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Down Syndrome Candidate Region 1 Isoform 1L regulated tumor growth by targeting both angiogenesis and tumor cells

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Abstract

Angiogenesis is critical for solid tumor growth beyond its minimal size. Previously, we reported that Down Syndrome Candidate Region 1 isoform 1L (DSCR1–1L) was one of the most up-regulated genes in endothelial cells induced by VEGF and histamine, and regulated endothelial cell proliferation, migration and angiogenesis. However, it was not known whether DSCR1–1L played a role in tumor growth. In this study, we found that DSCR1–1L shRNAs significantly inhibited the growth of transplanted melanoma in mice and its associated tumoral angiogenesis. In the gain of function assay, overexpression of DSCR1–1L cDNA in mouse endothelium is sufficient to significantly increase the tumor initiation induced by carcinogen, the growth of xenografted tumor, and the tumor metastasis in our endothelially-expressed DSCR1–1L transgenic mice, in which angiogenesis was induced. It was the first time to find that DSCR1–1L was also expressed in various tumor cells. DSCR1–1L shRNAs inhibited, but overexpression of DSCR1–1L cDNA increased, the tumor cell proliferation and migration. Most recently, we reported that

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Author Statement

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DSCR1–1L modulated angiogenesis by down-regulation of VE-cadherin expression. Here, we found that DSCR1–1L down-regulated the expression of E-cadherin. Hence, DSCR1–1L is an excellent therapeutic target for cancers by regulation of both the endothelial and tumor cells through down-regulating (V)E-cadherin. DSCR1–1L shRNAs have the potential to be developed for clinical application.

Keywords

Down Syndrome Candidate Region 1 isoform 1L; angiogenesis; tumor; metastasis; E-cadherin

Background

Angiogenesis is a hallmark of many diseases, including cancer, wound healing, inflammation, and ischemic heart disease. Among many angiogenic factors, vascular endothelial growth factor (VEGF) plays a central role in tumor angiogenesis and associated microvascular permeability to plasma proteins (1–5). Anti-VEGF neutralizing antibodies and VEGFR kinase/multiple kinase inhibitors have been successfully developed and widely used in the clinic (review in (6)). However, anti-angiogenic therapy faces the problems of insufficient efficacy (7–16), resistance and intrinsic refractoriness (14,17,18), in addition to their toxic side effects (19). Therefore, it is desirable to identify other targets of angiogenesis.

In our gene profiling study, we identified that Down Syndrome Candidate Region 1 isoform 1L (DSCR1–1L) was one of the most up-regulated genes in human umbilical vein endothelial cells (HUVECs) induced by VEGF for one hour (20). The DSCR1 gene (aliases: DSCR1, ADAPT78, CSP1, DSC1, MCIP1, RCN1) encodes four different mRNA transcripts / isoforms that contain various N-terminuses encoded by each of the first four exons, and a common C-terminus encoded by exons 5–7 (21,22). The transcription of DSCR1 isoform 4 (DSCR1–4) is controlled by a promoter located between exon 3 and exon 4, which is different from the promoter that regulates the transcriptions of isoforms 1, 2 and 3 (21–24). The N-terminuses of DSCR1 isoform 1 (DSCR1–1), isoform 3 (DSCR1–3) and isoform 4 (DSCR1–4) proteins contain 84, 3, and 29 amino acid residues, respectively, while the mRNA transcript of isoform 2 is most likely not translated into protein because it lacks a methionine starting site (21,22).

The DSCR1 isoforms have different expression patterns, functions and regulatory mechanisms (21,22). DSCR1–1 was found to play a protective role against cell stress (25–27). DSCR1–4 played an inhibitory role in cardiac and skeletal muscle hypertrophy and angiogenesis (20,28–34). Prior to our previous reports (20,35), nothing was known about the role of DSCR1–1L in angiogenesis. We systematically analyzed the function of DSCR1 isoforms 1L, 3 and 4 in angiogenesis by overexpressing their respective proteins and their specific siRNAs (20). We found that DSCR1–1L and DSCR1–4 had antithetical effects on the angiogenic responses (20). The overexpression of DSCR1–1L protein significantly increased HUVEC proliferation and Matrigel angiogenesis in the presence and absence of VEGF, whereas the HUVEC proliferation and Matrigel angiogenesis induced

by VEGF were strikingly inhibited with a DSCR1–1L-specific siRNA (20). In contrast, the overexpression of DSCR1–4 protein strikingly inhibited HUVEC proliferation and Matrigel angiogenesis induced by VEGF, whereas DSCR1–4-specific siRNA (D4Si) stimulated such responses in the presence and absence of added VEGF (20). The overexpression of DSCR1–3 protein had no effect on the HUVEC proliferation and Matrigel angiogenesis in either the presence or absence of VEGF (20). DSCR1–1L was highly expressed in human tumor vasculature, but not detected in tumor cells or in normal tissues (ovaria and kidney) (20,23). Most recently, we reported that knocking down DSCR1–1L inhibited angiogenesis induced by VEGF in mice (35). DSCR1–1L controled angiogenesis by downregulation of VE-cadherin expression via decreasing the VE-cadherin promoter activity, but not 3'untralation region (3'UTR) activity (35). DSCR1–1L inhibited the formation of a transciprtion complex that contained a novel oligonucleotide element in the VE-cadherin promoter (35). However, it was not known whether DSCR1–1L regulated tumor growth.

In this study, we found that DSCR1–1L shRNAs significantly inhibited the growth of transplanted melanoma and its associated tumoral angiogenesis in mice. In the gain of function assay, the tumor initiation induced by carcinogen, the growth of xenografted tumor, and the tumor metastasis were significantly increased in our endothelially-expressed DSCR1–1L transgenic mice, in which angiogenesis was induced (35). Unexpectedly, DSCR1–1L was expressed in tumor cells of human melanoma tissues and in several human cancer cell lines, and regulated the proliferation and migration of tumor cells, in addition to endothelial cells. Therefore, DSCR1–1L is an excellent therapeutic target for cancer by regulation of both tumor cell biology and tumor microenvionment.

Materials and Methods

Materials

VEGF was purchased from R&D Systems (Minneapolis, MN, USA). Trypsin/EDTA, and Trypsin Neutralization Solution were purchased from Lonza (Walkersville, MD, USA). Antibodies against E-cadherin, CD31, and β-actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). A customized DSCR1–1L antibody was produced by NeoBioLab (Woburn, MA) and validated as described in our most recent report (35).

Cell culture

Primary HUVECs purchased from Lonza (Walkersville, MD) were cultured on plates coated with 30 μg/ml vitrogen (Collagen Biomaterials, Palo Alto, CA) in endothelial basic medium (EBM) with the EGM-SingleQuots Kit (Lonza, Walkersville, MD). The HUVECs at passages 5 were used for all experiments. All cancer cell lines were products of The American Type Culture Collection (Manassas, VA) and cultured in DMEM or RPMI1640 with 10% FBS followed the instruction from the Company.

The construction of DSCR1–1L shRNAs

This study utilized three DSCR1–1L shRNAs, hu-shD1L, mu-shD1L and hu/mu-shD1L, that specifically knocked down DSCR1–1L in the species of human, mouse, both of

human and mouse, respectively, as described in our most recent publication (35). The shRNA oligonucleotides were cloned to the lentiviral vector pLKO.1 to produce lentiviruses following the instructions provided by Addgene (Cambridge, MA). HUVECs and mouse melanoma B16F1 cells were transduced with the lentiviruses expressing shGFP as a control, hu-shD1L, mu-shD1L, and hu/mu-shD1L, respectively. Sixty hours later, the RNAs were isolated and subjected to quantitative real-time reverse transcription-PCR (RT-PCR). After confirmation of the specificity, each DSCR1–1L shRNA was cloned to the pENTR1A-stuffer vector and transferred to the adenovirus pAd/PL-DEST vector to prepare adenoviruses following the instructions provided by Invitrogen (Carlsbad, CA). The adenoviruses expressing DSCR1–1L shRNAs were used to infect human and mouse cells, respectively.

Proliferation assay

As described previously (36), cells were seeded in 96-well plates. Twenty-four hours later, the cells were transduced with the viruses as indicated. Forty-eight hours later, the cells were serum-starved with EBM, DMEM and RPMI1640 containing either 0.1% or 1% fetal bovine serum (FBS) for 48 hours, and treated with and without VEGF or EGF (10 ng / ml) for 24 hours, respectively. Cell Counting Kit-8 reagents (Dojindo Molecular Technologies, Inc. Washington. D.C) were added to each well. The plates were incubated for 3 hours before the absorbance at 450 nm was measured using a microplate reader.

Monolayer migration assay

Monolayer migration assay was carried out as described previously (36). Briefly, cells (6×10^4 cells/well) were seeded in 6-well plates. Twenty-four hours later, the cells were transduced with the viruses as indicated. Forty-eight hours later, the cells were serum-starved with EBM or DMEM containing 0.1% or 1% FBS for 24 h, respectively. Scratch wounds were generated with a 200 μl pipette tip and photographed immediately at 0 h. The cells were stimulated either with or without VEGF or EGF (10 ng/ml) for 16 h and photographed. The cells that migrated to the wound area were counted. The results were expressed as mean ± SD from 6 views.

Transplanted tumor assay

All tumor cells were mixed in an amount of 50μl Matrigel and injected s.c to the mouse flank skin. To study whether DSCR1–1L shRNAs inhibited tumor growth, A375 melanoma cells (0.5×10^4 cells) were used in nude mice (males and females). One week after the tumor cell transplantation, the mice were injected i.v. via the tail vein with 2×10^{11} OFU of the non-replicating adenoviral vectors as indicated twice a week. The tumor sizes and mouse body weights were measured daily and the animals were sacrificed when the tumors reached a size of nearly 2,000 mm^3. The tumor sizes were calculated as the result of a^2 (length) ×b (width) / 2. To study the growth of tumor in transgenic mice, LAP0297 lung tumor cells (0.5×10^4 cells) that were syngeneic in Fvb mice (37) were injected to the EC-FH-DSCR1–1L mice (35) and their respective control littermates that had been provided with tetracycline-free water for 6 days.

The carcinogen-induced tumor formation

Seven days after provided with tetracycline-free water, the EC-FH-DSCR1–1L mice (35) and their respective control littermates were injected i.p. once with carcinogen azoxymethane (AOM, 10 mg/Kg body weigh) as described previously (38). Seven days later, the mice were provided with drinking water containing 2% Dextran Sulfate Sodium (DSS) for 7 days and then with normal drinking water for 2 weeks. This DSS-water cycle was repeated twice. The animals were sacrificed using CO_2 at the end of the third cycle. The colons were removed and opened longitudinally to measure tumor numbers.

Animal welfare

All of the animal experiments were performed in compliance with the Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee.

Statistical analysis

Results were presented as mean ± SD. ANOVA and student's t-test were employed to determine the statistical significance. The p values less than 0.05 were considered as statistically significant.

Results

DSCR1–1L shRNAs inhibited the tumor growth in vivo.

Most recently, we reported that DSCR1–1L siRNA inhibited, but overexpression of DSCR1–1L cDNA in mouse endothelium induced, angiogenesis, respectively (35). Because angiogenesis plays critical roles in tumor growth, we then studied the impact of DSCR1–1L shRNAs on tumor growth with the transplanted melanoma model. Human molenoma cells were injected s.c in flank skin of nu/nu mice. One week later when the tumors were solid, mice were randomly grouped and injected iv. via mouse tail vein with the adenoviruses expressing shGFP as a control, mu-shD1L or hu/mushD1L (35) twice per week. The growth of melanoma was significantly inhibited by mu-shD1L or hu/mu-shD1L (Fig.1A, left panel). The tumor mass was significantly less with DSCR1–1L treatment than that with control shRNA at day 42 (Fig.1A, right panel). The RNA was extracted from the tumors and the skin tissues surrounding the tumors, which were angiogenic tissues supporting tumor growth. The Realtime RT-PCR data with the DSCR1–1L primers showed that the expression of DSCR1–1L was greatly knocked down in both the tumor tissues and the skin tissues surrounding the tumors in the mice treated with mu-shD1L and hu/mu-shD1L (Fig.1B). Because the adenoviruses were injected i.v. via the tail vein, and adenoviruses are known to be delivered and metabolized in the liver, the RNA was isolated from the liver tissues and subjected to Realtime RT-PCR with the DSCR1–1L primers. The DSCR1–1L mRNA expression was significantly knocked down by hu/mu-shD1L and mu-shD1L in the liver tissues, respectively (Fig.1B). However, the mice did not show any sick syndromes. With H&E staining on the liver tissues, there was no obvious damage seen with the treatment of DSCR1–1L shRNAs (Fig.1C). By immunohistochemical staining with CD31 antibody on tumor tissues, the numbers and the area of vessels were significantly decreased in the tumors treated with mu-shD1L and hu/mu-shD1L (Fig.1D). Our data clearly demonstrated

that knocking down the expression of DSCR1–1L greatly inhibited tumor growth and tumor angiogenesis.

The overexpression of DSCR1–1L in mouse endothelial regulated tumor development and progression.—Most recently, we reported that overexpression of DSCR1–1L in mouse endothelium induced angiogenesis and its associated microvessel permeability. We then studied the impact of overexpression of DSCR1–1L on tumor growth with the mouse LAP0297 lung tumor model. LAP0297 lung tumor cells that was syngeneic in Fvb mice (37) were subcutaneously injected into the flank skin of EC-FH-DSCR1–1L mice and their respective control littermates that had been on tetracycline-free drinking water for 6 days to induce the expression of transgene. The tumor masses were greatly and significantly increased in the EC-FH-DSCR1–1L mice compared to that in their respective control littermates during the first 3 weeks after tumor transplantation (Fig.2A). The tumor tissues from week 3 were immunohistochemically stained with an antibody against CD31. The vessel density and vessel area were measured from ~40 views of each condition and analyzed by Image J software to obtain quantitative results. The vessel densities and vessel areas were greatly increased in the EC-FH-DSCR1–1L mice (Fig.2B). However, the tumor masses in EC-FH-DSCR1–1L mice were not significantly different from that in their respective control littermates at week 5 after tumor transplantation (Fig.2A). The lungs from the mice, in which tumors had been implanted for 4 weeks were dissected and photographed. Metastasized tumors were detected in EC-DSCR1–1L mice, but not in their control wildtype littermates (Fig.2C). We further studied whether the expression of DSCR1–1L in the mouse endothelium favored tumor development with carcinogenic treatment. Previously, we found that DSCR1–1L up-regulated the calcineurin-NFAT axis (20), and positively regulated NF-κB activity (39). It is well known that both NFAT and NF-κB play critical roles in angiogenesis and inflammation. Therefore, we chose the AOM/DSS-induced mouse colorectal tumor model, a well-studied chronic inflammation-induced mouse tumor model, for our studies. In this model, AOM, a classic chemical carcinogen, and a low dosage of repeated DSS treatments triggered chronic inflammatory response. All of the mice developed colorectal tumors in this model (40). The EC-FH-DSCR1–1L mice and the control wildtype littermates that had been provided with tetracycline-free drinking water for 6 days to induce the transgenic expression were injected i.p. with AOM, and provided with DSS in drinking water as described previously (38). The colons were then dissected. The tumor numbers in colons were significantly increased in the EC-FH-DSCR1–1L mice as compared to that in the wild type control mice (Fig.2D). Our data indicated that the expression of DSCR1–1L in mouse endothelium regulated carcinogen-induced tumor development, tumor growth, and metastasis *in vivo*.

DSCR1–1L was expressed in tumor cells, in addition to endothelial cells.

Previously, we reported that DSCR1–1L was highly induced in endothelial cells stimulated by VEGF and histamine, and was expressed in the vasculature, but not detected in the cancer cells, of human ovarian and kidney cancer tissues, nor in normal ovarian or kidney tissues (20,24,41). To generalize these findings to other human cancers, we tested the expression of DSCR1–1L in human melanoma tissues. DSCR1–1L was detected in the cancer tissues (Fig.3A, T in panel I), but not in the normal para-tumor tissues (Fig.3A, P in panel I).

There was no positive signal detected on human melanoma tissues stained with IgG as a control (Fig.3A, panel II). Unexpectly, the positive stainings were detected in the tumor cells (Fig.3A, arrow heads on panel I), in addition to the vessels (Fig.3A, arrows on panel I). To further confirm that DSCR1–1L was expressed in both vasculature and tumor cells, we carried out double immunofluoresent stainings with antibodies against DSCR1–1L and CD31, a vessel marke. The data clearly showed that DSCR1–1L was detected in both the vessles (Fig. 3A, arrow on panel III) and the cancer cells (Fig.3A, arrow head on panel III) in the human melanoma cancer tissues. To confirm that the unexpected detection of DSCR1–1L in cancer cells in melanoma tissues was not due to the new antibody used, we carried out immunostaining with this new antibody on human ovarian cancer tissues. This new antibody gave out strong signals in the vessels in cancer tissues (Fig. 3B, arrows on panel I), but not in the cancer cells (Fig. 3B, arrow heads on panel I), consistent with that we reported previously (20). Then, we examined whether DSCR1–1L was expressed in human cancer cell lines. Cellular extracts from cells as indicated were immunoblotted with DSCR1–1L antibody. In addition to various kinds of endothelial cells, DSCR1–1L was detected in fibroblast cells and all of the human cancer cell lines tested, including those of ovary, breast, fibrosarcoma, colon, cervix, liver, kidney, lung, glioblastoma, lymphocyte, melanoma, pancreas and prostate (Fig.3C). These results indicated that DSCR1–1L was expressed in cancer cells, in addition to in endothelial cells.

DSCR1–1L functioned in tumor cells.

Previously, we reported that DSCR1–1L regulated HUVEC proliferation induced by VEGF and histamine (20,24). Since DSCR1–1L was expressed in the tumor cells, we would like to study whether DSCR1–1L played a role in tumor cells. The cell proliferation and the monolayer migration assays were carried out in serum-starved HUVECs and A375sm cells that were transduced with or without hu-shD1L, hu/mu-shD1L, shGFP as a control, DSCR1–1L cDNA, or Lac Z as a control, in the presence and absence of VEGF or EGF, respectively. The results indicated that knocking down the expression of DSCR1–1L with its shRNAs almost completely inhibited the proliferation and migration of HUVECs and melanoma A375sm cells induced by VEGF and EGF, respectively (Fig.4A). The overexpression of DSCR1–1L induced the proliferation and migration of both HUVECs and A375sm cells, even in the absence of VEGF or EGF stimulation (Fig.4A). These data indicated that DSCR1–1L played important roles in melanoma cells.

Next, we test whether DSCR1–1L played a role in other tumor cells. Human pancreatic cancer Aspc1 cells, prostate cancer PC3 cells, melanoma A375sm, renal cancer A498 cells and colorectal cancer HCT116 and HT29 cells were transduced with shGFP as a control, hu-shD1L and hu/mu-shD1L. The expression of DSCR1–1L in these cancer cells was greatly knocked down by hu-shD1L and hu/mu-shD1L (Fig.4B). hu-shD1L and hu/mu-shD1L inhibited the proliferation of Aspc1, PC3, A753, A498, HCT116 and HT29 cells (Fig.4C). Our results showed that DSCR1–1L regulated the proliferation of several tumor cells.

DSCR1–1L down-regulated the expression of E-cadherin.

Most recently, we reported that the expression of DSCR1–1L cDNA down-regulated, and shRNAs up-regulated the VE-cadherin in both protein and mRNA levels, respectively (35).

With the loss-of-function assay, the overexpression of VE-cadherin cDNA significantly inhibited the proliferation and migration in HUVECs induced by DSCR1–1L (35). Therefore, we studied whether DSCR1–1L regulated the expression of E-cadherin. Cellular extracts were isolated from A375sm cells that were transduced with Lac Z as control, DSCR1–1LcDNA, shGFP as control, hu-shD1L, hu/mu shD1L. Immunoblot analysis with an antibody against E-cadherin showed that the protein levels of E-cadherin in A375sm cells were greatly down-regulated or up-regulated by DSCR1–1L cDNA and shRNAs, respectively (Fig.5). Our data clearly indicated that DSCR1–1L down-regulated the expression of E-cadherin.

Discussion

So far, most studies about DSCR1 gene have been focused on DSCR1–4. Previously, we reported that DSCR1–1L was upregulated by angiogenic factors, including VEGF and histamine, and was expressed in the vasculature of human cancer tissues, but was not detectable in the normal vessels (20,23,24). Further, knocking down DSCR1–1L inhibited HUVEC proliferation and migration induced by VEGF and histamine (20,24). Most recently, we reported that knocking down the expression of DSCR1–1L with novel DSCR1–1L shRNAs inhibited the angiogenesis induced by VEGF in mice (35). The expression of DSCR1–1L in mouse endothelium was sufficient to induce angiogenesis (35). The studies suggested that DSCR1–1L plays important roles in diseases, in which angiogenesis was an important factor, including cancer and ischemic diseases.

Here, we found that DSCR1–1L shRNAs inhibited the tumor growth and tumoral angiogenesis in mice. Overexpression of DSCR1–1L in mouse endothelium was sufficient to promote tumor initiation, growth and metastasis. These data demonstrated that DSCR1–1L regulated tumor growth through angiogenesis.

We were, the first, to find that DSCR1–1L was expressed in tumor cells, in addition to vessels, in human melanoma tissues, but not detected in normal para-tumor tissues. DSCR1–1L was highly expressed in several endothelial cells that were commonly used, and more than thirty human cancer cell lines. DSCR1–1L shRNAs inhibited the proliferation of several human cancer cells. Our data suggested that DSCR1–1L was an excellent therapeutic target for cancer by targeting both of the endothelial and cancer cells.

Previously, we found that DSCR1–1L up-regulated the calcineurin-NFAT axis (20). Here, tumor formation was increased in the chronic inflammation-induced mouse tumor model in EC-FH-DSCR1–1L mice. These data suggested that DSCR1–1L might play a role in inflammation.

So far, the functions of DSCR1–1L have been overlooked, not even to mention the molecular mechanism underlying it. Previously, we reported that DSCR1–1L regulated angiogenesis by activating the calcineurin-NFAT axis, unlike DSCR1–4 that inhibited this pathway (20,28–34). Most recently, we found that DSCR1–1L down-regulated VE-cadherin in HUVECs (35). In the loss-of-function assay, the VE-cadherin overexpression prevented the HUVEC proliferation and migration induced by the expression of DSCR1–1L (35).

Our data indicated that VE-cadherin was another down-stream target of DSCR1–1L (35). It was known that E-cadherin plays important roles in tumor growth. Here, we found that DSCR1–1L down-regulated the expression of E-cadherin in tumor cells. Therefore, our data suggested that E-cadherin is a down-stream target of DSCR1–1L in tumor cells. The molecular mechanism, by which DSCR1–1L down-regulates E-cadherin, will be studied in the future.

In summary, DSCR1–1L played an important role in tumor progression by regulating both of the angiogenesis and tumor cells, suggesting it may represent a novel excellent therapeutic target for cancer. DSCR1–1L shRNAs will be developed into therapeutics. The findings that DSCR1–1L down-regulated E-cadherin contribute significantly to the field of mechanism studies. Therefore, these studies have significant impacts on basic research and have translation potentials.

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Abbreviations

DSCR1–1L	Down Syndrome Candidate Region 1 isoform 1L
DSCR1–3	Down Syndrome Candidate Region 1 isoform 3
DSCR1–4	Down Syndrome Candidate Region 1 isoform 4
HUVECs	human umbilical vein endothelial cells
VEGF	vascular endothelial growth factor

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Highlights

- DSCR1–1L shRNAs inhibited the tumor growth in vivo.
- The overexpression of DSCR1–1L in mouse endothelial regulated tumor development and progression.
- DSCR1–1L was expressed in tumor cells, in addition to endothelial cells.
- DSCR1–1L regulated the proliferation of several tumor cells.
- DSCR1–1L down-regulated the expression of E-cadherin in tumor cells.

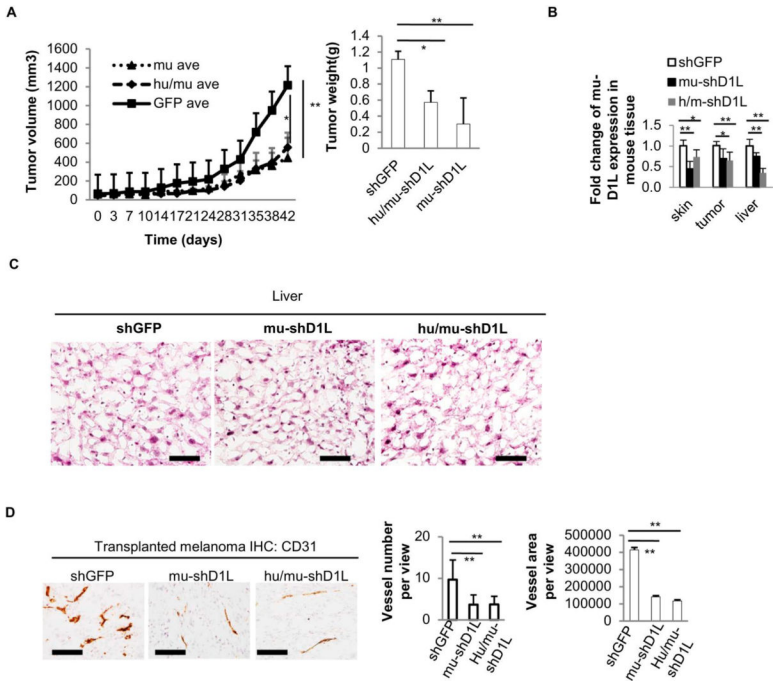

Figure 1. The melanoma growth in mice were inhibited by DSCR1–1L shRNAs.

A) The growth courses of tumors in the mice treated with the adenoviruses expressing hu/mu-shD1L mu-shD1L and shGFP as a control (left panel). The tumors were dissected and weighed on day 42 (right panel, n=20 mice / group); **B)** The RNA isolated from the tumors and the skin tissues surrounding the tumors and the livers were subjected to Realtime RT-PCR with the DSCR1–1L primers (n=20); **C)** Liver tissues from mouse bearing tumors were stained with H&E; Magnification bars are 50 μm. **D)** Tumor tissues were immunohistochemically stained with CD31 antibody (left panels). Magnification bars are 50 μm. Vessel density (middle panel) and area (right panel) were measured and plotted (n=20 mice in each group). *p < 0.05, ** p < 0.01, ***p < 0.001.

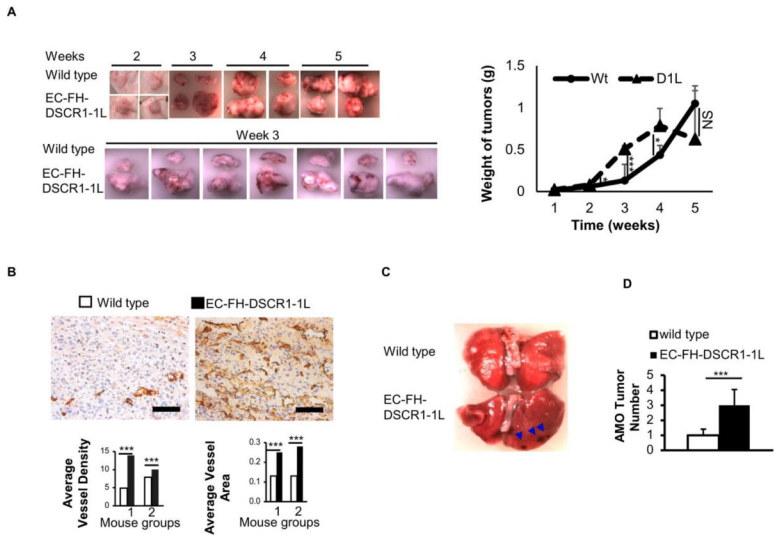

Figure 2. The expression of DSCR1–1L in mouse endothelium accelerated tumor initiation, growth and metastasis.

A) The images of tumors (left panels) at various weeks (top panel) and week 3 (bottom panel), and the curve of tumor growth at various weeks (n = 7 for week 3, n=4 for others,); **B**) The immunohistochemically staining of tumor tissues at week 3 with CD31 antibody (top panels), the average vessel density and the vessel area (bottom panels), (n=20 views, SDs were too small to be seen). Magnification bars are 50 μm; **C)** The images of lungs, which represent 1 of 6 different lungs in each group; **D)** The numbers of AOM-induced tumors in the wild type mice and the EC-FH-DSCR1–1L mice. *p < 0.05, ** p < 0.01, ***p < 0.001, NS, no significant difference.

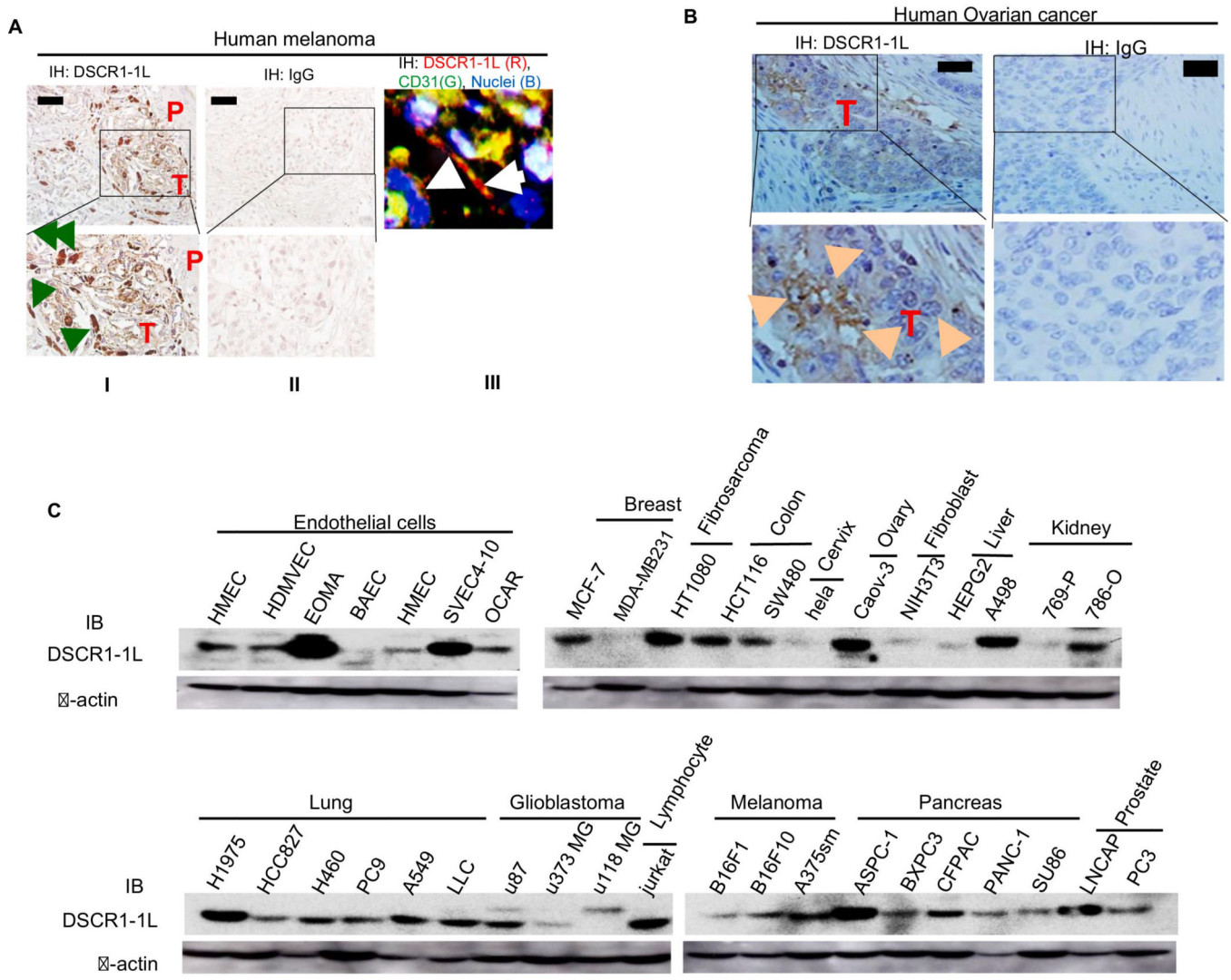

Figure 3. DSCR1–1L was expressed in human tumor cells.

A) Human melanoma tissues were immunohistochemically stained with DSCR1–1L antibody (panels I), IgG as a control (panel II), and the antibodies against DSCR1–1L (red) and CD31 (green) (panel III). DSCR1–1L was detected in vessels (arrows) and tumor cells (arrow heads) in tumor area (T), but not in para-tumor normal tissues (P); **B)** Human ovarian cancer tissues were immunohistochemically stained with DSCR1–1L antibody (panels I), and IgG as a control (panel II). DSCR1–1L was detected in vessels (arrows), but not in tumor cells (arrow heads) in tumor area (T). Magnification bars are 50 μm. The images represent 1 of 6 different patient samples, all of which exhibited similar staining; **C**) The cellular extracts that were isolated from cells as indicated were subjected to immunoblotting with the antibodies against DSCR1–1L (top panels) and β-actin for protein equal loading control). The experiments were repeated three times.

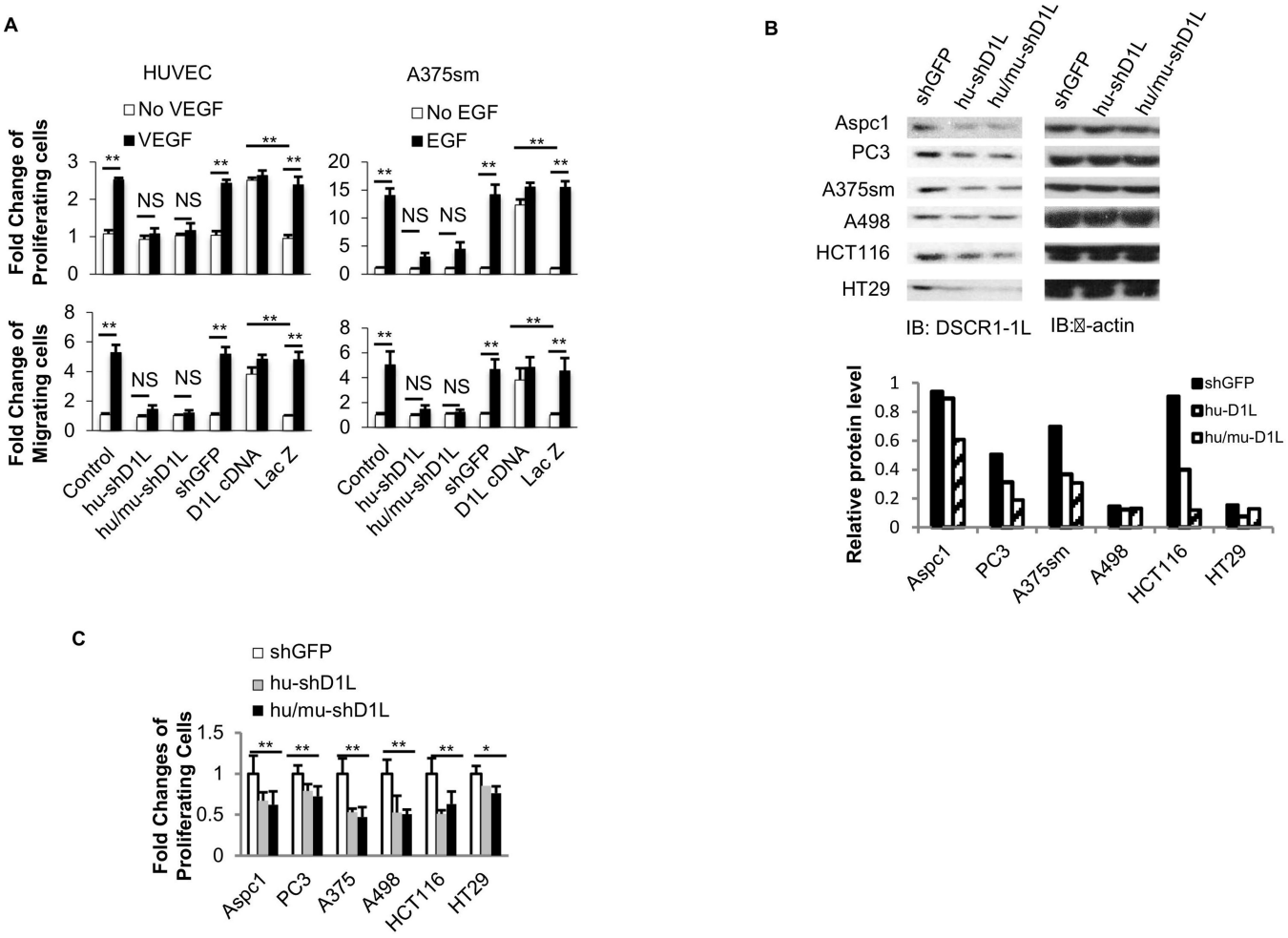

Figure 4. DSCR1–1L functioned in tumor cells.

A) The serum-starved HUVECs (left panels) and A375sm cells that were transduced without (control), and with hu-shD1L, hu/mu-shD1L, shGFP as a control, DSCR1–1L cDNA and Lac Z as a control were stimulated with and without VEGF (left panels) or EGF (right panels) for proliferation assay (top panels) and migration assay (bottom panels), (n=4); **B) and C)** Tumor cells as indicated were transduced with shGFP, hu-shD1L, and hu/mu-shD1L and subjected to immunoblotting with antibodies against DSCR1–1L (**B**, top left panel), β-actin for protein equal loading control (**B**, top right panel) and the quantification of Western blot (B, bottom panel), and proliferation assay (**C,** n = 6); ** $p < 0.01$, * $p < 0.05$, NS, no significant difference. All experiments were repeated three times.

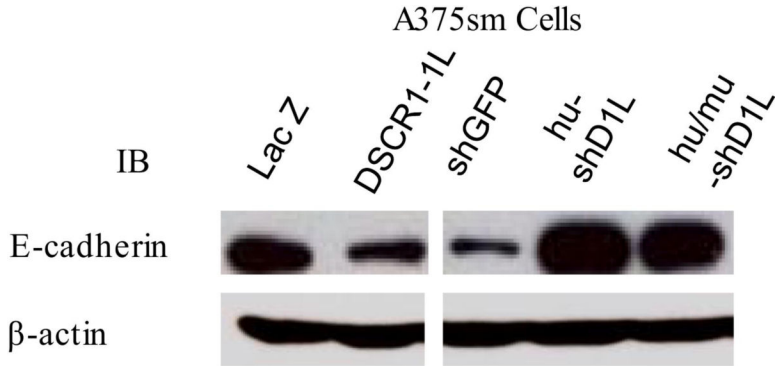

Figure 5. DSCR1–1L downregulated the expression of E-cadherin.

The cellular extracts isolated from the A375sm cells that were transduced with Lac Z as a control, FH-DSCR1–1L, shGFP as a control for shRNAs, hu-shD1L and hu/mu-shD1L were subjected to Immunoblotting with the antibodies against E-cadherin 1L (top panel), and β-actin as the equal protein loading control (bottom panel). The experiments were repeated three times.