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VDUP1 POTENTIATES RAS-MEDIATED ANGIOGENESIS VIA ROS PRODUCTION IN ENDOTHELIAL CELLS

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Abstract – Vitamin D3 up-regulated protein 1 (VDUP1) is a tumor suppressor of which expression is reduced in a variety of cancer cells, and enforced expression inhibits the tumor cell proliferation. It inhibits the activity of thioredoxin, thus contributing cellular ROS generation. Since ROS is a critical factor for angiogenesis, we investigated the role of VDUP1 in angiogenesis and endothelial proliferation. The expression of VDUP1 was upregulated by overexpression of an oncogene, Ras. Enforced expression of VDUP1 increases ROS production and proliferation of Ras-overexpressing endothelial cells. Overexpression of VDUP1 increases the resistance to the anchorage-dependent cell death and tube formation of the Ras-overexpressing endothelial cell. In addition, the removal of ROS by ROS scavenger attenuates the effect of VDUP1 on tube formation. These results suggest that VDUP1 is involved in Ras-mediated angiogenesis via ROS generation in endothelial cells.

Key words: Angiogenesis, VDUP1, ROS, Anoikis, Ras.

INTRODUCTION

Angiogenesis is a cellular process involving the growth of new blood vessels from pre-existing vessels. This process is a normal process for growth and development to keep the supply of oxygen to tissues at a desirable level. However, this is also a critical step for the malignant tumorigenesis or metastasis (28).

Abbreviations: NAC, N Acetyl L Cysteine; **ROS,** Reactive oxygen species; **Trx,** thioredoxin; **VDUP1,** Vitamin D3 up-regulated protein 1; **ASK-1,** Apoptosis signal-regulating kinase 1; **PAG,** Proliferation-associated gene; **DMEM,** Dulbecco's Modified Eagle's Medium; **FBS;** Fetal bovine serum.

In addition, abnormal regulation of angiogenic process may cause certain diseases such as diabetes, macular degeneration, and rheumatoid arthritis, where too many blood vessels can lead to tissue death (1, 4, 26, 29).

Ras and Ras-related proteins are often involved in cancers, leading to increased invasion and metastasis and decreased apoptosis (5, 13, 18, 21). In addition, Ras has been known as a potent angiogenic oncogene that induce angiogenesis via reactive oxygen species (ROS) (15). ROS generated by Ras induces the expression of VEGF in endothelial cells (26), which in turn directly regulates the proliferation of endothelial cells and angiogenesis (7, 22, 27).

Vitamin D3 up-regulated protein 1 (VDUP1) is a redox regulator that is involved in ROS generation by inhibiting thioredoxin (TRX) activity (8, 30). TRX regulates the protein-nucleic acid interactions through the redox regulation of cysteine residues (23). VDUP1 directly interacts with redox active domain of TRX, and blocks the reducing activity of TRX and as well as interactions between TRX and other factors such as ASK-1 and PAG (14, 23). Because TRX system regulates cellular redox by scavenging intracellular ROS, VDUP1 functions as a negative regulator of TRX, thus increasing the intracellular level of ROS.

In this study, we investigated the regulation of VDUP1 expression in endothelial cells by oncogenic Ras and its role in angiogenesis. Unexpectedly, we found that VDUP1, which has been known as a tumor suppressor, induced proliferation of endothelial cells and functioned as an angiogenic factor via ROS generation. Although ROS often functions as a toxic agent of the cells, recent studies revealed that ROS-inducible oxidative stress is a critical factor for many cellular phenomena including proliferation, cell-cycle arrest, and apoptosis (2, 19, 31). In addition, ROS plays important roles in tumorigenesis and angiogenesis (6). In fact, VDUP1 plays diverse roles in ROS-mediated cellular processes depending on the cell types (16). Our novel findings will add further insights into the functions of VDUP1, contributing to a new effective strategy in anti-cancer therapy and cure of cardiovascular diseases.

MATERIALS AND METHODS

Antibodies, Cell lines and transfection

Pancreatic islet endothelial cell lines, MS-1 and SVR were purchased from ATCC (ATCC CRL-2279 and 2280, respectively). SVR is a derivative of MS1, transduced with a retrovirus encoding H-*ras* (3, 20). These endothelial cell lines were maintained in DMEM media supplemented with 5% FBS and the appropriate antibiotics.

For overexpression of VDUP1 in endothelial cells, murine full-length cDNA of VDUP1 was amplified by RT–PCR, inserted with C-terminal HA tag to *Eco*RI and *Xho*I sites of pcDNA3.1(+) (Invitrogen, Carlsbad, CA, USA), and transiently transfected to MS-1 or SVR cell lines using Lipofectamine™ Reagent (#50470, Invitrogen) following the manufacturer's protocol. For Western blot analysis of VDUP1 expression, anti-VDUP1 antibody (Code No. K0204-3) was purchased from MBL international corporation (Woburn, MA, USA).

RT-PCR analysis

Total cellular RNA was extracted using TRIzol®Reagent (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's instructions. Aliquots (3 μg) of total RNA were transcribed into cDNA at 37°C for 1 h in a total volume of 20 μl with 2.5 U of Moloney murine leukemia virus reverse transcriptase (Roche). Reverse-transcribed cDNA samples were added to a PCR mixture consisting of 10 × PCR buffer, 0.2 mM dNTP, 0.5 U Taq DNA polymerase (Takara, Tokyo, Japan), and 10 pmol of primers for each gene. The primer sequences were as follows: VEGF, 5'-ACCTCACCAAAGCCAGCACA-3' and 5'-GGCGTGGTGGTGACATGGTT-3', VDUP1, 5'-CATGAGGCCTGGAAACAAAT-3' and 5'-GCCATTGGCAAGGTAAGTGT-3', mouse β actin, 5'-GTGGGCCGCTCTAGGCACCAA-3' and 5'-CTTTGATGTCACGCACGATTTC-3'. Amplifications were conducted with 25 cycles for β-actin and at 30 cycles for the others.

Measurement of ROS

Cells were incubated with 20 μM of 2′,7′-dichlorofluorescein diacetate (H2-DCFDA) for 30 min, washed with PBS, trypsinized and collected in 1 ml of PBS, and subjected to fluorescence-activated cell sorting (Beckton Dickinson FACScan).

Proliferation assay

Endothelial cells were seeded at 2 x 10^3 cells/well into a 96-well plate, then incubated for 24 h at 37°C in a humidified atmosphere of 5% CO_2. Cells were washed twice with PBS, then exposed for 6 h to HuMedia-EB2 containing 2% FBS. Cell proliferation was estimated by measuring cell metabolic activity using a Cell Counting Kit-8 (CCK-8) according to the manufacturer's protocol (Dojindo, Kumomato, Japan).

Anoikis Experiments

A 2-ml volume of growth medium containing 0.66% Bacto agar (Difco) was poured into a 6-well dish to form the bottom layer. A total of $1x10^4$ cells were suspended in 2 ml of medium containing 0.4% agar, and this suspension was overlaid on the hardened bottom layer. A 1-ml volume of fresh medium containing 0.4% agar was added to the dish every week. Colonies were visualized after 3 weeks of incubation.

Tube formation assay

For tube formations assay, 48 well culture plates were coated with 200μl of Matrigel™ basement Membrane Matrix (Cat. No. 356234, BD Biosciences) per wells, then allowed to polymerize for 20 min at 37°C. Cells were seeded on coated plates at a density of $2x10^5$ cells per well in 500μl DMEM medium containing 10% FBS at 37°C. Endothelial tube formation was observed after 9 hours.

Statistical analysis

Values are expressed as mean ± SEM. Statistical nonhomogeneity was tested with Student's unpaired *t*-test. A p value of <0.05 was considered significant.

RESULTS

Oncogenic Ras induces VDUP1 expression in endothelial cells

Ras has been known as an angiogenic factor that induces ROS generation and proliferation of angiogenic endothelial cells (15), and VDUP1 is a potent inducer for cellular ROS

(23, 24, 32). Thus, to investigate the relationship between Ras and a redox regulator VDUP1, we measured the expression level of VDUP1 in Ras-overexpressing endothelial SVR cells. RT-PCR and immunoblot analysis showed that VDUP1 expression is significantly increased in SVR cells both in mRNA and protein levels (Fig. 1 A and B, respectively). To investigate the role of VDUP1 in Ras-mediated ROS generation in endothelial cells, we next measured the effect of VDUP1 in ROS production in Ras-activated endothelial cell line (SVR). FACS analysis showed that the level of ROS in the Ras-activated endothelial SVR cells was higher than normal endothelial cells (MS-1). In addition, overexpression of VDUP1 further increased ROS level in SVR (Fig. 1C). These results suggested that the Ras-mediated ROS production in endothelial cells is, at least partially, due to the increased expression of VDUP1 induced by Ras.

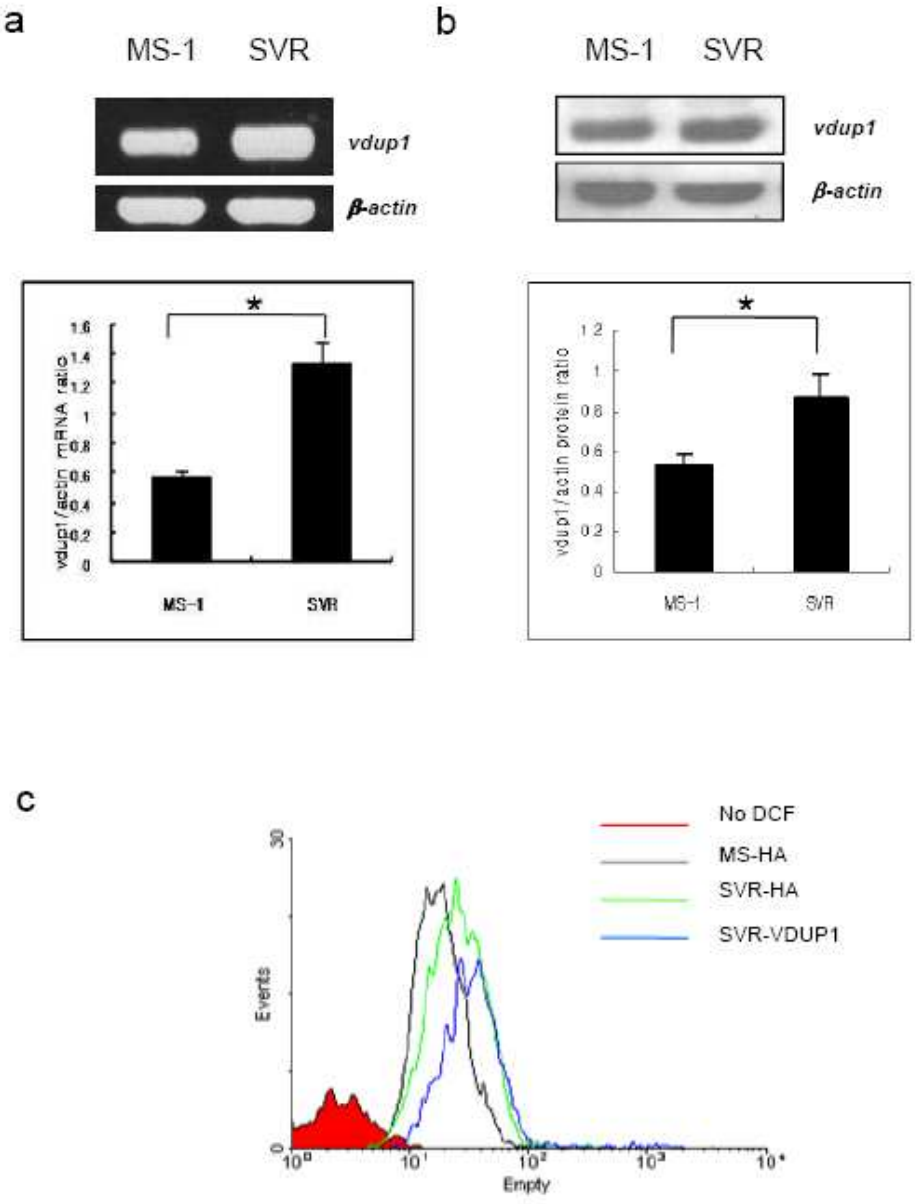


Figure 1. Level of VDUP1 expression and ROS in Ras-overexpressing endothelial cells. (A) Total RNA was isolated from normal (MS-1) and Ras-overexpressing (SVR) pancreatic islet endothelial cell lines, and the expression of VDUP1 was determined via RT-PCR. (B) Protein level of VDUP1 in each cell line was determined by immunoblot. The results are representative of three individual experiments. The levels of mRNA and proteins were quantified by densitometric analysis normalized to actin (*p<0.05). (C) To investigate the effect of VDUP1 on Ras-mediated ROS generation, SVR cell line was transfected with pcDNA3.1-VDUP1 (SVR-VDUP1), and generation of ROS was measured by H_2DCFDA technique. For control, MS-1 and SVR cell lines were transfected with pcDNA3.1. One representative experiment of three is shown.

Effect of VDUP1 in VEGF expression and proliferation of Ras-overexpressing endothelial cells

Ras has been previously known to induce VEGF expression via ROS generation (15). Thus, we next investigated the effect of VDUP1 on VEGF expression in endothelial cells. RT-PCR analysis showed that VEGF expression was significantly increased by VDUP1 in endothelial cells (MS-1), and the induction of VEGF by VDUP1 was further increased when Ras was overexrpessed (SVR) (Fig. 2A, B). VEGF is a potent angiogenic factor that induces proliferation of endothelial cells. Since VDUP1 increased VEGF expression in endothelial cells, we next investigated the effect of VDUP1 on endothelial proliferation. CCK-8 assays showed that enforced expression of VDUP1 was able to increase the proliferation of endothelial cells, and the effect was further emphasized in Ras-overexrpessing endothelial cells (SVR) (Fig. 2C), that is coincident with the expression of VEGF (Fig. 2A). Taken together, these results suggested that VDUP1 plays a critical role in Ras-mediated endothelial proliferation via induction of VEGF expression.

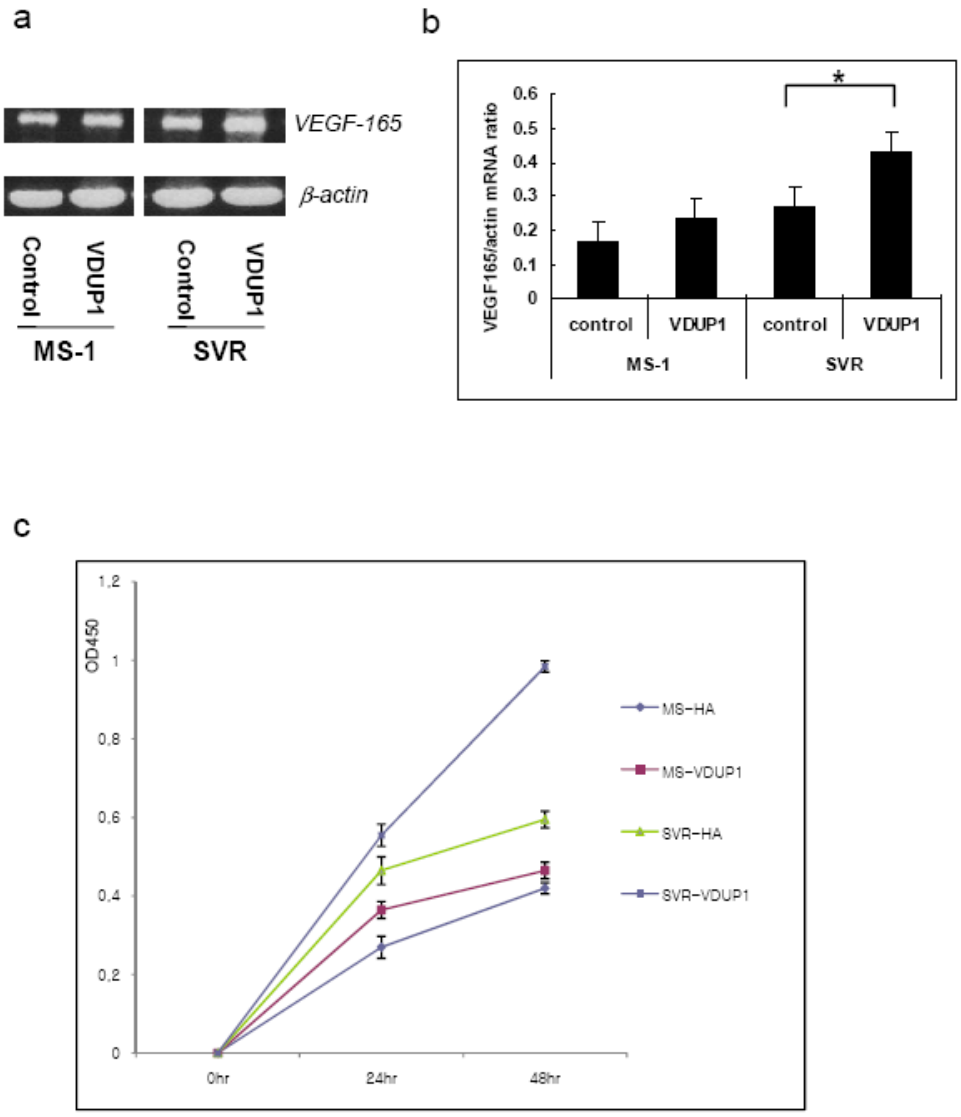


Figure 2. VDUP1 increases VEGF expression and proliferation of Ras-activated endothelial cells. (A) MS-1 or SVR cell lines were transfected with pcDNA3.1-VDUP1 or vector controls, and the expression of VEGF variants were analyzed by RT-PCR. Expression of VEGF-165 is shown as a representative of VEGF isoforms. β-actin was used as a control. One representative experiment of three is shown. (B) mRNA levels of VEGF-165 band was determined by densitometric analysis normalized to actin (*p<0.05). (C) Cellular proliferation was measured by using the cell counting kit (CCK-8). Note that overexpression of both Ras and VDUP1 synergistically increased rate of cell proliferation.

VDUP1increases proliferation and inhibits the anoikis of Ras-overexpressing endothelial cells

VEGF has been reported to inhibit the anchorage-dependent apoptosis (anoikis) of endothelial cells (11). Thus, we next performed soft agar assay to investigate the effect of VDUP1 on anoikis of the endothelial cells. As expected from the previous reports (15, 17, 18, 21), the results showed that anoikis of the endothelial cells was significantly reduced when the Ras was overexpressed. Interestingly, the survival rate of the SVR cell line was further increased by the enforced expression of VDUP1 (Fig. 3). These results suggested that VDUP1 regulates not only the Ras-mediated endothelial proliferation but also the survival of the endothelial cells, via induction of VEGF that is a critical factor for angiogenesis.

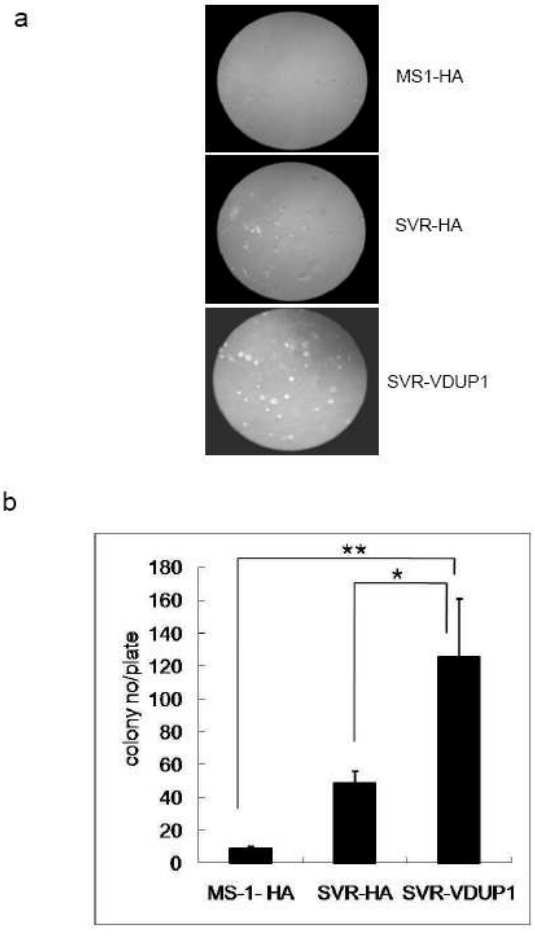


Figure 3. VDUP1 inhibits anoikis of Ras-overexpressing endothelial cells. MS-1 and SVR cell lines were transfected with pcDNA3.1-VDUP1 or vector control (HA), and single cell suspensions were seeded into soft-agar and photographed at ×50 magnification (left) and the number of the colonies were counted (right) 1 week later. Representative figures (A) and statistical analysis (B) from three individual experiments are shown (*p<0.05, **p<0.01).

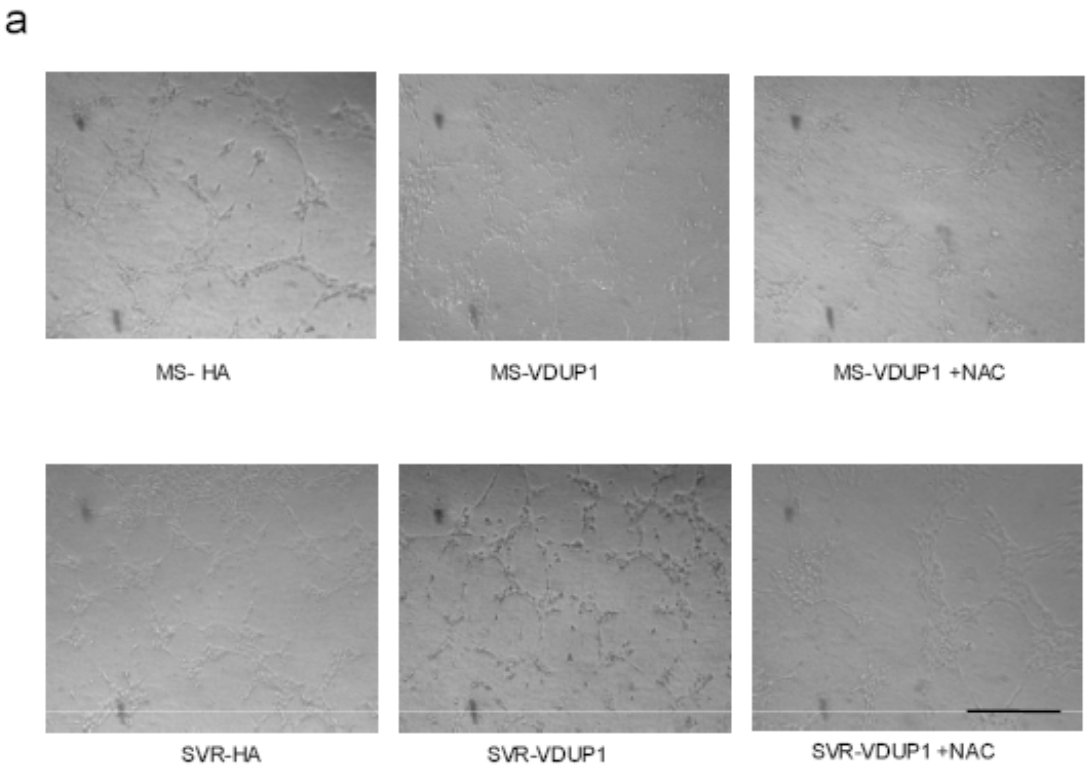
VDUP1 enhances the tube formation of Ras-overexpressing endothelial cells via ROS generation

Next, to investigate the direct involvement of VDUP1 in angiogenesis, we performed tube formation assays with MS-1 and SVR cell lines in the absence or presence of VDUP1 overexpression. In consistent with proliferation or survival patterns (Fig. 2B and 3), overexpression of VDUP1 prominently induces the tube formation of Ras-overxpressing SVR, while VDUP1 moderately increase tube formation in normal endothelial MS-1 (Fig. 4). Furthermore, the effect of VDUP1 on endothelial tube formation was inhibited by the addition of ROS scavenger, N Acetyl L Cysteine (NAC) (Fig. 4). These results strongly suggested that VDUP1 plays an important role in Ras-mediated angiogenesis via ROS generation in endothelial cells.

DISCUSSION

Ras is an oncogene that is involved in the cellular proliferation and tumorigenesis (13, 5, 9, 25, 33, 12, 10). In endothelial cells, Ras functions as a potent angiogenic factor that promotes the proliferation of endothelial cells via ROS generation (31). In this study, we first found that VDUP1 is upregulated by overexpression of Ras in endothelial cells. Since VDUP1 is a redox regulator that increases cellular ROS (8, 30), the fact that the expression of VDUP1 is induced by Ras may imply the involvement of VDUP1 in Ras-mediated ROS generation in endothelial cells. In addition, ROS has been reported to induce VEGF expression in endothelial cells. Thus, increased level of ROS by VDUP1 in endothelial cells may affect the proliferation of endothelial cells or angiogenesis. In fact, enforced expression of VDUP1 increases ROS generation and VEGF expression in endothelial cells, leading to the increased cellular proliferation. The induction of VEGF expression by VDUP1 was maximal when the Ras was overexpressed in endothelial cells, suggesting that VDUP1 and Ras may be functionally interrelated in endothelial cells. Indeed, VDUP1 and Ras exhibited synergistic effect on proliferation of endothelial cells.

Ras has been reported to suppress anoikis of endothelial cells (21) to which resistance is a prerequisite for the development and progression of cancers or angiogenesis. With the similar pattern of proliferation, VDUP1 synergistically



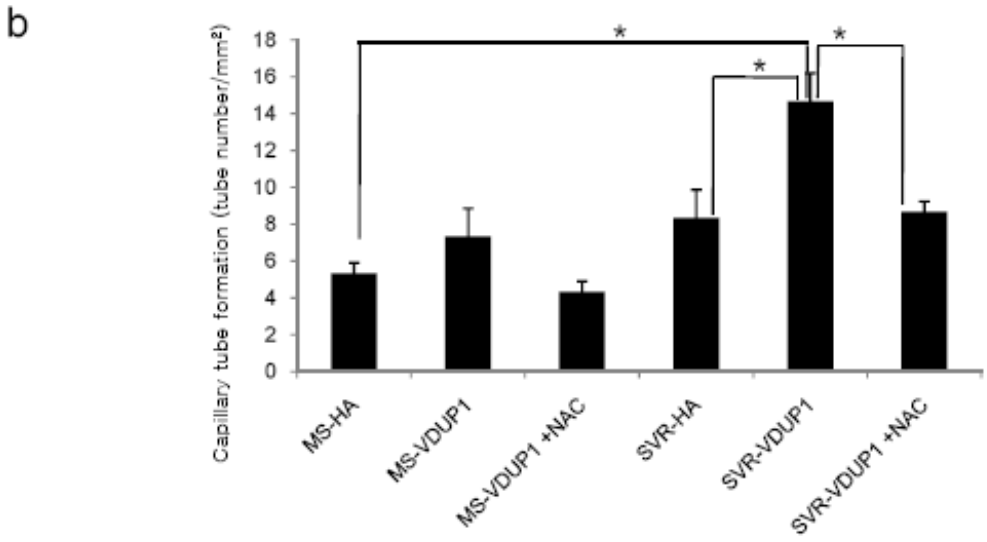


Figure 4. VDUP1 induces tube formation of Ras-overexpressing endothelial cells. Capillary tube formation was measured with MS-1 and SVR cells transfected with pcDNA3.1-VDUP1 or vector control in the absence or presence of NAC as indicated. Pictures of capillary tubes were taken by a microscope after 9-hour incubations. (A) Each figure is representative of three independent experiments (Bar, 50 □m). (B) The results of the experiments are expressed in terms of the number of capillary tubes formed per mm^2, and presented as the mean±SEM from six different experiments (* $p<0.05$).

increased resistance to endothelial anoikis with Ras. As a result, endothelial tube formation was prominently increased when both VDUP1 and Ras were overexpressed in the endothelial cells, supporting the direct involvement of VDUP1 in Ras-mediated angiogenesis. The fact that removal of cellular ROS inhibited the tube formation implies that VUDP1 is involved in angiogenic process via generation of ROS.

VDUP1 is a multifunctional factor that regulates a variety of cellular physiology including proliferation, apoptosis and differentiation (8, 16). However, for the proliferation, VDUP1 has been known only as a tumor suppressor that negatively regulates the cellular proliferation until recently. In our study, a novel function of VDUP1 has been revealed as an angiogenic factor in endothelial cells via regulating Ras-mediated ROS generation. Thus, modulation of VDUP1 activity depending on the cell types may be a new effective strategy not only in anti-cancer therapy, but also against other angiogenic diseases such as rheumatoid arthritis, systemic sclerosis, diabetic retinopathy, and cardiovascular diseases.

In conclusion, the expression of VDUP1 is upregulated by oncogenic Ras in endothelial cells, mediating ROS generation. Enforced expression of VDUP1 enhances proliferation and survival of endothelial cells. ROS produced by VDUP1 directly regulates Ras-mediated angiogenesis.

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