thrombin receptor (PAR-1) gene silencing in DU 145 prostate cancer cells

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Abstract The efficiency of small interfering RNA (siRNA)-induced gene knockdown is hampered by low transfection efficiency. We established a novel and simple double transfection method using specific siRNA duplexes targeted against human thrombin receptor PAR-1 in DU 145 prostate cancer cells. The initial siRNA transfection of cell suspensions followed by re-transfection of adherent cells on the following day resulted in undetectable PAR-1 mRNA and absent receptor protein. PAR-1 mRNA expression was silenced for up to five days. Functional studies showed that PAR-1 gene silencing in DU 145 cells abolished the modulating effects of thrombin on cell adhesion to the extracellular matrix proteins, fibronectin and laminin, thus demonstrating the essential role of PAR-1 in mediating thrombin effects on DU 145 cell adhesion.

Keywords: RNA interference; Small interfering RNA; Double transfection; Thrombin; PAR-1; Prostate cancer

1. Introduction

RNA interference (RNAi) is a gene-regulating process in which double-stranded RNA (dsRNA) intracellularily triggers post-transcriptional suppression of homologous gene expression. Although the mechanism of RNAi is not yet understood in full detail, a multi-step process seems to be involved. A ribonuclease III-related enzyme called Dicer cleaves long dsRNA into small interfering RNA duplexes (siRNAs), which participate in the formation of an effector complex (RNA-induced silencing complex or RISC) targeting complementarily homologous mRNA degradation and gene silencing [1].

Long dsRNA species (>30 bp) usually induce rapid and global gene knockdown in mammalian cells. Therefore, 21- to 28-nucleotide sequence-specific siRNAs with 3′-overhangs have been designed for improved specificity. Induction of siRNA in mammalian cells can inhibit expression of target mRNA and has been utilized as a powerful tool for functional genomics [2]. In addition, systemic delivery of siRNA inhibits gene expression in murine disease models [3].

Although RNAi techniques have been widely used in gene functional analysis, siRNA-mediated gene silencing is hampered frequently due to low delivery efficacy of either synthetic siRNAs or vector-based systems to target cells [4]. The efficacy of RNAi-induced gene silencing depends on cell type, RNAi target, amount of siRNA and delivery method with optimal transfection being critical for successful inhibition of gene expression. The identification of optimal transfection conditions usually requires time-consuming and expensive experiments.

Accumulating evidence indicates that the serine protease thrombin, in addition to its central role in hemostasis and thrombosis, is involved in a variety of aspects related to tumor pathogenesis [5]. The identification and molecular understanding of a subfamily of G protein-coupled receptors, referred to as protease-activated receptors (PARs) of which PAR-1, -3, and -4 are proteolytically activated by thrombin, provide mechanistic explanations with respect to the interplay between malignancy and thrombin [6]. PAR-1 is believed to mediate most if not all of the cellular effects exerted by thrombin and is expressed by a variety of established tumor cell lines and primary tumor tissues [5]. The role of PAR-1 can be confirmed by receptor activating peptides. However, molarity of peptides required is several orders of magnitudes higher than that of thrombin [7–9]. Moreover, the effects of thrombin on several cell types were not completely mimicked by PAR-1 activating peptides [10,11].

Expression of the major thrombin receptor PAR-1 was demonstrated in several widely used prostate cancer cell lines [9,12,13]. In addition, thrombin modulates Rho signaling [14], proliferation [13] and secretion of vascular endothelial growth factor [9] in prostate cancer cells. However, the exclusive contribution of PAR-1 to the effects observed after thrombin treatment in prostate cancer cells remains to be shown.

In the present study, we established a novel double transfection method using synthetic siRNA and demonstrated undetectable expression of PAR-1 for up to five days after transfection in DU 145 prostate cancer cells. In addition, the essential role of PAR-1 for thrombin-induced cell adhesion to extracellular matrix (ECM) proteins was conclusively established.

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Abbreviations: RNAi, RNA interference; siRNA, small interfering RNA; dsRNA, double-stranded RNA; PAR-1, protease-activated receptor 1; ECM, extracellular matrix; FN, fibronectin; LN, laminin

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2. Materials and methods

2.1. Cell culture

The human prostate cancer cell line DU 145 (ATCC, Manassas, VA, USA) was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 2% glutamine, and penicillin/streptomycin (Biochrom, Berlin, Germany). Subculturing was performed with 0.05% trypsin/0.02% EDTA. DU 145 cells in passages 25–40 were used for experiments.

2.2. Transfection with small interfering RNA

Transfection of adherent cells. The specific siRNA duplexes targeted against human thrombin receptor PAR-1, siRNA transfection reagent, and reduced-serum transfection medium were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The day before transfection, 4×10^4 cells were seeded in each well of 12-well cell culture plates in RPMI 1640 medium containing 10% FCS without antibiotics and incubated for 24 h. The next day, transfection complexes were prepared using PAR-1 siRNA, siRNA transfection reagent, and transfection medium according to the manufacturer's instructions and were delivered to cell monolayers in 600 μl fresh media with 50 or 100 nM final concentration of siRNA duplexes. A scrambled siRNA (Santa Cruz Biotechnology) was used as negative control.

Transfection of cell suspensions. A modified transfection protocol was used [15] with siRNA transfection complexes prepared as above. To compensate for cell proliferation from the time of seeding to transfection in adherent cells, increased cell numbers (8×10^4) in single cell suspensions prepared by trypsinization were gently mixed with transfection complexes containing either 50 or 100 nM final concentration of siRNA and plated in 12-well plates in growth media without antibiotics. Cells were incubated with transfection complexes for 5 h, followed by replacement of 90% of the transfection media with fresh growth media without transfection complexes.

Double transfection. DU 145 cells (8×10^4) were initially transfected with either 25 or 50 nM of siRNA in suspensions for 5 h before replacement of 90% volume of transfection media. After 24 h of incubation, adherent cells were re-transfected with siRNA at the same concentration as transfection performed in suspension. Thereafter, 50% of media volumes were replaced every 3 days with fresh culture media.

2.3. RNA isolation and semi-quantitative duplex RT-PCR

Cells cultured in 12-well plates were harvested at different time points as indicated and cellular RNA isolated using 0.5 ml TRIzol reagent (Life Technologies, Karlsruhe, Germany) for each well of cells according to the manufacturer's instructions. Total RNA was digested with 1 unit RQ1 RNase-free DNase (Promega, Madison, WI, USA) per μg RNA. 2 μg of total RNA was reverse-transcribed using random hexamers and the Superscript II preamplification system (Life Technologies). Amplifications were performed in 50 μl reaction mixtures containing PCR buffer (10 mM Tris–HCl, 1.5 mM $MgCl_2$, and 50 mM KCl, pH 8.3), 0.2 mM of each deoxynucleotide triphosphate, 2 units of Taq DNA polymerase (Qiagen, Hilden, Germany), 0.2 μM of each human PAR-1 primer (forward: GTG CTG TTT GTG TCT GTG CT, reverse: CCT CTG TGG TGG AAG TGT GA) [13], and 40 nM of each human glyceraldehydes-3-phosphate dehydrogenase (GAPDH) primer (forward: TCG GAG TCA ACG GAT TTG GTC GTA, reverse: AGC CTT CTC CAT GGT GGT GAA GA) [16]. After initial denaturation (94 °C for 5 min), 30 cycles were performed with 1 min denaturation at 94 °C, 1 min annealing at 55 °C and 2 min synthesis at 72 °C, followed by a 7 min final extension step. PCR products were electrophoresed on 2% agarose gels, stained with ethidium bromide, and visualized under UV light. The intensity of PCR product was analyzed by the NIH software ImageJ 1.28u.

2.4. Flow cytometry

After 72 h of siRNA transfection, cells were collected for detection of PAR-1 expression on the cell surface by flow cytometry analysis following established procedures [13] with slight modifications. In brief, cell monolayers were dissociated by exposure to 50 mM EDTA and single cell suspensions prepared in PBS containing 2% bovine serum albumin. Cells were incubated with 50 μg/ml of mouse monoclonal anti-human PAR-1 IgG1 (WEDE15, Beckman Coulter Immunotech, Krefeld, Germany) at 4 °C for 30 min. After washing, phycoerythrin-conjugated goat $F(ab')_2$ anti-mouse IgG was added. The cells were washed twice and 10 000 cells were analyzed by a FACScan flow cytometer (Becton Dickinson, Heidelberg, Germany). Isotypic murine monoclonal antibody (clone MsIgG1) was utilized as negative control.

2.5. Cell adhesion

DU 145 cells were transfected with siRNA in 12-well plates using the double transfection protocol. 72 h after the initial transfection, 96-well culture plates were precoated with 5 μg/cm^2 human fibronectin (FN, Roche, Mannheim, Germany) or 2 μg/cm^2 mouse laminin (LN, Roche) at 37 °C for 2 h. Untransfected or transfected cells with either PAR-1 siRNA or control siRNA were harvested with 50 mM of EDTA, resuspended in serum-free media containing different concentrations of thrombin and seeded in 96-well plates (2.5×10^4 cells per well in 100 μl medium). After 30 min of incubation, media and non-adherent cells were carefully removed and the plates were washed twice with PBS. After the addition of 100 μl fresh medium to each well, numbers of adhesive cells were estimated by incubation with 10 μl WST-1 (Roche) at 37 °C for 30 min and absorbance read at 450 nm as previously reported [13].

2.6. Statistics

Results are presented as means ± S.E.M. Differences between means were analyzed by Student's two tailed t test. Differences were regarded statistically significant at $P \leqslant 0.05$.

3. Results

3.1. Comparison of gene silencing efficacy of different transfection protocols

Initially, we used a target-specific siRNA against human thrombin receptor PAR-1 to transfect adherent DU 145 prostate carcinoma cells by a conventional transfection protocol. After 48 h of transfection with 50 nM PAR-1 siRNA, PAR-1 mRNA expression was downregulated to 27% residual level compared to untreated cells (Fig. 1). The scrambled control siRNA had no significant effect on PAR-1 expression. Increasing PAR-1 siRNA concentration to 100 nM was unsuccessful due to 80% cell detachment 48 h post transfection (data not shown).

Transfection of DU 145 cell suspensions was attempted. To minimize the prolonged presence of transfection complexes in cell suspensions causing cytotoxic effects, 90% volume of media containing transfection complexes were replaced with fresh media after 5 h to reduce the concentration of complexes and to avoid decreasing cell numbers. Transfection of siRNA at 50 nM in cell suspensions improved PAR-1 gene silencing (18% residual mRNA expression compared to untreated cells), which was however still incomplete. Transfection of control siRNA in suspensions did not inhibit expression of PAR-1 mRNA (Fig. 1). siRNA applied at 100 nM displayed pronounced cytotoxic effects in DU 145 suspensions within 5 h rendering this approach impossible.

Alternatively, we aimed to test a double transfection method with initial transfection of DU 145 cell suspensions followed by re-transfection of adherent cells on the following day. Double transfection with 25 nM siRNA suppressed PAR-1 mRNA expression to 22% residual level 48 h post the initial transfection, while application of 50 nM siRNA using the double transfection protocol resulted in undetectable PAR-1 mRNA (Fig. 1). Furthermore, gene silencing was demonstrated at the protein level by flow cytometry, indicating absent thrombin receptor PAR-1 on the cell surface 72 h after the initial transfection (Fig. 2).

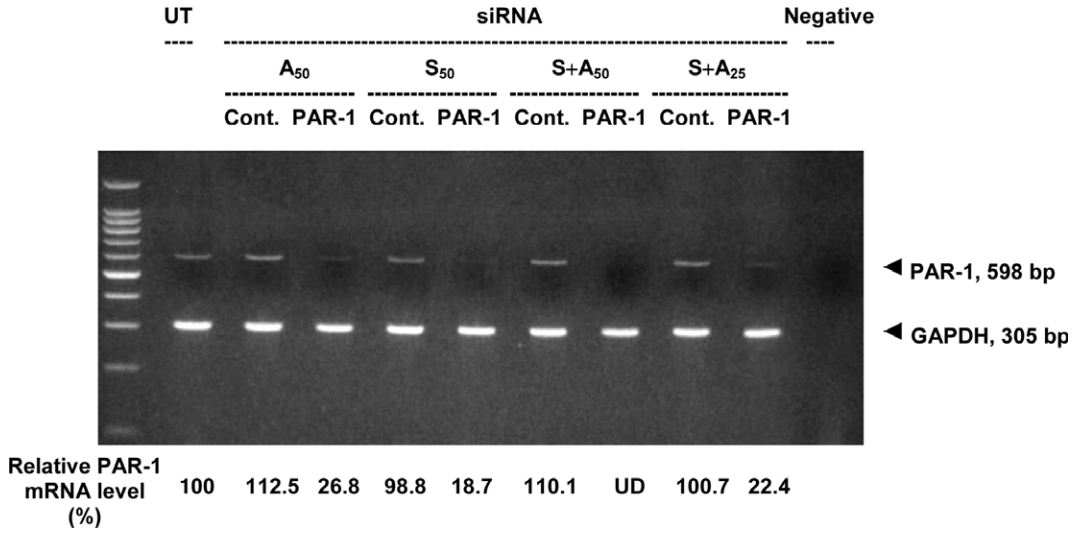

Fig. 1. Comparison of gene silencing effects of siRNA on thrombin receptor (PAR-1) expression of DU 145 cells using different transfection protocols. Cells were transfected with either 50 nM final concentration of PAR-1 siRNA or negative control siRNA (Cont.) in adherent cells (A_{50}) or in suspensions (S_{50}), respectively. Cells were also initially transfected in suspensions with either 25 or 50 nM siRNA followed by re-transfection of adherent cells after 24 h with the same siRNA concentration as used for the first transfection ($S+A_{25}$ and $S+A_{50}$). Total RNA was extracted 48 h post-(initial) transfection, RNA was reverse-transcribed and cDNAs amplified by duplex RT-PCR to determine the expression levels of PAR-1 mRNA. The intensity of PAR-1 signals was normalized to GAPDH expression using the NIH ImageJ software (UT, untreated; UD, undetectable).

3.2. Persistence of RNAi-mediated PAR-1 silencing

Using the double transfection protocol, PAR-1 transcripts in DU 145 cells were silenced by RNAi for up to five days post the initial transfection (Fig. 3). After seven days, PAR-1 mRNA re-emerges with 27% relative intensity compared to controls, which is equal to the inhibitory level obtained two days after transfection using the conventional method (Figs. 1 and 3).

3.3. Abolishment of thrombin-induced cell adhesion by PAR-1 silencing

The presence of thrombin in cell culture media induced cell adhesion to the ECM components, fibronectin (FN, Fig. 4A) and laminin (LN, Fig. 4B). The presence of thrombin in concentrations ranging from 0.1 to 1.0 U/ml stimulated DU 145 adhesion to FN compared to control cells without thrombin treatment (0.1 U/ml: $P = 0.022$; 0.5 U/ml: $P = 0.031$; and 1.0 U/ml: $P = 0.028$). DU 145 cell adhesion to LN was also stimulated by thrombin (0.1 U/ml: $P = 0.014$; 0.5 U/ml: $P = 0.036$; and 1.0 U/ml: $P = 0.054$). Maximal adhesion of DU 145 cells to ECM proteins was observed in the presence of 0.5 U/ml thrombin.

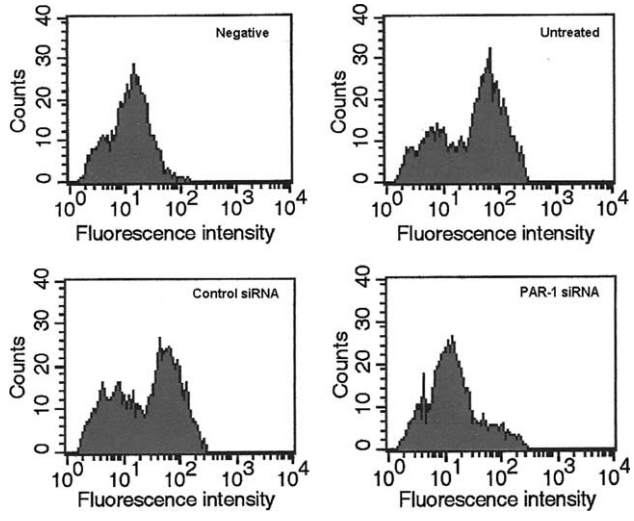

Fig. 2. Flow cytometric analysis of PAR-1 gene knockdown of DU 145 cells by double transfection with 50 nM of siRNA performed in cell suspensions and adherent cells. Cells were collected 72 h post initial transfection to detect PAR-1 protein expression on cell surface using the monoclonal antibody WEDE15 against PAR-1.

DU 145 cells with PAR-1 knockdown were used for adhesion assays 72 h after initial transfection. Lack of PAR-1 in transfected cells caused cellular unresponsiveness to 0.5 U/ml thrombin with respect to adhesion to either FN or LN (Fig. 5A and B), whereas control siRNA did not affect the modulating effects of thrombin on DU 145 adhesion to ECM proteins.

4. Discussion

Since the introduction of specific suppression of gene expression in mammalian cells using siRNA [17], RNAi techniques have been widely adopted in functional gene analysis. Delivery of synthetic siRNA or vector-based siRNA expression systems to target cells reverses the expression and function of the gene under consideration.

Similar to other nucleic acid-based approaches, optimal introduction of siRNA into target cells is critical for gene silencing. Complete gene silencing is difficult to achieve with low delivery rates. Incomplete siRNA-induced gene suppression may result from the presence of a fraction of mRNA in a protected compartment such as spliceosomes, other nuclear locations or non-transfected cells [18]. Improved RNAi-mediated gene silencing has been reported using several approaches, including the application of multiplex siRNAs against the same gene [19], conjugation of siRNA with membrane permeant peptides [20], and introduction of mismatches

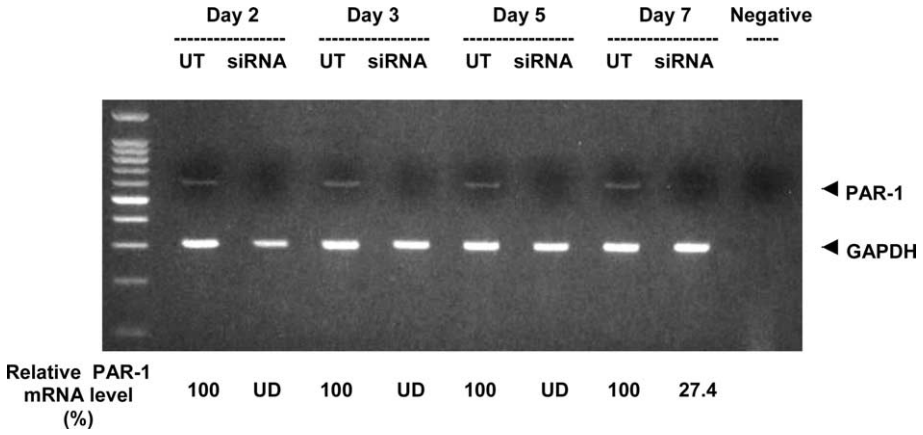

Fig. 3. Kinetics of gene silencing after double transfection of PAR-1 siRNA in DU 145 cells. Total cellular RNA was isolated at different time points and relative RT-PCR performed to examine PAR-1 mRNA levels (UT, untreated; UD, undetectable).

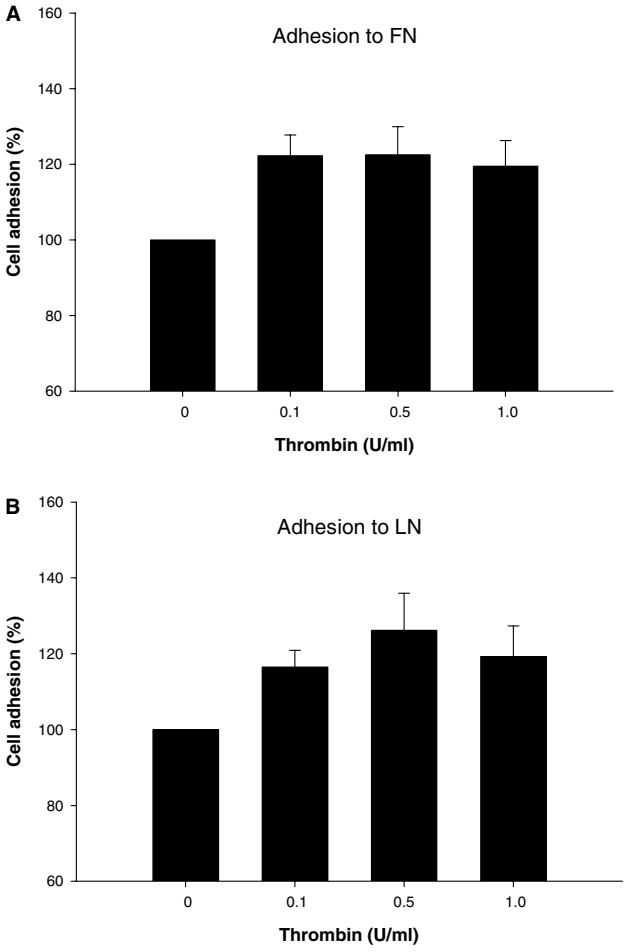

Fig. 4. Thrombin-induced modulation of DU 145 cell adhesion to extracellular matrix components. Cells suspended in serum-free media with different concentrations of thrombin were seeded into plates precoated with either fibronectin (FN, 5 μg/cm^2, A) or laminin (LN, 2 μg/cm^2, B). After 30 min of incubation, unbound cells were removed and adhesive cells assayed by WST-1 reagent. Data were obtained from four independent experiments.

into siRNA duplexes [21]. Enhanced gene silencing in refractory adherent cells was achieved by transfection of cell suspensions instead of attached cells [15].

In the present study, we utilized a single siRNA corresponding to the thrombin receptor PAR-1 in prostate carcinoma DU 145 cells using a double transfection method with initial transfection of cell suspensions for 5 h and re-transfection of adherent cells on the following day. PAR-1 mRNA was undetectable 48 h after the initial transfection and PAR-1 knockdown was confirmed at the protein level by flow cytometry. In contrast, transfection with PAR-1 siRNA of adherent DU 145 cells using a standard protocol inhibited only approximately 70% of PAR-1 expression and a single transfection of cell suspensions caused approximately 80% inhibition.

Using the double transfection protocol, highly efficient PAR-1 gene silencing was achieved with four additionally designed siRNA duplexes directed against different PAR-1 target sites, one of which totally abolished PAR-1 mRNA expression in DU 145 cells 48 h after transfection (data not shown). However, the general applicability of the double transfection approach remains to be demonstrated with respect to additional genes and other adhesive cell types.

It was suggested that trypsinization-induced cell surface availability to transfection complexes or cellular changes during cell attachment/detachment to or from culture plates may modulate uptake of siRNA [15]. Although the kinetics of siRNA uptake in DU 145 cells has not been studied, it should be noted that siRNA delivery to keratinocytic cells reached maximal level after transfection for 2–4 h [18]. In our studies, the deprivation of siRNA-containing media 5 h post transfection to minimize cytotoxic effects for adherent cells kept in suspension presumably resulted in a subpopulation of non-transfected cells and incomplete PAR-1 silencing. After overnight cellular attachment to cell culture plates, adherent DU 145 cells were subject to a second siRNA transfection to deliver additional siRNA to both transfected and non-transfected cells, which resulted in complete PAR-1 gene knockdown. Another possible mechanism involved is delayed recovery of mRNA levels by repeated exogenous applications of synthetic siRNA [22].

Synthetic siRNA-induced gene silencing in proliferating mammalian cell cultures is transient by its nature. However, the persistence of gene silencing is a critical parameter to perform experiments in gene-silenced cells. It was previously reported that the expression of targeted genes recovered to

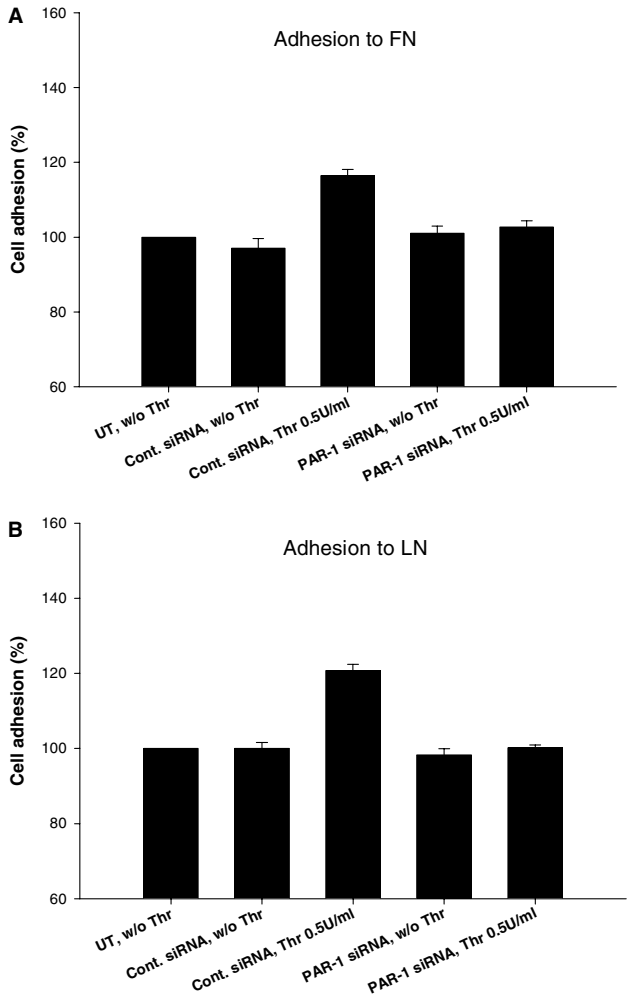

Fig. 5. PAR-1 silencing by RNAi using double transfection abolishes thrombin-induced DU 145 cell adhesion to extracellular matrix components. 72 h post the initial transfection of siRNA, untreated or transfected cells with control or PAR-1 siRNA were harvested in serum-free media and cell adhesion assayed with or without 0.5 U/ml thrombin for 30 min. Cell adhesion to extracellular matrix proteins, fibronectin (FN, A) and laminin (LN, B), was assayed. (UT, untreated; Thr, thrombin; w/o, without).

control level 4–5 days after transfection in mammalian cells [18,23]. Our data demonstrated undetectable PAR-1 mRNA in doubly transfected DU 145 cells up to 5 days after the initial transfection. After 7 days, PAR-1 mRNA re-emerged at a level comparable to that obtained 48 h after conventional transfection, thus indicating more effective gene silencing by double transfection compared to standard protocols. It seems reasonable to suggest that the modified transfection method induces longer persistence of reduced PAR-1 before expression returns to the normal level.

Although no attempts have been undertaken to specifically address the question if non-specific changes are induced in genes other than the target, it should be noted that the double transfection protocol using siRNA at 50 nM concentration improves PAR-1 silencing without causing significant cytotoxic effects for at least 7 days after transfection, thus excluding off-target gene modulation associated with major cytotoxicity or cell death [2].

Adhesion of tumor cells to platelets, vascular endothelium and ECM modulates their invasive and metastatic capacity. Thrombin has been reported to activate tumor cells to express various adhesion molecules and stimulate cellular adhesion to endothelial cells, platelets and ECM components [24]. PAR-1 activation peptide can mimic thrombin-induced adhesion, suggesting that the effect of thrombin is mediated by proteolytic activation of PAR-1 [8,25]. In addition, thrombin enhanced adhesion of B16F10 melanoma cells transfected with full-length PAR-1 sense cDNA to fibronectin compared to mock-transfected cells, demonstrating the importance of PAR-1 in thrombin-induced cell adhesion [26]. Results from the present study indicate that the presence of thrombin for 30 min stimulated prostate cancer cell adhesion to ECM proteins, fibronectin and laminin. This effect seems to be exclusively dependent on the presence of the thrombin receptor PAR-1, since PAR-1 silencing by RNAi completely abolished thrombin's effects on cell adhesion.

In conclusion, our data demonstrate that the novel double transfection method effectively improves gene silencing of thrombin receptor PAR-1 in DU 145 prostate cancer cell line. The absence of PAR-1 abolishes thrombin-modulated cell adhesion to ECM proteins, thus conclusively establishing the role of PAR-1 in mediating thrombin's effect. Highly efficient RNAi-induced gene silencing of PAR-1 provides a novel tool to characterize the complex interaction between thrombin and its receptor in prostate tumor biology. Compared with rather complex attempts to enhance effective inhibition of endogenous gene expression by RNAi especially in adhesive cells, the present study provides a simple alternative by applying a double transfection strategy. Further studies should be performed to evaluate the approach with respect to other cell types and genes under consideration.

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