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Inhibition of invasion and metastasis in oral cancer by targeting urokinase-type plasminogen activator receptor

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## **Abstract**

There have been reports of strong correlations between poor prognosis in various cancers and concomitant expression of urokinase-type plasminogen activator (uPA) and its surface receptor (uPAR). We and others have previously shown that the uPA system plays a significant role in a subset of head and neck squamous cell carcinoma. In the present study, we found that uPAR is required for invasion and metastasis of highly malignant oral cancer cells (OSC-19). Treating OSC-19 cells with antisense oligonucleotides (AS) targeting uPAR resulted in a dramatic decrease of uPAR mRNA expression. Furthermore, pretreatment with AS or siRNA targeting uPAR inhibited progression of OSC-19 cells in experimental models. These results suggest that overexpression of uPAR increases the invasiveness and metastasis of OSC-19 cells, and that uPAR is a promising therapeutic target for regulation of progression of oral cancer.

*Keywords:* Antisense oligonucleotide; small interfering RNA; Urokinase-type

plasminogen activator receptor; Oral squamous cell carcinoma

## Introduction

Several proteolytic enzyme systems, involving plasminogen activators (PAs), matrix metalloproteinases (MMPs) and other enzymes, are thought to be intimately involved in invasion and metastasis of tumor cells [1]. Urokinase-type PA (uPA), which plays an essential role in conversion of PA to active plasmin and proteolysis of extracellular matrix (ECM), has been implicated in cancer cell invasion and metastasis [2, 3]. In particular, the activity of receptor (uPAR)-bound uPA is an important factor in the degradation ECM [4, 5].

In a previous study, we found that cells overexpressing both uPAR and uPA mRNA were more invasive and aggressive in an *in vivo* experimental metastasis model [6]. In immunohistochemical studies, the uPA system has been found to be significantly associated with mode of invasion [7] and secondary regional lymph node metastasis of squamous cell carcinoma (SCC) in the oral cavity [8]. The majority of studies of the uPA system in oral SCC have shown that patterns of expression of the uPA system correlate with metastatic ability of cancer cells or clinicopathological observations. In

the present study, we examined overexpression of uPAR mRNA in OSC-19 cells derived from human oral SCC, and examined the function of uPAR using antisense oligodeoxynucleotides (AS-ODNs) and small interfering RNA (siRNA). We found that uPAR is required for invasion and metastasis of OSC-19 cells in the present *in vivo* models. Additionally, transfection of cells with uPAR siRNA inhibited degradation of fibronectin. These findings provide the first direct, conclusive evidence that reduction of uPAR expression by antisense nucleic acid can effectively inhibit progression of oral SCC.

## **Materials and Methods**

### Cell line and antibodies

OSC-19 cells were previously established from a metastatic tumor in a cervical lymph node of a patient with grade-4C (cordlike type) SCC of tongue. In the

present study, OSC-19 cells derived from human oral SCC were maintained in Eagle's minimum essential medium (EMEM; Nissui Seiyaku, Tokyo, Japan) supplemented with 10% fetal bovine serum (Filtron, Brooklyn, Australia), in an atmosphere of 5% CO<sub>2</sub> and 95% air at 37°C. The anti-actin monoclonal antibody was purchased from Molecular Probes, Inc. (Eugene, OR, USA). Fluorescein (FITC)- and Cy5-linked secondary antibodies were purchased from Molecular Probes, Inc. (Eugene, OR, USA).

## ODNs

An 18-mer ODN phosphodiester was used to inhibit uPAR gene expression [9]. The ODNs were protected against exonuclease cleavage by a double substitution at the 3'-end and 5'-end with phosphorothioate residues, and were purified by high-performance liquid chromatography (JBioS, Asagiri, JAPAN). The AS-uPAR corresponded to nucleotides 44 to 61 of the human uPAR gene cDNA sequence [10]. The controls consisted of sense (S)- and nonsense (NS)-ODNs (same base composition as AS-uPAR, but in random order) for uPAR. The sequence of the NS-uPAR was



5'-CCTGGGTCAGCGCGCTAG-3'. Cells were suspended in EMEM at a density of  $10^6$  cells/ml, and were incubated with medium alone or with 40  $\mu$ M ODNs for 60 min at 37°C. Cell viability exceeding 95% was confirmed by trypan blue exclusion before ODN-treated cells were used in experiments.

#### Reverse transcription (RT)-PCR

RT-PCR analysis was performed using the modifications of Conboy et al. [11]. Briefly, total RNA was extracted from cancer cells using a guanidinium-isothiocyanate-phenol-chloroform-based method (ISOGENE, Nippon Gene, Tokyo, Japan). The prepared RNA was mixed with the oligo-dT primer, incubated for 15 min at 68°C, and then quickly chilled on ice for 5 min. The RNA samples were reverse-transcribed into first-strand cDNA at 42°C for 60 min in RT solution [11]. The cDNA samples were amplified by adding a PCR mixture [11]. The amplification was performed as described previously by Noguchi-Takino et al. [12]. Primers for the uPAR and  $\beta$ -actin genes were used as described by Noguchi-Takino et al. [12]. The lengths for

the amplified fragments of the uPAR and  $\beta$ -actin genes were 455 and 592 bp, respectively.

For quantitation, PCR product values of the uPAR gene, falling within the linearity range, were normalized to that of the  $\beta$ -actin gene, which was used as an internal standard.

#### Orthotopic implantation model

The mode of cancer invasion in a tumor-host relationship closely correlates with clinical course. In a study of SCC of the oral cavity, Yamamoto et al. [7] graded mode of invasion, and showed that grade 4C and 4D (diffuse invasion) had poor prognosis. To evaluate the effect of AS-uPAR on mode of invasion, we injected cells pretreated with ODNs into the submucosa of the oral floor. The present xenograft model is described in detail elsewhere by Kawashiri et al. [13]. Briefly, 6-week-old female BALB/c nude mice (Charles River Japan, Yokohama, Japan) were used. Tumor cells ( $2 \times 10^5$  cells/mouse) were injected into the submucosa of the oral floor using a

microsyringe with a 27-gauge needle. After 7 days, the tumors were excised and examined histopathologically.

In this orthotopic implantation model, the OSC-19 cells deeply invade, forming small cordlike cancer nests, which indicate grade 4C mode of invasion [13].

#### Chick embryo assay

The metastatic ability of OSC-19 cell lines pretreated with ODNs was examined using the chick embryo assay. The assay for metastasized cells was performed as described previously by Endo et al. [14]. Briefly, fertilized chicken eggs were incubated in a forced-air incubator at 37°C in a humidified atmosphere. Tumor cells ( $10^6$  cells/egg) were injected into the choriallantoic membrane (CAM) vein of 10-day-old chick embryos. The embryos were sacrificed 7 days after tumor cell inoculation. The embryonic liver and lung were then dissected, and DNA was extracted from them. A fragment of the human  $\beta$ -globin gene in human tumor cells that metastasized into chick embryonic organs was specifically amplified by PCR. The

amplified fragment was separated electrophoretically in a 1.5% agarose gel. The specific primers for the  $\beta$ -globin gene were Hu $\beta$ -1 (5'-AGAGCCATCTATTGCTTACA-3') and Hu $\beta$ -8 (5'-TATGACATGAACTTAACCAT-3'), and the length of the target fragment was 576 bp [14].

#### siRNA transfection

The 21-nucleotide-long siRNAs were chemically synthesized, desalted, deprotected and purified using polyacrylamide-gel electrophoresis. Two oligoribonucleotides (5'-GGUGAAGAAGGGCGUCCAATT-3' and 5'-UUGGACGCCCUUCUUCACCTT-3') labeled with the fluorescent dye TAMRA at the 5' end were used to inhibit uPAR synthesis (Ambion, Austin, TX, USA). As a control, we used 2 nonsense siRNA oligoribonucleotides: 5'-UUCUCCGAACGUGUCACGUTT-3' and 5'-ACGUGACACGUUCGGAGAATT-3' (Qiagen, Valencia, CA, USA). Cells were transfected with the oligoribonucleotides

using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The transfection was performed overnight on subconfluent cells, with a final siRNA concentration of 40 nM. In each experiment, a series of cells was used for RT-PCR to evaluate inhibition of uPAR expression.

#### *In vitro* fibronectin degradation/invasion assays

We investigated the possibility that uPAR is involved in invasiveness of OSC-19 cells, using the fibronectin degradation/invasion assay with OSC-19 cells transfected with siRNA targeting uPAR. Cells transfected with the oligoribonucleotides were collected by trypsinization, and  $10^5$  cells were added to FITC-fibronectin-coated crosslinked gelatin films on an 18-mm round glass cover slip in a 12-well plate, as described elsewhere [15, 16]. This technique allows us to measure the fibronectin degradation and the invasiveness in terms of foci of invadopodial extensions and surface indentations in the crosslinked gelatin film. Invasive cells locally degrade the FITC-fibronectin-coated matrix, leaving a dark area easily detectable by

immunofluorescence microscopy in the *in vitro* fibronectin degradation/invasion assay, as described in detail elsewhere [15, 16]. After the cells were allowed to grow on the films for the indicated periods of time, they were examined using an MRC-1024 confocal laser-scanning microscope (Bio-Rad, UK). To quantify the degraded area of FITC-fibronectin, we used NIHimage to calculate the area of the black region (30 pixel <) and the black dots (30 pixel >), which indicated fibronectin degradation.

## Statistics

All experiments were repeated 3 times, and similar results were obtained each time. The statistical significance of differences between groups was evaluated using a single sample two-tailed Student's *t* test.

## Results

## Effect of treatment with AS-uPAR on uPAR mRNA level

The pretreatment of OSC-19 cells for 60 min with 40  $\mu$ M AS-uPAR resulted in a dramatic decrease of uPAR mRNA levels (Figure 1). The cells treated with S- or NS-uPAR had the same uPAR/ $\beta$ -actin ratio as the control cells. The cells treated with AS-uPAR had a uPAR/ $\beta$ -actin ratio that was 57% less than that of the control cells.

## Effects of ODNs for uPAR on mode of invasion

In control groups that were untreated or were pretreated with S- or NS-uPAR, all oral floor tumors were Grade 4C. In contrast, in the group pretreated with AS-uPAR, diffuse invasion was inhibited and the tumors were Grade 3; i.e., they consisted of groups of cells without a distinct borderline (Figure 2). In the group treated with AS-uPAR intratumorally on day 3 after the injection of untreated tumor cells, the tumors were the same as in the control groups.

## Effects of pretreatment with ODNs for uPAR on experimental metastases

In control groups, metastasized cells were observed in the chick liver (Figure 3). The pretreatment of OSC-19 cells for 60 min with 40  $\mu$ M AS-uPAR resulted in a dramatic decrease of liver metastases (Figure 4). Liver metastases were not affected by pretreatment with S- or NS-uPAR. In cells treated with AS-uPAR, metastasis was inhibited by 86%. Additionally, AS-uPAR exhibited a significant antitumor effect against lung metastases (data not shown).

## Effect of transfection with siRNA targeting uPAR on in vitro fibronectin degradation/invasion assay

The transfected cells were detectable under immunofluorescence microscopy (Figure 5A), and were morphologically similar to control cells (Figure 5B). Transfection of cells with uPAR siRNA resulted in a dramatic decrease of fibronectin degradation (Figure 6A). Fibronectin degradation was not affected by transfection of nonsense



siRNA. In cells transfected with uPAR siRNA, degradation was inhibited by 77% (Figure 6B).

## **Discussion**

In the present study, we showed that AS-uPAR inhibits overexpression of uPAR in highly malignant oral cancer cells (OSC-19). We also demonstrated that uPAR plays an important role in the progression of oral cancer cells. We observed an especially strong relationship between the uPA system and initial invasion of oral cancer cells.

A specific cell surface receptor for the ligand uPA catalyzes the formation of plasmin from PA, thus generating the proteolytic cascade that contributes to the breakdown of ECM, which is a key step in cancer metastasis [2, 3]. Recent studies by Aguirre-Ghiso et al. have demonstrated signaling via uPAR in cancer cells *in vivo* [17, 18]. They showed that a large reduction in the uPAR level induced a protracted state of

tumor dormancy, but did not affect *in vitro* growth [17]. Further analysis revealed that the overexpressed uPA/uPAR complex interacted strongly with  $\alpha 5\beta 1$  integrin, resulting in powerful and persistent activation of the Mek-ERK pathway upon binding to fibronectin [17, 18]. When uPAR was downregulated or the uPAR- $\alpha 5\beta 1$  interaction was disrupted, the ERK pathway became deactivated, the cell arrested in the G0/G1 phase of the cell cycle *in vivo*, and tumor dormancy was triggered *in vivo* [17, 18]. In the present study, AS-uPAR inhibited invasion and metastasis *in vivo* but did not inhibit *in vitro* growth. This suggests that the same mechanism is at work in the present *in vivo* experimental models and the studies by Aguirre-Ghiso et al.  $\alpha 5\beta 1$  integrin is the fibronectin receptor, and the uPAR siRNA decreased the fibronectin degradation.

Recent reports suggest that regulation of uPAR expression is a viable method of controlling cancer progression *in vivo*. Crowley et al. [19] reported that a mutant uPA molecule that binds to uPAR but lacks enzyme activity inhibited metastasis of PC3 prostate cancer cells in an immunodeficient mouse model. Similarly, blocking uPAR with the epidermal growth factor-like domain of murine uPA suppresses neovascularization and growth of B16 melanomas [20]. Recently, researchers have

developed various agents that target uPAR, including AS-ODN, siRNA, protein and peptide-based drugs [21-23]. These reports suggest that uPAR is a promising therapeutic target for regulation of cancer progression. The present results indicate that our strategy for targeting uPAR is an effective method of inhibiting progression of oral SCC. However, in previous *in vivo* studies, cancer progression was only weakly inhibited by antisense uPAR mRNA. This discrepancy may be due to the activity of other proteinases (including MMPs) that play central roles in the process of cancer invasion and metastasis. OSC-19 cells express high levels of some MMPs (data not shown). Therefore, in addition to reduction of uPAR expression, other strategies may be needed to obtain more effective and reliable inhibition of cancer progression.

The uPAR promoter region is located in a 188-bp region between bases -141 and +47, relative to the transcription start site [3]. Binding of transcription factors (Sp1, AP-2, NF- $\kappa$ B and 2 molecules of AP-1) to the uPAR promoter region activates basal transcription of the gene. NF- $\kappa$ B also induces expression of proteinases (uPA, MMP-1, and -9) [24-26]. Thus, drugs that inhibit activity of NF- $\kappa$ B may inhibit cancer invasion and metastasis. We have previously shown that parthenolide, an active ingredient of

herbal remedies such as feverfew (*tanacetum parthenium*), decreases NF- $\kappa$ B DNA-binding activity and expression of target genes of NF- $\kappa$ B [27, 28]. In studies of the uPAR promoter, Shiratsuchi et al. [29] and Hapke et al. [30] investigated reduction of uPAR expression via methods involving AP-1 and a *PEA3/ets* transcriptional element, respectively. Their results suggest that methods targeting transcriptional elements could be useful complementary therapies together with reduction of uPAR expression.

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## Figure Captions

Fig. 1. Gene expression of uPAR in OSC-19 cells treated with ODNs. Cells suspended in EMEM at a density of  $10^6$  cells/ml were incubated with 40  $\mu$ M ODNs for 60 min at 37°C. An RNA sample from each group was analyzed by RT-PCR. The lengths of the uPAR and  $\beta$ -actin fragments were 455 and 592 bp, respectively.

Fig. 2. Histologic photomicrographs of the oral floor tumor. (A) This tumor exhibited the grade-4C mode of invasion (control group). (B) This tumor exhibited the grade-3 mode of invasion, suggesting reduced invasiveness as a result of antisense therapy (AS-uPAR group). H & E stain. Bar = 0.1 mm.

Fig. 3. Histologic photomicrographs of embryonic liver on day 7 after OSC-19 cell inoculation (control group). H & E stain. Bar = 0.4 mm.

Fig. 4. Detection of metastasized OSC-19 cells in chick embryo liver. Tumor cells ( $10^6$  cells/egg) pretreated with ODNs were injected into the CAM vein of 10-day-old chick embryos. After 7 days, 6 embryos from each group were sacrificed and the liver DNA was extracted. The sample DNA was analyzed by PCR using a primer pair specific to the human  $\beta$ -globin gene.

Fig. 5. Immunofluorescence photomicrographs of control cells and the cells transfected with uPAR siRNA. (A) The transfected cells were red colored (siRNA labeled with the fluorescent dye TAMRA), but control cells were not. (B) The transfected cells were morphologically similar to control cells (blue: actin staining labeled with Cy-5). Bar = 10  $\mu$ m.

Fig. 6. The uPAR siRNA inhibited fibronectin degradation. (A) Transfection of cells with uPAR siRNA resulted in a dramatic decrease of fibronectin degradation (black region). (B) Fibronectin degradation was not affected by transfection with nonsense siRNA, whereas degradation was inhibited in cells transfected with uPAR siRNA.

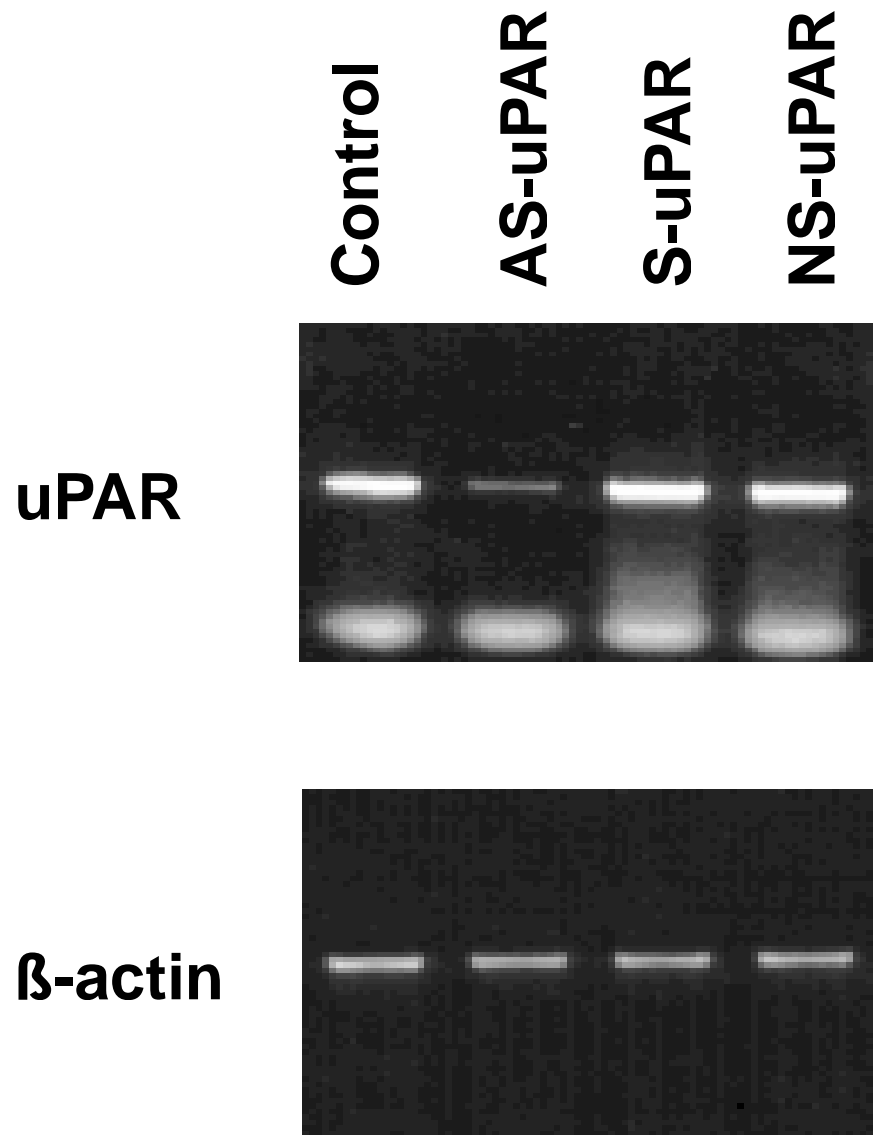


Figure 1

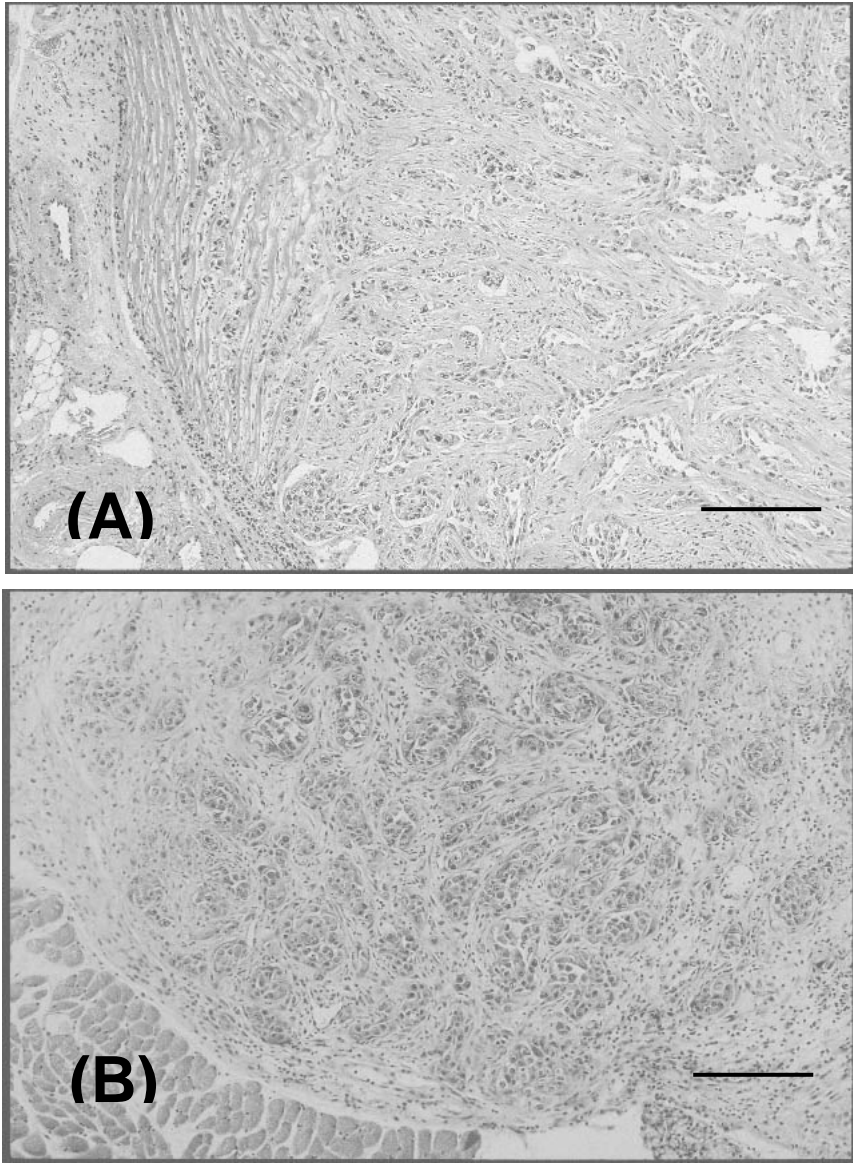


Figure 2

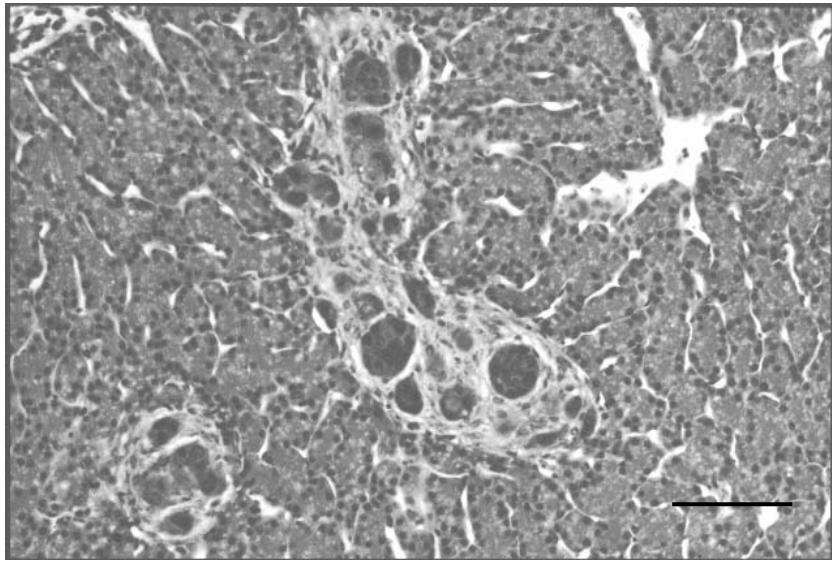


Figure 3

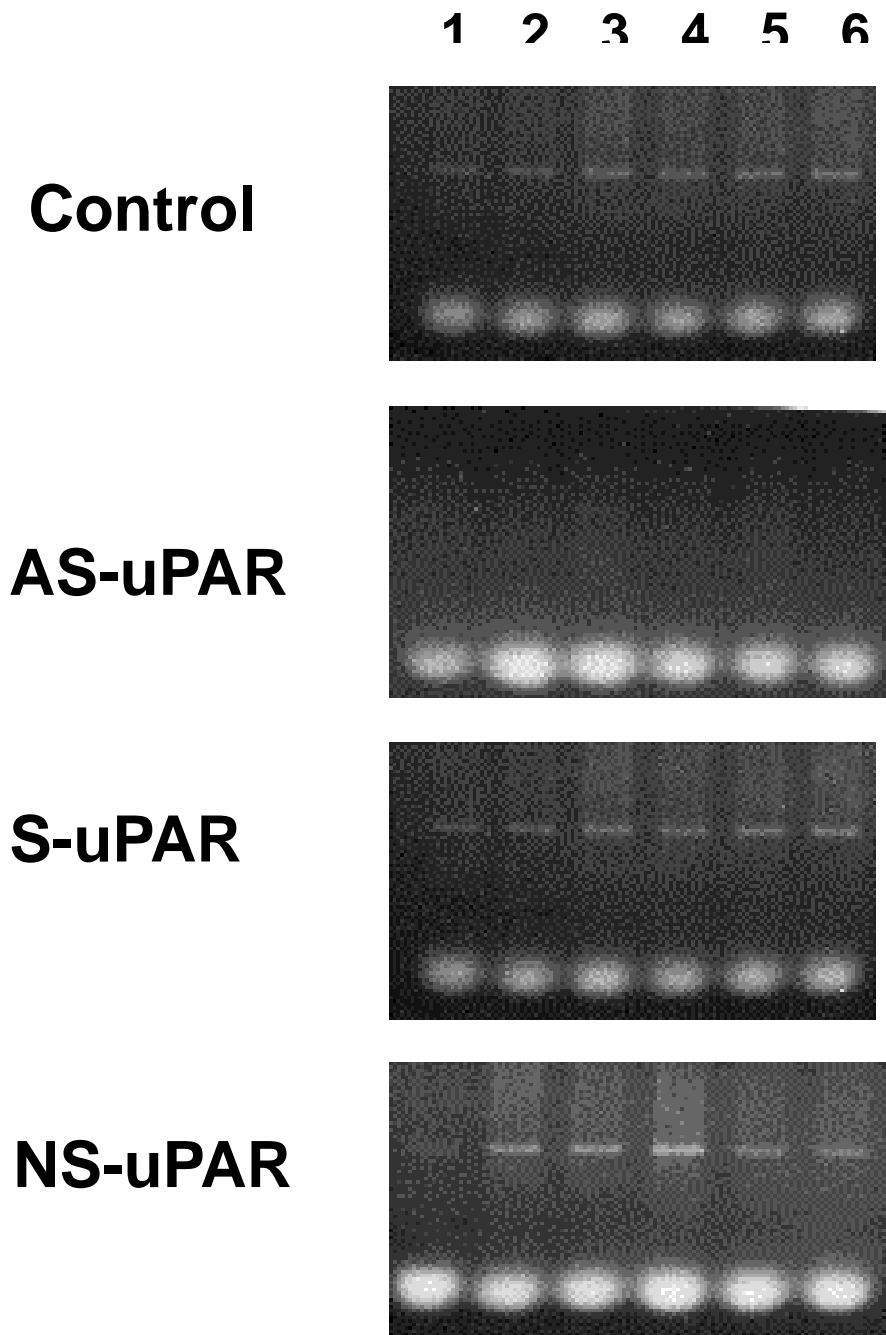


Figure 4



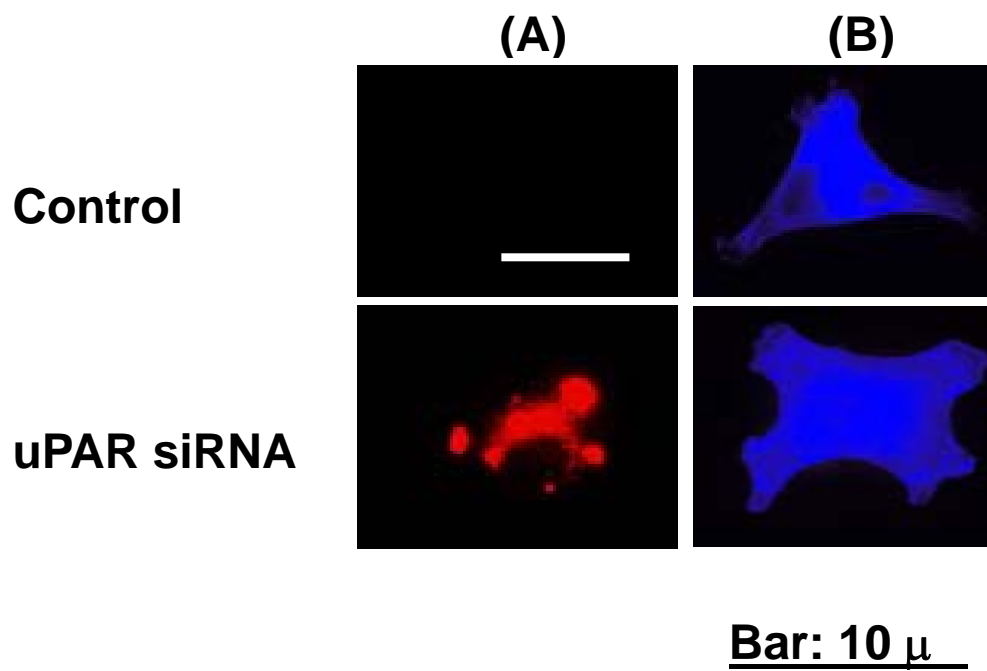


Figure 5

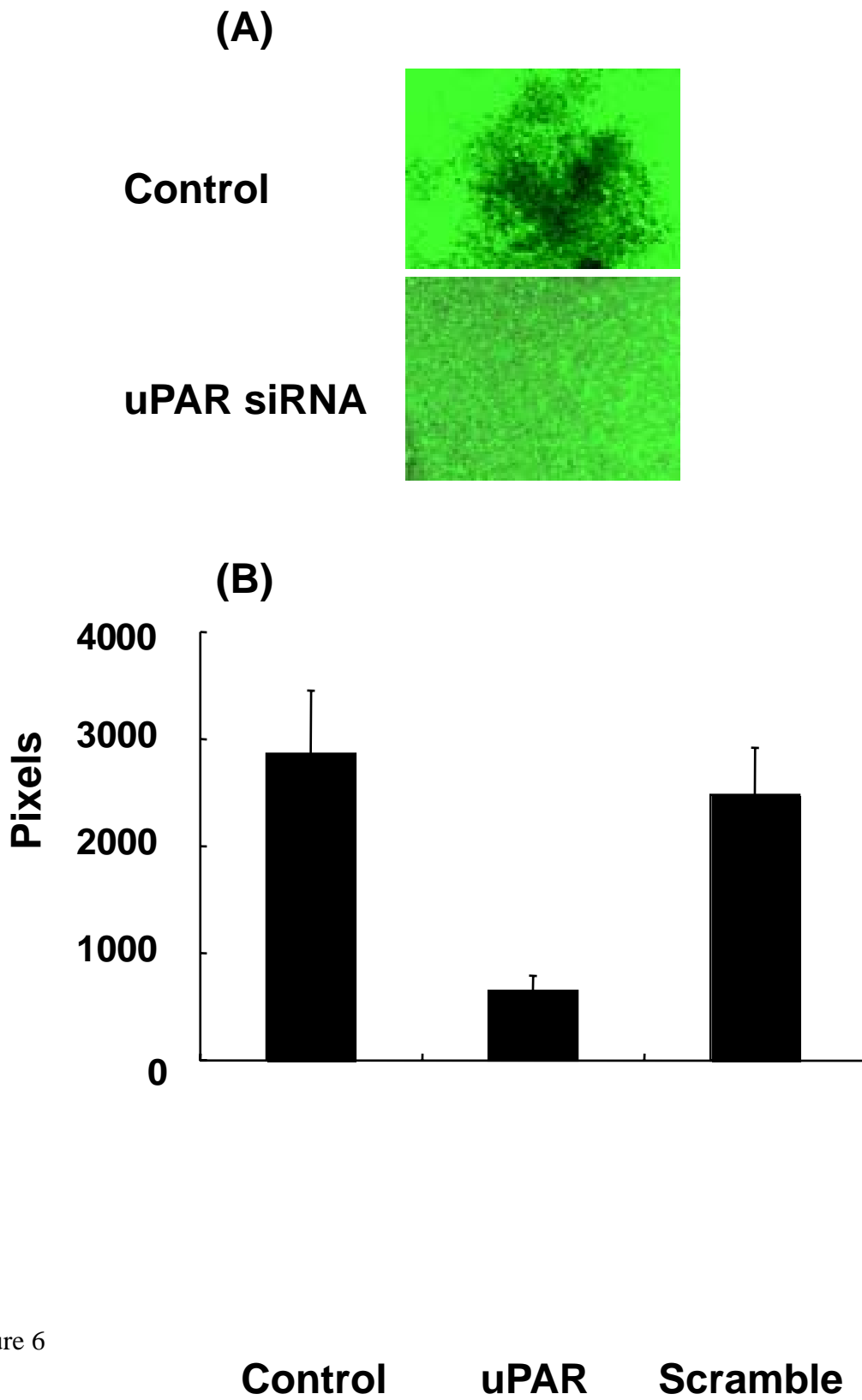


Figure 6