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Original Article

Effect of Human S100A13 Gene Silencing on FGF-1 Transportation in Human Endothelial Cells

Renxian Cao,[1] Bin Yan,[2] Huiling Yang,[2] Xuyu Zu,[2] Gebo Wen,[1]* Jing Zhong[2]*

Background/Purpose: The S100 protein is part of a Ca^{2+} binding protein superfamily that contains an EF-hand domain, which is involved in the onset and progression of many human diseases, especially the proliferation and metastasis of tumors. S100A13, a new member of the S100 protein family, is a requisite component of the fibroblast growth factor-1 (FGF-1) protein release complex, and is involved in human tumorigenesis by interacting with FGF-1 and interleukin-1. In this study, experiments were designed to determine the direct role of S100A13 in FGF-1 protein release and transportation.

Methods: We successfully constructed the lentiviral vectors containing shRNA targeting the human S100A13 gene. Human umbilical vein endothelial cells (HUVECs) were transfected with lentiviral RNAi vectors for S100A13. Then immunofluorescence staining, real-time quantitative polymerase chain reaction and Western blotting were used to detect the inhibition efficiency of the vectors and to monitor the release and transportation of FGF-1 protein.

Results: Lentiviral RNAi vectors induced suppression efficiency of S100A13 gene by 90% in HUVECs. FGF-1 protein was found to be transported from the cytoplasm to the cell membrane, and then released from cells when HUVECs were deprived of serum. The release of FGF-1 protein was blocked by the down-regulation of S100A13, but the transportation was not affected, suggesting that S100A13 is a key cargo protein for FGF-1 release.

Conclusion: S100A13 promotes the release of FGF-1 protein, but does not affect the transportation of FGF-1 protein in HUVECs.

Key Words: FGF-1, lentiviral vector, RNAi, S100A13, shRNA

[1]Department of Pathophysiology, University of South China and [2]Clinical Medical Research Institute of the First Affiliated Hospital of the University of South China, Hengyang, China.

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***Correspondence to:** Dr Gebo Wen, Department of Pathophysiology, University of South China, Hengyang, China.
E-mail: wengb@nhu.edu.cn
OR
***Correspondence to:** Dr Renxian Cao, Department of Pathophysiology, University of South China, Hengyang, China.
E-mail: caorenxian@hotmail.com

The S100 protein family is a Ca^{2+} binding protein superfamily. This family contains an EF-hand domain, which is involved in the onset and progression of a wide range of human diseases, such as cholecystic fibrosis, cardiomyopathy, rheumatic arthritis and tumorigenesis.[1,2] So far, 25 members of this family have been found in humans.[3] S100A13, an acidic, 11-kDa protein, is the only member of the S100 protein family that is widely expressed in various tissues. Compared with other S100 proteins, S100A13 has specific physical and functional characteristics, including broad Ca^{2+} sensitivity, unusual metal-free state and the absence of a surface-exposed hydrophobic patch when in the Ca^{2+}-saturated state.[4] Following analysis of differentially expressed genes, using suppression subtractive hybridization, in normal thyroid tissue and thyroid tumors, we found that S100A13 was highly expressed in thyroid cancer. We therefore constructed a S100A13 eukaryotic expression vector to investigate the function of this protein. Recently, S100A13 has attracted interest for its involvement in non-classical pathway transmembrane transport of fibroblast growth factor-1 (FGF-1) and interleukin-1a, which are involved in angiogenesis, tumor growth, and cell proliferation and differentiation.[5,6]

FGF-1 is a member of the fibroblast growth factor family, which acts as a broad-spectrum mitogen and potent angiogenic agent.[7,8] Thus, FGF-1 appears to play a significant role, not only in normal development and wound healing, but also in tumor development and progression. FGF-1 has also evoked interest as a candidate oncogene as it can potentially initiate and promote tumorigenesis.[7] These biological effects are mediated through the activation of fibroblast growth factor receptors (FGFRs), with the participation of heparan sulfate proteoglycans, and consequently require the release of the polypeptide to produce an action.[9] However, FGF-1 lacks classical signal peptide sequences at the N-terminal, and therefore cannot be secreted via the classical pathway mediated by the endoplasmic reticulum-Golgi complex; it is instead released by novel secretion mechanisms. Some studies have suggested that S100A13, p40Syt-1 and FGF-1 form the release complexes to allow the transmembrane transport of FGF-1 during its release.[10,11] A detailed study of the contribution that S100A13 has on the secretion of FGF-1 is still required.

In this study, we successfully constructed a shRNA lentiviral vector targeting the human S100A13 gene using the Gateway technique. This vector was used to investigate the role of S100A13 in the intracellular translocation and release of FGF-1 induced by serum deprivation stress. These studies were achieved through effective downregulation of the S100A13 gene in human umbilical vein endothelial cells (HUVECs).

Materials and Methods

Cell line and reagents

HUVECs were cultured in high glucose DMEM medium containing 10% fetal bovine serum in a 5% CO_2 incubator at 37°C. BLOCK-iT™ Lentiviral RNAi Expression System, SuperScript™III cDNA reverse transcription kit and Trizol reagent were purchased from the Invitrogen (Carlsbad, CA, USA); the plasmid extraction kit was from Promega (Annadale, NSW, Australia); the BCA protein quantification kit was from KeyGEN Biology Inc. (Nanjing, China); rabbit anti-human S100A13 antibody was a gift from Claus W. Heizmann; rabbit anti-human β-actin polyclonal antibody, goat anti-rabbit secondary antibody labeled with CYC3 fluorescein and goat anti-rabbit secondary antibody labeled with horseradish peroxidase were purchased from Santa Cruz Inc. (Santa Cruz, CA, USA); BlueRanger pre-stained protein marker and fluorescence assay kit were from Hyclone-Pierce (South Logan, UT, USA); Premix Ex Taq kit was from Takara Bio Inc. (Otsu, Shiga, Japan). DMEM medium and fetal bovine serum were purchased respectively from Hyclone Company and Hangzhou Sijiqing Biological Engineering Materials Co. Ltd. (Qingtaimen, Hangzhou, China). Taqman probes and primers for real-time quantitative polymerase chain reaction (PCR) were purchased from Takara Bio Inc.

Generation of the S100A13 shRNA-pENTR/U6 entry vector

We designed and synthesized two complementary DNA oligonucleotides according to the nucleotide sequence of S100A13 reported in GenBank (NM 001024210), and following the designing principles for shRNA given by Invitrogen. Top strand oligo: 5′-CACCGGAAGATGTAATG CACCTTGAC-GAATCAAGGTGC ATTACATCTTCC-3′; Bottom strand oligo: 5′-AAAAGGAAGATGTAATGCACCT-TGATTCGTCAAGGTGCATTACATCTTCC-3′. Top and bottom strand oligos were annealed to generate double-stranded (ds) oligos. The ds oligo was then cloned into the pENTR/U6 entry vector by T4 DNA Ligase, and the resulting entry clone contained the U6-S100A13 RNAi cassette (human U6 promoter + ds oligo + PolIII terminator). The pENTR/U6-S100A13 entry construct was verified by DNA sequencing (Shanghai Sangon Biotech, Shanghai, China).

Construction of the S100A13 pLenti6/ BLOCK-iT expression vector

With the catalysis of LR clonase II, the U6-S100A13 RNAi cassette in pENTR/U6 was transferred into the pLenti6/BLOCK-iT-DEST Vector using the LR recombination reaction to generate the pLenti6-GW/U6-S100A13^{shRNA} expression construct. The pLenti6-GW/U6-S100A13^{shRNA} expression construct was identified by PCR.

Transfection of the lentiviral RNAi vectors for the S100A13 gene into HUVECs

One day before transfection, cells were plated in growth medium without antibiotics at a density allowing 50% confluence for the time of transfection. RNAi vectors for S100A13 were transfected into HUVECs according to the manufacturer's protocol for Lipofectamine 2000. Cells were harvested 72 hours later.

Real-time quantitative PCR analysis of S100A13 gene expression

Total RNA was extracted with Trizol reagent from four groups of cells: (1) the control cells, (2) SR-PSOX lentiviral RNAi vector (generation in our laboratory) transfected cells, (3) S100A13 lentiviral RNAi entry vector transfected cells and (4) S100A13 lentiviral RNAi expression vector transfected cells. cDNA was then synthesized from this RNA using the SuperScriptIII cDNA reverse transcription kit. Taqman probes and primers for the S100A13 and GAPDH genes were used. S100A13 Taqman probe: 5′-CGCTGAGGCTATCCTTCCGGCC-3′; forward primer: 5′-TTCTTCACCTTTGCAAGGC-3′, reverse primer: 5′-GAGAGCCCACATCCTTGAGC-3′. GAPDH Taqman probe and primers were purchased from Takara Inc. A total of 1×10^6 copies of cDNA from normal HUVECs were diluted by a factor of 10 and used to generate standard curves of S100A13 and GAPDH genes. Real-time quantitative PCR reaction buffer was made using the Premix Ex Taq kit.

Western blot analysis for S100A13 protein expression

Total protein was extracted from cells in the four groups (as listed above). Protein concentration was determined using a BCA protein quantification kit. After samples were boiled for 10 minutes, proteins were subjected to SDS-PAGE, and then transferred to PVDF membrane. The membrane was blocked with TBST containing 5% skim milk powder for 2 hours. Rabbit anti-human S100A13 antibody and rabbit anti-human β-actin antibody at a concentration of 1:1000 and 1:400, respectively, were used to probe the membrane, which was incubated overnight at 4°C. The following day, the membrane was exposed to goat anti-rabbit secondary antibody labeled with horseradish peroxidase and incubated at 37°C for 45 minutes. Bands were then visualized using a protein fluorescence detection kit (Hyclone-Pierce).

Indirect immunofluorescence analysis

HUVECs were divided into four groups: (1) control cells, (2) S100A13 RNAi cells, (3) HUVECs deprived of serum for 3 hours and (4) S100A13 RNAi cells deprived of serum for 3 hours. These four groups and the four groups listed earlier were cultured in 12-well plates. The following day, cells were fixed with methanol/glacial acetic acid (3:1 v/v) for

15 minutes, permeabilized with 0.25% Triton + 5% DMSO for 20 minutes, and blocked for 2 hours. Cells were exposed to rabbit anti-human FGF-1 antibody (1:100) and rabbit anti-human S100A13 antibody (1:1000) primary antibodies and incubated overnight at 4°C. The following day goat anti-rabbit secondary antibody labeled with CYC3 fluorescence (1:3000) was added to the cells, and incubated for 45 minutes. The results were observed and photographed using fluorescence microscopy.

Enzyme-linked Immunosorbent assay (ELISA)

Cells were divided into the four groups listed for indirect immunofluorescence. A total of 1×10^5 cells/well were plated into a 12-well plate and grown to 60–70% confluence within 24 hours. Cell culture medium was collected and analyzed according to the manufacturer's instructions given in the ELISA kit. To eliminate the effect of cell number on concentration of FGF-1 in the cell medium, cells in each group were trypsinized, counted and the ELISA value was corrected for cell density.

Co-immunoprecipitation

Cells were lysed in a buffer composed of 50 mM Tris, pH 7.6, 150 mM sodium chloride, 1% nonidet P-40, 10 mM sodium phosphate, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 2 mM phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 10 μg/mL pepstatin. After centrifugation, 50 μg of the clarified cell lysate was incubated with 1 μg of FGF-1 antibody or 1 μg of S100A13 antibody at 4°C overnight, followed by incubation with a slurry of Sepharose protein A/G beads (40 μL) at 4°C for 1 hour with gentle shaking. Beads were collected by brief centrifugation and washed five times with lysis buffer. Proteins were eluted with 50 μL of 100 mM glycine (pH 2.5) and separated on an 8–12% SDS-PAGE, followed by Western blotting as described above. Rabbit IgG was used as a control.

Statistical analysis

These procedures were repeated three times, and data are presented as mean ± standard deviation. Differences among groups were determined using analysis of variance and SNK-q test. A p value of less than 0.05 was considered to be statistically significant.

Results

Downregulation of S100A13 induced by S100A13-specific shRNA

The results of sequencing of shRNA-pENTR/U6-S100A13 entry vector demonstrated that no mutation and deletion occurred in the S100A13 ds oligo insert. Thus, the S100A13 lentiviral RNAi entry vector was constructed successfully. The pLenti6-GW/U6-S100A13^{shRNA} expression construct was identified using PCR, and results showed that the U6 RNAi cassette was transferred correctly from pENTR/U6 into the pLenti6/BLOCK-iT-DEST vector.

mRNA levels of the S100A13 gene were normalized against the housekeeping gene, GAPDH. In control cells and SR-PSOX RNAi cells, mRNA levels of S100A13 were higher and not significantly different from control, suggesting that the mRNA expression of S100A13 in the HUVECs was not inhibited by SR-PSOX shRNA (control shRNA). By contrast, mRNA levels of S100A13 were clearly downregulated in cells transfected with S100A13-specific lentiviral RNAi entry plasmid and expression plasmid. When compared with control and SR-PSOX RNAi cells, the inhibition efficiency reached approximately 90% (Figure 1). These results suggested that the specific shRNA lentiviral entry and expression vector of the S100A13 gene can specifically and efficiently inhibit the mRNA expression of the S100A13 gene in HUVECs.

To further test the inhibitory efficiency of the vectors on protein expression of S100A13 in HUVECs, Western blotting was performed. As shown in Figure 2, the high expression level of S100A13 protein was detected in the control and SR-PSOX RNAi cells, while the expression of S100A13 protein was downregulated in the two S100A13 RNAi groups. After normalization against β-actin, the protein level of S100A13 in cells

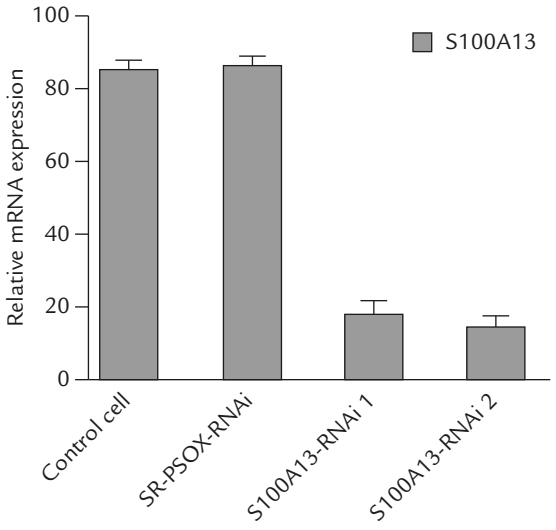

Figure 1. Expression for S100A13 mRNA after S100A13 RNAi knockdown. Normalized S100A13 mRNA expressions are summarized in the bar chart. Control cell = normal human umbilical vein endothelial cells (HUVEC); SR-PSOX-RNAi = SR-PSOX shRNAi transfected cells; S100A13-RNAi 1 = HUVEC transfected with S100A13 entry vector; S100A13-RNAi 2 = HUVEC transfected with S100A13 expression vector.

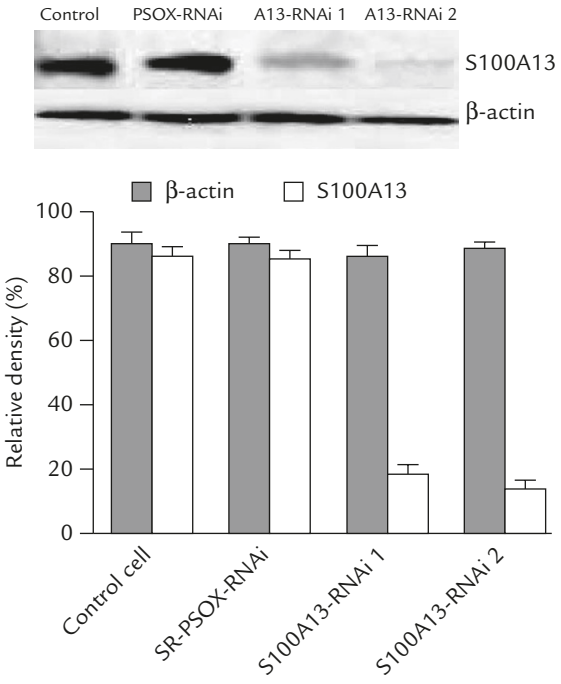

Figure 2. Expression of S100A13 protein after S100A13 RNAi knockdown**.** Control cell = normal human umbilical vein endothelial cells (HUVEC); SR-PSOX-RNAi = SR-PSOX shRNAi transfected cells; S100A13-RNAi 1 = HUVEC transfected with S100A13 entry vector; S100A13-RNAi 2 = HUVEC transfected with S100A13 expression vector.

transfected with S100A13-specifc shRNA lentiviral entry and expression vectors was inhibited with an efficiency of approximately 90%. Our results suggest that the protein level of S100A13 in the HUVECs was not affected by the SR-PSOX RNAi, but inhibited specifically and efficiently by the specific shRNA lentiviral entry and expression vector for the S100A13 gene.

Immunofluorescence staining was also used to determine protein expression of S100A13 (Figure 3). Results showed that S100A13 protein was expressed mainly in the cytoplasm and perinuclear area of the HUVECs, and expression was dramatically inhibited by lentiviral RNAi entry and expression vectors for the S100A13 gene, but no impact was observed using SR-PSOX RNAi.

Serum-deprivation induced release of FGF-1 was blocked by S100A13 gene silencing

It was revealed that FGF-1 was evenly distributed in the cytoplasm, especially in the perinuclear region (Figure 4A), and S100A13 RNAi did not affect this distribution (Figure 4B). After cells were serum-deprived, FGF-1 was distributed mostly adjacent to the cell membrane but not the perinuclear region, and the protein level of FGF-1 in cells was evidently decreased compared with the untreated cells (Figure 4C). Cells treated with S100A13 RNAi and serum-deprivation, FGF-1 protein was similarly redistributed, from a diffuse cytosolic pattern to an area near the inner surface of the plasma membrane, but protein level in cells was not significantly changed compared with control cells (Figure 4D). We further collected the cell culture medium of the four groups to perform ELISA (Table and Figure 5). The protein concentration of FGF-1 in cell medium in control and S100A13 RNAi cells was very low, while in cells deprived of serum, FGF-1 was obviously increased. When S100A13 gene was downregulated by RNAi, the serum-deprivation induced release of FGF-1 was blocked, and the protein concentration of FGF-1 in the cell medium was very low and almost equal to the control and S100A13 RNAi cells. To uncover the mechanism underlying S100A13

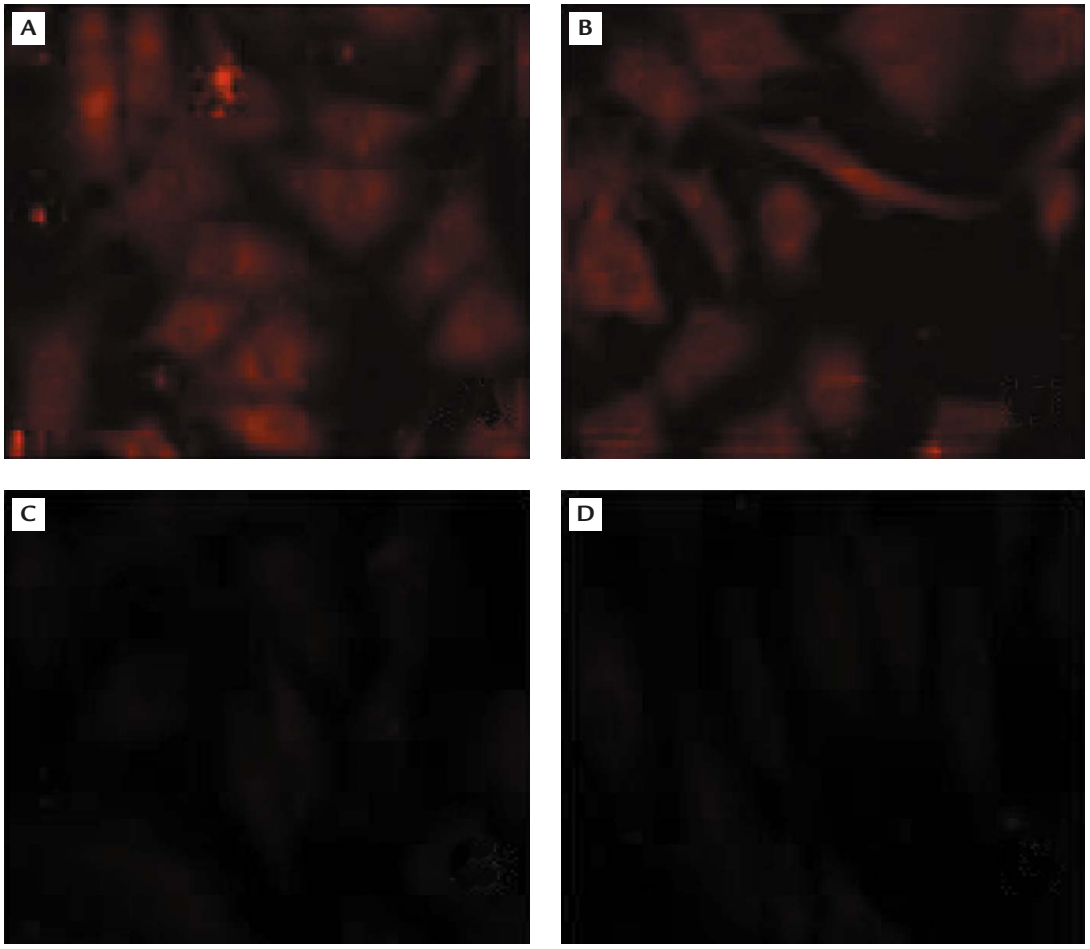

Figure 3. Immunofluorescence staining of S100A13 protein after S100A13 RNAi knockdown. (A) Control human umbilical vein endothelial cells (HUVEC); (B) SR-PSOX shRNAi transfected HUVEC cells; (C) HUVEC transfected with S100A13 entry vector; and (D) HUVEC transfected with S100A13 expression vector.

knockdown induced block of FGF-1 release from the cellular membrane, co-immunoprecipitation between S100A13 and FGF-1 was performed in HUVECs infected with S100A13 RNAi and SR-PSOX RNAi lentivirus, respectively. As shown in Figure 6, no physical interaction was observed between S100A13 and FGF-1 in both S100A13 RNAi and SR-PSOX RNAi lentivirus infected HUVECs. These results suggested that S100A13 gene silencing did not affect the redistribution of FGF-1 protein, but may have blocked serum-deprivation induced FGF-1 release from the plasma membrane.

Discussion

The S100 protein family is the largest protein superfamily with an EF-hand domain. This family is involved in many biological processes, including the cell cycle, cell differentiation and oncogenesis, through their regulation of the interaction with Ca^{2+} and a target protein.[12–14] Many members of the S100 family have abnormal expression in tumors and are involved in the metastasis of tumors. S100A4, S100A6, S100A7 and S100B, for example, are overexpressed in a variety of tumors and are associated with an increase in malignant tumor cells and tumor metastasis.[15] S100A13 is a new member of the S100 gene family, but its exact role in many biological activities remains to be determined. Specifically, the function of S100A13 in the non-classical (non-vesicular) release route of FGF-1 is currently being investigated. FGF-1, as a potent mitogen and angiogenic protein, has attracted increasing attention as a participant in the initiation, development and metastasis of tumors.[16–18]

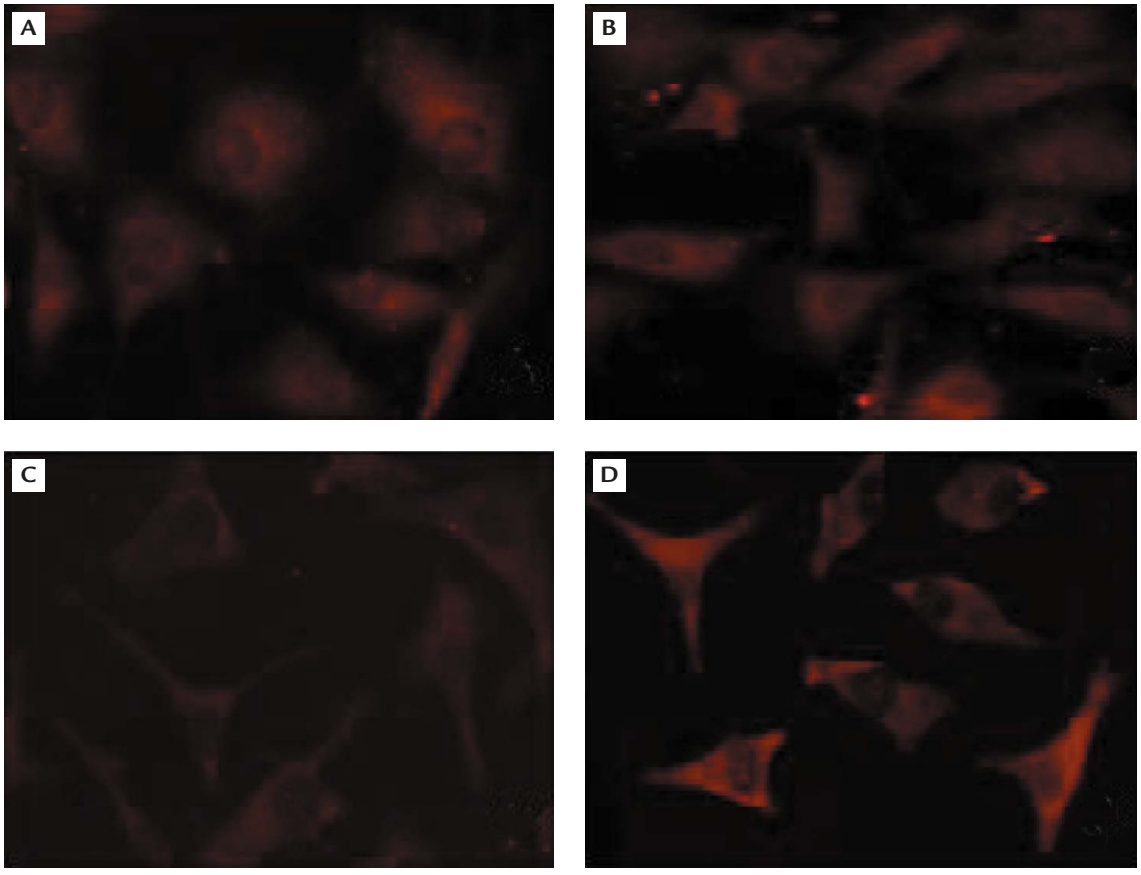

Figure 4. Immunofluorescence staining of FGF-1 subjected to serum deprivation for 3 hours. (A) Normal human umbilical vein endothelial cells (HUVEC); (B) S100A13 RNAi transfected HUVEC; (C) Serum-deprived HUVEC; and (D) Serum-deprived S100A13 RNAi transfected HUVEC.

Table. Protein concentration of FGF-1 in culture medium of human umbilical vein endothelial cells (HUVEC)

	Normal HUVEC	S100A13 RNAi transfected HUVEC	Serum-deprived HUVEC	Serum-deprived S100A13 RNAi transfected HUVEC
Concentration (pg/mL)	26.97 ± 2.39	28.98 ± 1.79	121.79 ± 5.42	26.87 ± 2.71

To produce a biological effect, FGF-1 needs to be released from cells. FGF-1 lacks conventional signal sequencing in the amino terminal, and is therefore released via the ER-Golgi-independent non-classical route, which requires the association of FGF-1 with S100A13 and p40Syt-1.[19,20] This observation implies that RNAi targeted at the S100A13 gene can block the release of FGF-1, thus inhibit the development of tumors and the angiogenesis in tumors.

RNAi, a sequence-specific post-transcriptional silencer mediated by dsRNA, is an important protective mechanism against transposon and virus transfection, and also a means for regulating the expression of endogenous genes in the process of growth and development.[21] As a simple and effective alternative for the gene knockout technique, RNAi has been widely used in experimental research and clinical practice. In this study, we used lentiviral vector mediated shRNA to suppress the expression of the S100A13 gene. The whole S100A13 gene was analyzed with specialized design software for shRNA, and gene 215–235bp was selected as our intervention target. 42.86% in G/C, CAAC (5′ end), AAAA (3′ end), CGAA hairpin loop and TTCG antisense hairpin loop were

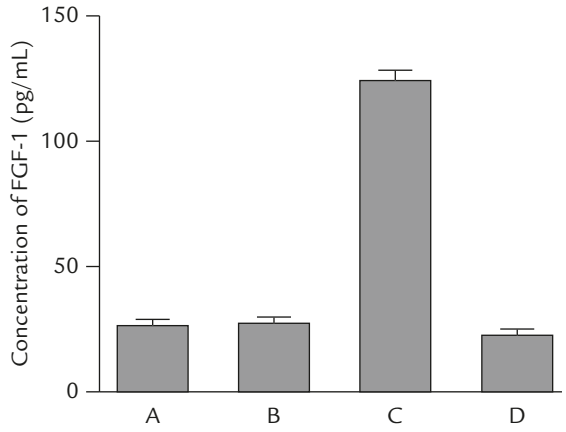

Figure 5. Enzyme linked immunosorbent assay of the FGF-1 protein in culture medium. (A) Normal human umbilical vein endothelial cells (HUVEC); (B) S100A13 RNAi transfected HUVEC; (C) Serum-deprived HUVEC; and (D) Serum-deprived S100A13 RNAi transfected HUVEC.

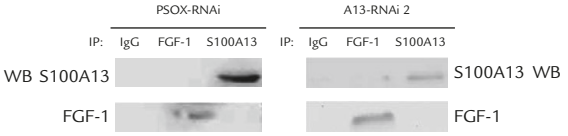

Figure 6. Co-immunoprecipitation of S100A13 and FGF-1 in human umbilical vein endothelial cells. IgG was used as a control. IP = Immunoprecipitation; WB = Western blot.

added to the two complementary single-stranded shRNA for cloning. The pENTR/U6 entry vector provided a rapid and efficient way to add dsDNA into a vector containing a RNA PolIII-dependent expression cassette for use in RNAi analysis. Using the Gateway technique the U6 RNAi cassette is easily recombined into the DEST vector, which then generates replication-incompetent lentivirus to silence the target gene. After construction of the S100A13 shRNA expression vector, the interference effect on the S100A13 gene was observed using RT-PCR, Western blotting and immunofluorescence staining. The results suggested both the entry vector and the expression vector of the S100A13-shRNA could downregulate the expression of S100A13, with an interference efficiency of 90%. The construction of this S100A13-shRNA lentiviral vector is essential for the further investigation into the biological effects of S100A13.

Maciag et al first demonstrated that S100A13 and p40 synaptotagmin-1 can be co-purified from brain homogenates with FGF-1, and further demonstrated that heat shock stress causes a co-release of FGF-1, S100A13 and Syt-1 from NIH3T3 cells.[19,20] In the present study we attempted to examine the effect of the S100A13 gene on serum deprivation induced release of FGF-1 by the shRNA silencing of the S100A13 gene. Our results suggested that FGF-1 was expressed in endothelium cells in normal culture condition, and is diffusely distributed in the cytosol, which is consistent with other studies.[22] Under normal conditions, cells do not release FGF-1 protein. Several stresses, however, such as heat shock, serum starvation, hypoxia and low-density lipoprotein treatment, can induce the release of FGF-1.[23] Our study demonstrated a change in location of FGF-1 following serum deprivation with FGF-1 mostly localizing in the inner surface of the cell membrane, and not the perinuclear region of the cytosol. Furthermore, FGF-1 almost disappeared from the cells after the endothelium was subjected to serum deprivation stress. Some researchers have suggested that FGF-1 is connected with S100A13, p40Syt-1 and Cu^{2+} to form multiprotein release complexes, which allows the transport of FGF-1 to the cell medium.[24] Although biochemical evidence suggests that the formation of a multiprotein release complex containing S100A13 and p40Syt-1 is important for the release of FGF-1, the role of the S100A13 gene in the process of FGF-1 release is still unclear. We attempted to determine the involvement of S100A13 in serum-deprivation induced FGF-1 release through silencing the expression of the S100A13 gene by using specific S100A13 shRNA. We observed that S100A13 RNAi did not affect the expression and distribution of FGF-1 in the control and serum-deprived cells, but blocked serum-deprivation induced FGF-1 release from the inner surface of the plasma membrane to the cell medium. Co-immunoprecipitation results showed that no interaction occurred between S100A13 and FGF-1 in HUVECs, indicating an indirect manner by which S100A13 functions as a cargo protein of FGF-1. These findings suggest

that S100A13 RNAi may block the release process for FGF-1. S100A13 may be a key cargo protein in the final export mechanism in the non-classical release of FGF-1 during serum-deprivation stress. Further functional studies investigating the mechanisms involved, and the role of S100A13 in these processes, are needed.

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