

Connexin 43 overexpression induces lung cancer angiogenesis *in vitro* following phosphorylation at Ser279 in its C-terminus

ZIZI ZHOU, WENXIANG CHAI, YI LIU, YAO LIU, HUIYU PAN, QIANG WU and XIAOMING ZHANG

Department of Cardio-Thoracic Surgery, Shenzhen University General Hospital, Shenzhen, Guangdong 518055, P.R. China

Received January 20, 2022; Accepted April 27, 2022

DOI: 10.3892/ol.2022.13413

Abstract. Blocking angiogenesis can inhibit tumor growth and metastasis. However, the mechanism underlying regulation of lung cancer angiogenesis remains unclear. The gap junction protein connexin 43 (Cx43) is implicated in angiogenesis. The aim of the present study was to determine the role of Cx43 in angiogenesis *in vitro* and its signaling pathways. Human pulmonary microvascular endothelial cells were transfected with Cx43-targeting siRNA or Cx43-overexpressing recombinant plasmid vector. Reverse transcription-quantitative polymerase chain reaction and western blotting were performed to determine Cx43, zonula occludens-1 (ZO-1), E-cadherin, β -catenin, von Willebrand factor (vWF), and plasminogen activator inhibitor-1 (PAI-1) mRNA and protein expression levels, respectively. Tyr265, Ser279, Ser368, and Ser373 phosphorylation levels in the C-terminus of Cx43 and intracellular and membranal Cx43 contents were determined using western blotting. Additionally, immunofluorescence, tube formation, Cell Counting Kit-8, and Transwell migration assays were performed. The results revealed that compared with that in the control samples, Cx43, ZO-1, E-cadherin, β -catenin, vWF, and PAI-1 mRNA and protein expression were significantly increased in the Cx43 overexpression group and significantly decreased in the Cx43-knockdown group. Moreover, the phosphorylation level of Ser279 as well as cell proliferation and migration rates were markedly increased in the Cx43 overexpression group, and tube formation revealed that the potential of angiogenesis was also increased. Conversely, in the Cx43-knockdown group, the phosphorylation level of Ser279 and cell proliferation and migration rates were reduced, and the potential of angiogenesis was greatly impaired. Under Cx43 overexpression, membranal Cx43

content was significantly increased, whereas under Cx43 knockdown, it was significantly reduced. Therefore, Cx43 overexpression could induce pulmonary angiogenesis *in vitro* by promoting cell proliferation and migration and activating ZO-1, E-cadherin, β -catenin, vWF, and PAI-1. This may be achieved by promoting phosphorylation and activation of the intracellular signal site Ser279 at the C-terminus of Cx43.

Introduction

Although lung cancer is a malignant tumor with the highest rates of morbidity and mortality in the world (1), its pathogenesis remains unclear. Recurrence and metastasis are the main hurdles in lung cancer treatment. Angiogenesis plays a key role in tumor growth, development, and metastasis (2). Thus, blocking angiogenesis can inhibit tumor growth and metastasis and induce tumor cell dormancy and apoptosis. However, the mechanism underlying the regulation of lung cancer angiogenesis remains unclear.

Gap junctional intercellular communication (GJIC) plays an important role in the control of cell growth, differentiation, homeostasis, and morphogenesis (3). Gap junction proteins, also called connexins (Cx), coordinate the functions of various cells by forming gap junctions and affect cell growth and differentiation by regulating gene expression (4). Cxs are four-joint integral membrane proteins assembled by hexamers to form a hemichannel, which connects cells in a head-to-head manner to form gap junctions (5). The functions of gap junctions, including exchange of metabolites and electrical signals between cells, are highly diverse and also include functions not related to cell-cell communication (6). Gap junctions allow inorganic ions (Na^+ , K^+ , and Ca^{2+}) and molecules <1,000 Da to directly diffuse between adjacent cells (5,7-10), which is important for the development of cells, tissues, and organs as well as for normal metabolism and homeostasis (11-13). Recently, the association between gap junctions and angiogenesis has become a research hotspot. The activity of gap junction channels is regulated through changes in voltage, calcium ion concentration, pH, phosphorylation level, and protein interactions (6,8). Among them, phosphorylation is deeply involved in the regulation process during gap junction protein synthesis, transport, assembly, membrane insertion, internalization, and degradation as well as protection of gap junction hemichannels and complete channels (7,8,12).

In a previous study by the authors, it was determined that the expression of Cx43 was improved in vascular endothelium

Correspondence to: Professor Xiaoming Zhang, Department of Cardio-Thoracic Surgery, Shenzhen University General Hospital, 1098 Xueyuan Avenue, Nanshan, Shenzhen, Guangdong 518055, P.R. China

E-mail: whzyzxm@sina.com

Key words: connexin 43, human pulmonary microvascular endothelial cells, angiogenesis, phosphorylation activation, C terminus

of lung cancer compared with the normal control (Zhou *et al.*, unpublished data). Previous research has revealed that Cx43 may participate in angiogenesis mainly by regulating branching of capillaries (14,15), and formed gap junctions play an important role in determining the fate of endothelial cells, necessary for vascularization during the process of damage repair (16-18). Moreover, decreased expression of Cx43 can cause vascular dysfunction and impair the process of neovascularization (18-20); it can also directly inhibit endothelial cell migration, proliferation, and angiogenesis and reduce vascular endothelial growth factor (VEGF) transcription and translation (20). Recently, it was revealed that Cx43 plays an important role in angiogenesis (21). Based on these studies, it was hypothesized that Cx43 was the key regulator factor involved in angiogenesis. However, its specific mechanism in angiogenesis especially in lung cancer angiogenesis remains to be elucidated.

The C-terminal region is the primary region of Cx43 that is phosphorylated. It contains 21 serine and 2 tyrosine residues, which are the targets of phosphorylation by several protein kinases, such as mitogen-activated protein kinase (MAPK), protein kinase A (PKA), protein kinase C (PKC), p34 (Cdc2)/cyclin B kinase, casein kinase 1, and pp60 (src) kinase (12,22,23). Tyr265 is phosphorylated by Src, Ser279 by MAPK, Ser368 by PKC, and Ser373 by AKT/PKB (24).

Based on the available evidence, it was hypothesized that several downstream proteins could be involved in angiogenesis, and activation of the phosphorylation sites on the C-terminus of Cx43 may trigger this regulatory pathway. In the present study, a specific small-interfering RNA (siRNA) to inhibit Cx43 intracellularly and Cx43-overexpressing recombinant plasmid were used to explore the role of Cx43 in regulating angiogenesis in lung cancer. Furthermore, it was explored whether Cx43 modulates angiogenesis depending on the activation of its C-terminal phosphorylation sites and its possible association with downstream zonula occludens-1 (ZO-1), E-cadherin, β -catenin, von Willebrand factor (vWF), and plasminogen activator inhibitor-1 (PAI-1) signaling proteins. Through *in vitro* experiments, the effect and underlying action mechanism of Cx43 in angiogenesis was explored. Finally, it was determined whether Cx43 is a proangiogenic effector and the corresponding signaling pathway was explored, which will provide new insight for the treatment of lung adenocarcinoma by regulating Cx43-related angiogenesis.

Materials and methods

Reagents and instruments. The main reagents and instruments used the present study included the following: Human pulmonary microvascular endothelial cells (HPMECs; cat. no. HUM-iCell-a001; iCell Bioscience, Inc.), Endothelial Cell Medium (ECM; ScienCell Research Laboratories, Inc.); Lucifer Yellow CH (Invitrogen; Thermo Fisher Scientific, Inc.), Matrigel (Corning, Inc.), Cell Lysis Buffer (product no. 9803; Cell Signaling Technology, Inc.), vWF antibody (product no. 65707; Cell Signaling Technology, Inc.), PAI-1 antibody (product code ab66705; Abcam), phosphorylated (p)-Cx43 (Tyr265) antibody (cat. no. AF2306; Affinity Biosciences), p-Cx43 (Ser279) antibody (cat. no. AF8228; Affinity Biosciences), p-Cx43 (Ser368) antibody (cat. no. AF3199;

Affinity Biosciences), p-Cx43 (Ser373) antibody (cat. no. PA5-64670; Invitrogen; Thermo Fisher Scientific, Inc.), Cx43 antibody (cat. no. AF0137; Affinity Biosciences), ZO-1 antibody (product code ab96587; Abcam), E-cadherin antibody (product no. 3195; Cell Signaling Technology, Inc.), β -catenin antibody (product no. 8480; Cell Signaling Technology, Inc.), Na⁺/K⁺-ATPase (product code ab76020; Abcam), GAPDH (product code ab37168; Abcam), TRIpure Total RNA Extraction Reagent (cat. no. EP013), EntiLink™ 1st Strand cDNA Synthesis Kit (cat. no. EQ003), EntiLink™ PCR Master Mix (cat. no. EQ004), Gel DNA Purification Kit (cat. no. EP006) and EndoFree Plasmid Miniprep Kit [cat. no. EP004; all from Elk (Wuhan) Biotechnology Co., Ltd.], an inverted microscope (OLYMPUS IX51; Olympus Corporation), a fluorescence microscope (OLYMPUS BX51; Olympus Corporation), a CO₂ incubator (model no. SCO6WE; SHEL LAB; Sheldon Manufacturing, Inc.), a biological safety cabinet (model no. SW-CJ-1FD; Suzhou Antai Airtech Co., Ltd.), an imaging system (MicroPublisher; QImaging), a microplate reader (model no. DR-200Bs; Diatek), scanner (model no. LiDE110; Canon, Inc.), and a real-time PCR system (StepOne™ Real-Time PCR System; Life Technologies; Thermo Fisher Scientific, Inc.).

Plasmid constructs and transfection of siRNA and plasmid-DNA

Design, synthesis, and silencing efficiency of siRNA targeting Cx43 gene. siRNAs targeting the Cx43 gene (GenBank ID: NM_000165.5) were designed. Multiple siRNA candidates were obtained, of which three candidates with strong specificity were selected for further analysis using the Basic Local Alignment Search Tool (BLAST; <https://blast.ncbi.nlm.nih.gov/Blast.cgi>). The three candidate siRNAs [siRNA-1 base sequence (5'-3'): sense, GCGCCUAGGCAAACUCCUUGACAATT and antisense, UUGUCAAGGAGUUUGCCUAGGCGCTT; siRNA-2 base sequence (5'-3'): sense, CCACACUCUUGUACCUAGGCUCAUGUTT and antisense, ACAUGAGCCAGGUACAAGAGUGUGGTT; siRNA-3 base sequence (5'-3'): sense, AGUACGGUAUUGAAGAGCAUGGUAATT and antisense, UUACCAUGCUCUUCUAAUACCGUACUTT] and siRNAs with no homologous sequence to the Cx43 fragment (i.e., siRNA control group; base sequence (5'-3'): sense, GCGGAUUAACGCUCAGUUCACCCAATT and antisense, UUGGGUGAACUGAGCGUUAUCCGCTT) were chemically synthesized by HYcell Biotechnology Company. The siRNAs were then individually transfected into HPMECs cells, and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was used to detect the expression of Cx43 in the cells after 24 h. The silencing efficiency of each candidate siRNA was determined, and the siRNA sequence [base sequence (5'-3'): sense, CCACACUCUUGUACCUAGGCUCAUGUTT and antisense, ACAUGAGCCAGGUACAAGAGUGUGGTT] with the highest interference efficiency was selected for subsequent experiments.

Construction and identification of recombinant plasmid. To biosynthesize the Cx43 gene, pcDNA3.1-CX43-HA plasmid (GenScript) was constructed using the PCR-based Accurate Synthesis method (25). The plasmid was transferred to a TOP10 cloned strain, which was cultured on Luria Bertani

medium containing ampicillin overnight at 37°C. A single colony was selected and inoculated in Luria Bertani broth containing ampicillin and cultured overnight at 37°C and 13,000 x g. Following incubation, *Escherichia coli* cells containing the pcDNA3.1-CX43-HA plasmid were obtained [EndoFree Plasmid Miniprep Kit; Elk (Wuhan) Biotechnology Co., Ltd.], and the Cx43 fragment was amplified with bacterial plasmid as a template. After the plasmid was extracted [Gel DNA Purification Kit, Elk (Wuhan) Biotechnology Co., Ltd.] from the *E. coli* cells, it was identified through *XhoI* and *KpnI* enzyme digestion, and the whole recombinant plasmid was sequenced [EntiLink™ PCR Master Mix, Elk (Wuhan) Biotechnology Co., Ltd.].

Transfection. Log-phase HPMECs were seeded in a 6-well plate at 2×10^5 cells/well and cultured at 37°C in a 5% CO₂ incubator. After the cells were observed to adhere to the walls for 24 h and reached 80-90% confluence (inverted microscope; Olympus Corporation), they were transfected for 6 h at 37°C following the Lipofectamine™ 2000 transfection procedure of Invitrogen; Thermo Fisher Scientific, Inc.

Cell culture and experimental design. Frozen vials of primary cells were removed from a liquid nitrogen storage tank and thawed quickly by placing the vials in a 37°C water bath and simultaneously shaking them vigorously. The cell suspensions (1 ml) from the vials were transferred to 175-cm² culture flasks containing ECM (ScienCell Research Laboratories, Inc.). The flasks were incubated at 37°C and 5% CO₂. Subsequently, the medium was changed to fresh standard cell culture medium to remove dimethyl sulfoxide and incubated overnight. The medium was replaced every other day until the cells reached 80-90% confluence (inverted microscope; Olympus Corporation). Then, the cells were digested with trypsin/EDTA solution and neutralized using complete medium containing 5% fetal bovine serum (ScienCell Research Laboratories, Inc.). Next, the cells were transferred to a 50-ml centrifuge tube and centrifuged for 3 min at 220 x g at room temperature, and the supernatant was discarded. The cells were resuspended in standard cell culture medium and counted using disposable hemocytometers. The cells were seeded at 30,000 cells per cm² at 37°C in a 5% CO₂ humidified incubator for rapid growth. Passage numbers 3-5 were used for subsequent experiments. RT-qPCR, western blotting, Scrape loading/dye transfer assay, Cell Counting Kit-8 (CCK-8) assay, Transwell migration assay, and immunofluorescence assay were performed in a biological safety cabinet (Suzhou Antai Airtech Co., Ltd.). An angiogenesis 96-well microplate was used for the tube formation assay.

Tube formation assay. Matrigel (Corning, Inc.) was placed on ice and refrigerated (-4°C) overnight the day before cells were seeded. Matrigel (10 µl) was then applied to each inner well, and the slide was covered with a lid. Next, the slide was placed in an incubator for polymerization (60 min). In addition, the cell suspension was prepared. For a final density of 15,000 cells/well, a cell suspension of 3×10^5 cells/ml was used from each group (normal control group, connexin 43 group and small-interfering RNA group; resuspended in antibiotic-free standard medium) and pipetted up and down to mix thoroughly. The slide was removed from the incubator and placed on a

rack, and 50 µl of cell suspension was added to each top well of the slide (repeated six times for each group). The slide was covered with the lid and observed for well-proportioned cells. The cells were then incubated at 37°C for 20 h in a humidified incubator. Finally, images were acquired using a microscope (inverted microscope; Olympus Corporation; magnification, x50) and preserved. Tube network formation was assessed by measuring all parameters with ImageJ Angiogenesis Analyzer (ImageJ bundled with 64-bit Java 1.8.0_172; National Institutes of Health), including the number of branches and nodes and the length and spacing of the branches, which were selected for evaluating the angiogenic potential.

Scrape loading/dye transfer and characterization of gap junction channel function. Cells were cultured in a 6-well plate to 100% confluency (inverted microscope; Olympus Corporation) as aforementioned. The medium was discarded via suction with a pipette, and Dulbecco's phosphate-buffered saline (DPBS) (Ca/Mg) (product no. 14040133; Gibco; Thermo Fisher Scientific, Inc.) was used to wash the cells three times (2 ml/well). Cells in each well were then covered with LY dye solution (Lucifer Yellow CH; Invitrogen; Thermo Fisher Scientific, Inc.; 1 ml); the dye was loaded onto the cells by scratching one line with a blade tip of a no. 15 surgical scalpel. Subsequently, the plates were kept on a clean bench at room temperature for up to 4 min, allowing the LY dye to pass across several adjacent cell layers through functional gap junctions. To minimize fluorescence photobleaching of the dye, the plates were concurrently covered with aluminum foil. LY dye solution was then discarded from the plate wells. The cells were rinsed twice with DPBS (Ca/Mg) to discard the extracellular dye, and 2 ml of normal medium was then added into every well. The cells were immediately observed using a fluorescence microscope (Olympus Corporation) at a 100-fold magnification. The fluorescence distance of the dye spread was measured using ImageJ (ImageJ bundled with 64-bit Java 1.8.0_172; National Institutes of Health) This experiment was performed eight times. Four images and associated results were randomly selected from the normal control group, connexin 43 group and small-interfering RNA group.

CCK-8 assay. Once the cells reached 90% confluence (inverted microscope; Olympus Corporation), conventional hemocytometer-based cell counting was performed. Each of the 60 wells in the center of a 96-well plate was pipetted with 100 µl of cells (concentration 2×10^5 cells/ml), and the surrounding wells were pipetted with an equal volume of PBS (100 µl). Cells were labeled and incubated at 37°C for 24 h. After 24 h of serum deprivation in an equal volume of serum-free medium, the cells were washed once with PBS. Then cells were grouped (normal control group, connexin 43 group and small-interfering RNA group). Six replicates were performed for each of the groups, and the cells were cultured for 24 h after treatment. A 10% CCK-8 solution was prepared by adding 1.2 ml of CCK-8 (Dojindo Molecular Technologies, Inc.) into 12 ml of Endothelial Cell Medium (ScienCell Research Laboratories, Inc.). The treated cells were washed with PBS, and 100 µl of 10% CCK-8 was added to each well. Following incubation for 3 h, the absorbance (OD value) at 450 nm was measured using a microplate reader.

Table I. Sequences of the primers and lengths of the corresponding PCR products.

Gene	Primer	Base sequence (5'-3')	Tm	GC (%)	Product size (bp)	
GAPDH	NM_001256799.2	Sense	CATCATCCCTGCCTCTACTGG	59.4	57.1	259
		Antisense	GTGGGTGTCGCTGTTGAAGTC	60.1	57.1	
CX43	NM_000165.5	Sense	AAGGTTCAAGCCTACTCAACTGC	59.9	47.8	188
		Antisense	ACATGAGAGATTGGGAAAGACTTG	59.4	41.7	
ZO-1	NM_001301025.1	Sense	CAAGCCTGCAGAGTCCAAGC	60.7	60	139
		Antisense	TGAAGGTATCAGCGGAGGGA	60.3	55	
E-CAD	NM_001317184.1	Sense	TTCTTCGGAGGAGAGCGG	58.3	61.1	234
		Antisense	CAATTTTCATCGGGATTGGC	58.5	47.4	
β -catenin	NM_001098209.1	Sense	GCCAAGTGGGTGGTATAGAGG	58.7	57.1	192
		Antisense	GGGATGGTGGGTGTAAGAGC	59	60	
vWF	NM_000552.5	Sense	TGGAGAAACAGTGAAGATTGGC	59.4	45.5	168
		Antisense	ACCAGAACGTACTGGCACTCC	59	57.1	
PAI-1	NM_000602.4	Sense	ATTACTACGACATCCTGGAAGTGC	59.6	45.8	106
		Antisense	AGAATGTTGGTGAGGGCAGAG	59.1	52.4	

Tm, melting temperature, the melting temperature (Tm) by definition is the temperature at which one half of the oligo (primer) duplex will dissociate to become single stranded and indicates the duplex stability; GC (%), the GC content is the number of G's and C's in the primer as a percentage of the total bases; CX43, connexin 43; ZO-1, zonula occludens-1; vWF, von Willebrand factor; PAI-1, plasminogen activator inhibitor-1.

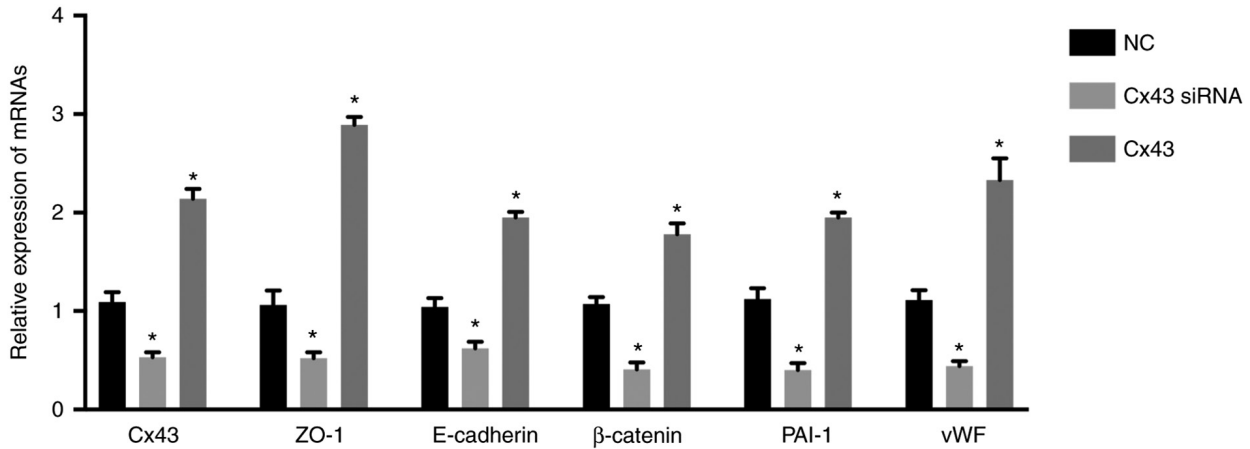


Figure 1. Relative expression levels of various mRNAs in different groups determined using RT-qPCR (the resulting data are presented as the mean \pm standard deviation; * $P < 0.01$ vs. NC; $n = 3$ in each group). RT-qPCR, reverse transcription-quantitative polymerase chain reaction; NC, normal control group; Cx43, connexin 43; ZO-1, zonula occludens-1; vWF, von Willebrand factor; PAI-1, plasminogen activator inhibitor-1; siRNA, small-interfering RNA.

Transwell migration assays. Cells were counted using a hemocytometer, and the concentration was adjusted to 1×10^5 cells/ml. In a Transwell plate (pore size, $3 \mu\text{m}$; thickness, $10 \mu\text{m}$; diameter, 12mm), $200 \mu\text{l}$ of solution of each group (normal control group, connexin 43 group and small-interfering RNA group) was respectively added to corresponding chamber; $800 \mu\text{l}$ complete medium was added to each of the three wells. Cells were labeled and cultured for 24 h; after a single PBS wash, the non-migrated cells were then swabbed with cotton; whereas, migrated cells were fixed using paraformaldehyde (concentration, 4%; duration, 30 min; room temperature). After 20 min of 0.1% crystal violet staining at room temperature, the migrated cells were

washed thrice with PBS and the entire chamber was cleaned to remove any residual dye. The filter membrane was air-dried, cut and examined under a microscope (inverted microscope; Olympus Corporation) on a glass slide. Three fields were selected at random for cell counting.

Western blotting. Total protein was extracted from the three groups of cells using Cell Lysis Buffer (Cell Signaling Technology, Inc.), and the protein concentration was measured using a BCA protein assay kit (cat. no. AS1086; Wuhan Aspen Biotechnology Co., Ltd.). Subsequently, $50 \mu\text{g}$ of total protein from each sample was added to 10% polyacrylamide gel electrophoresis gels. According to the

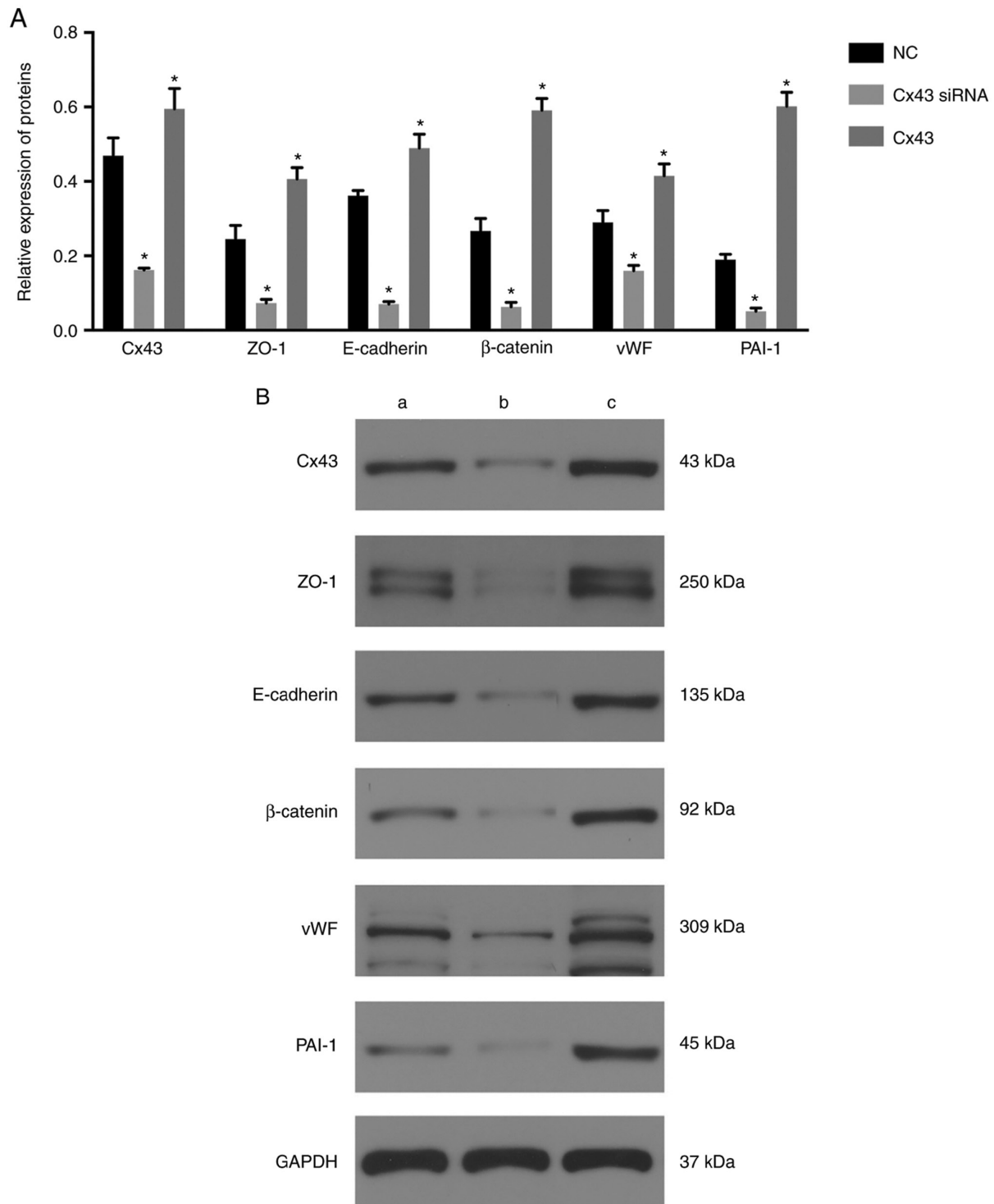


Figure 2. Relative expression levels (normalized with NC) of various proteins in different groups as determined through western blotting (the resulting data are presented as the mean \pm standard deviation; * $P < 0.01$ vs. NC; $n = 5$ in each group). (A) Densitometric analysis and (B) representative western blots. Groups: a, NC; b, Cx43-siRNA; and c, Cx43. NC, normal control group; Cx43, connexin 43; ZO-1, zonula occludens-1; vWF, von Willebrand factor; PAI-1, plasminogen activator inhibitor-1; siRNA, small-interfering RNA.

pre-stained marker display, after judging that the target protein was sufficiently separated, electrophoresis was terminated. The proteins were then electroblotted onto a PVDF membrane and the membrane was blocked with 1% western blocking reagent (product no. 11921681001; Roche Diagnostics GmbH) at room temperature for 1 h. Subsequently, the membrane was successively incubated

in the primary antibody solutions for the targeted proteins [p-Cx43 (Tyr265) antibody (Affinity Biosciences), p-Cx43 (Ser279) antibody (Affinity Biosciences), p-Cx43 (Ser368) antibody (Affinity Biosciences), p-Cx43 (Ser373) antibody (Invitrogen; Thermo Fisher Scientific, Inc.), Cx43 antibody (Affinity Biosciences), ZO-1 antibody (Abcam), E-cadherin antibody (Cell Signaling Technology, Inc.), β -catenin

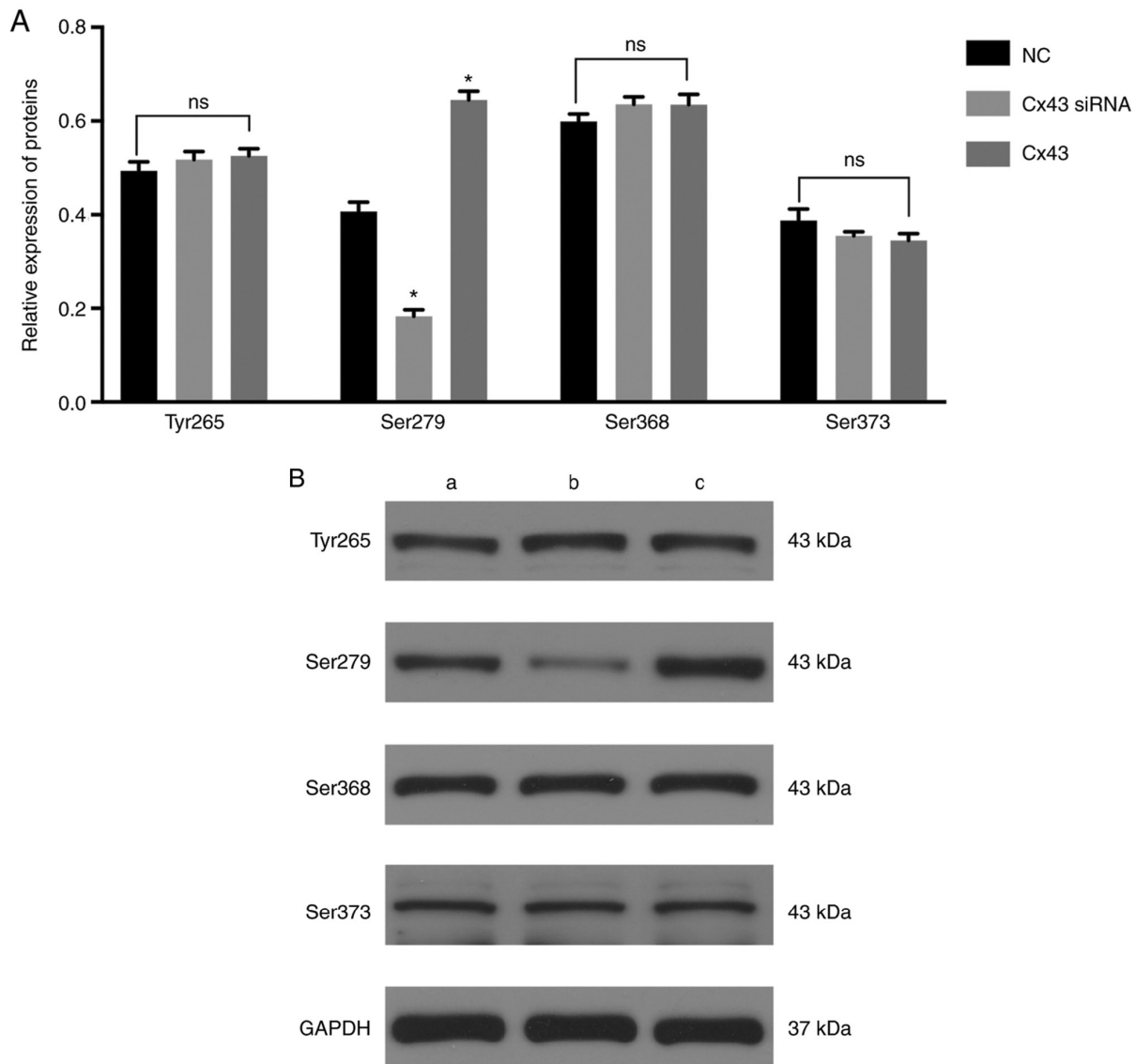


Figure 3. Relative expression levels (normalized with NC) of various proteins in different groups as determined using western blotting (the resulting data are presented as the mean \pm standard deviation; * $P < 0.01$ vs. NC; ns, $P > 0.05$ vs. NC; $n = 5$ in each group). (A) Densitometric analysis and (B) representative western blots. Groups: a, NC; b, Cx43-siRNA; c, Cx43. NC, normal control group; Cx43, connexin 43; siRNA, small-interfering RNA; ns, not significant.

antibody (Cell Signaling Technology, Inc.), vWF antibody (Cell Signaling Technology, Inc.), PAI-1 antibody (Abcam), Na^+/K^+ -ATPase (Abcam) and GAPDH (Abcam); the antibodies were diluted [p-Cx43 (Tyr265), 5% skim milk, 1:500; p-Cx43 (Ser279), 5% skim milk, 1:500; p-Cx43 (Ser368), 5% skim milk, 1:500; p-Cx43 (Ser373), 5% skim milk, 1:500; Cx43, 5% skim milk, 1:3,000; ZO-1, 5% skim milk, 1:500; E-cadherin, 5% skim milk, 1:2,000; β -catenin, 5% skim milk, 1:1,000; vWF, 5% skim milk, 1:1,000; PAI-1, 5% skim milk, 1:1,000; Na^+/K^+ -ATPase, 5% skim milk, 1:1,000; GAPDH, 5% skim milk, 1:10,000] for one night at 4°C and then incubated in the HRP-conjugated secondary antibody solution [HRP-Goat anti Rabbit (cat. no. AS1107; Wuhan Aspen Biotechnology Co., Ltd.; the antibody was diluted (5% skim milk, 1:10,000)] for 30 min at 37°C. An appropriate amount of ECL substrate solution (cat. no. AS1059; Aspen Biotechnology Co., Ltd.) was then added to each membrane, and the membrane was incubated for several minutes. After the bands appeared, filter paper was used to absorb the excess

substrate solution. The membrane was then exposed to an X-ray film. The film was placed in the developer solution for development and fixing solution for fixing (scanner; Canon, Inc.). Densitometry was performed using AlphaEaseFC™ 4.0 software (ProteinSimple).

RT-qPCR. TRIpure Total RNA Extraction Reagent [cat. no. EP013; Elk (Wuhan) Biotechnology Co., Ltd.] was used to extract the total RNA of the cells from each group (normal control group, connexin 43 group, and small-interfering RNA group), and 3.75 μg of total RNA was used for reverse transcription of RNA to cDNA [EntiLink™ 1st Strand cDNA Synthesis Kit; cat. no. EQ003; Elk (Wuhan) Biotechnology Co., Ltd.], and the cDNA was subjected to PCR to detect the target gene. GAPDH was used as a reference gene. The conditions of the reverse transcription reaction were: 42°C for 60 min and 95°C for 5 min. The semi-quantitative reaction conditions were: 94°C for 4 min, 94°C for 30 sec, 56°C for 30 sec, and 72°C for 25 sec for 30 cycles. Real-time PCR was performed again,

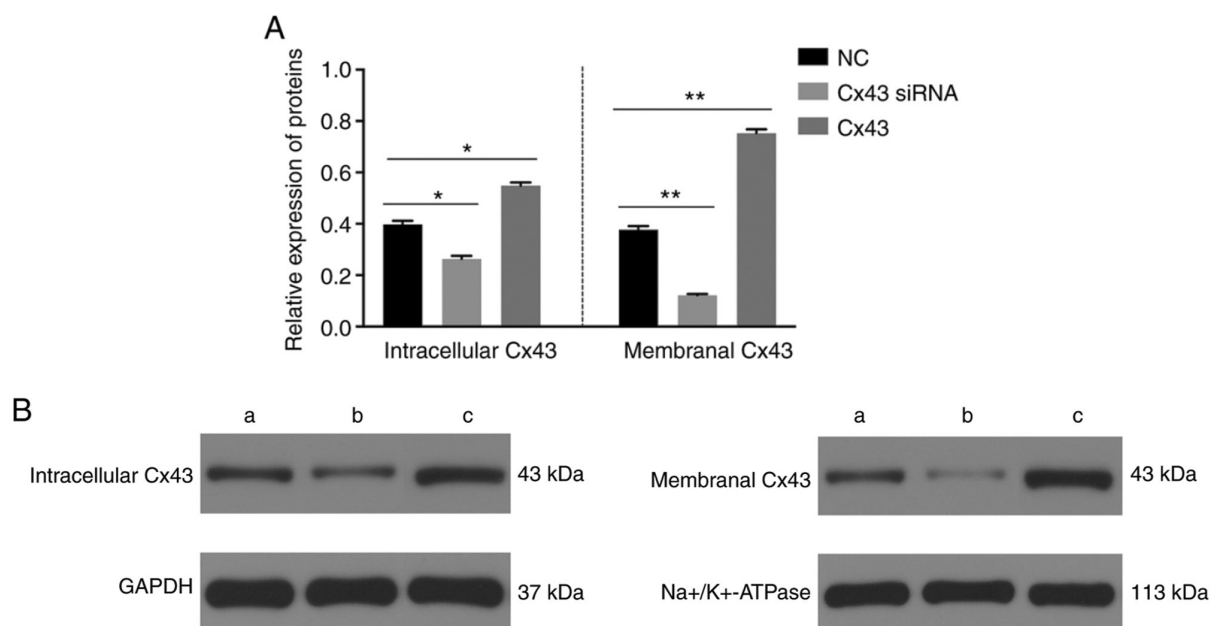


Figure 4. Relative intracellular and membranal expression levels of Cx43 in different groups as determined using western blotting (the resulting data are presented as the mean \pm standard deviation; * P <0.05 vs. NC; ** P <0.01 vs. NC; n =5 in each group). (A) Densitometric analysis and (B) representative western blots. Groups: a, NC; b, Cx43-siRNA; c, Cx43. NC, normal control group; Cx43, connexin 43; siRNA, small-interfering RNA.

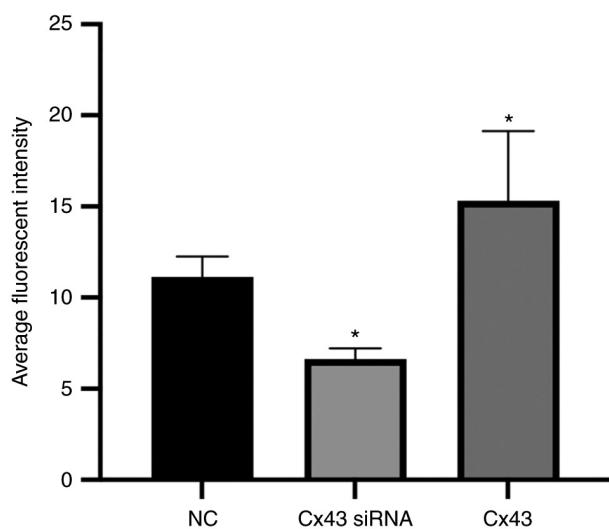


Figure 5. Average fluorescence intensity of various groups following immunofluorescence assay (the resulting data are presented as the mean \pm standard deviation; * P <0.01 vs. NC; n =5 in each group). NC, normal control group; Cx43, connexin 43; siRNA, small-interfering RNA.

cDNA was diluted 10 times, and the reaction conditions were: 50°C for 2 min, 95°C for 10 min, 95°C for 30 sec, and 60°C for 30 sec for 40 cycles. The PCR products were analyzed by electrophoresis in agarose gels (1% gel) stained with ethidium bromide, and the gel was imaged using a gel imaging system and then scanned in grayscale. Integral optical density (IOD) was used to represent the expression level (real-time PCR system; Thermo Fisher Scientific, Inc.). 6-carboxyfluorescein (product code c1360; Invitrogen; Thermo Fisher Scientific, Inc.) was used as a fluorophore in the process. The ratio of the expression of target gene to that of GAPDH was analyzed, and the relative expression level of the target gene was calculated

using $2^{-\Delta\Delta Cq}$ method (26). The sequences of the primers are listed in Table I. The experiment was performed in triplicate.

Statistical analysis. SPSS 22.0 (IBM Corp.) software was utilized for statistical analysis. The data are presented as the mean \pm standard deviation. Experimental groups were compared using one-way analysis of variance followed by Student-Newman-Keuls post hoc test. P <0.05 was considered to indicate a statistically significant difference.

Results

Expression of Cx43 and other angiogenesis-related mRNAs in HPMECs. Following transfection of pcDNA3.1-CX43-HA plasmid, the mRNA level of Cx43 in the Cx43 overexpression group (i.e., Cx43 group) increased by approximately two-fold, whereas following the transfection of Cx43-specific siRNA in the Cx43-knockdown group (i.e., Cx43 siRNA group), it decreased to $48.62 \pm 0.50\%$ compared with that in the normal control group (i.e., NC group) (P <0.01) (Fig. 1). The mRNA levels of CX43, ZO-1, E-cadherin, β -catenin, PAI-1, and vWF were significantly increased in the Cx43 group and significantly decreased in the Cx43-siRNA group compared with those in the normal control group (P <0.01) (Fig. 1). These results suggested that overexpression of Cx43 stimulated the mRNA expression levels of ZO-1, E-cadherin, β -catenin, PAI-1, and vWF, while silencing of Cx43 suppressed their expression levels.

Silencing of Cx43 suppresses and overexpression of Cx43 induces ZO-1, E-cadherin, β -catenin, vWF, and PAI-1 expression in HPMECs. The expression levels of Cx43, ZO-1, E-cadherin, β -catenin, vWF, and PAI-1 were significantly decreased in the Cx43 siRNA group and significantly increased in the Cx43 group compared with those in the normal control group (P ≤0.01) (Fig. 2). These results suggested

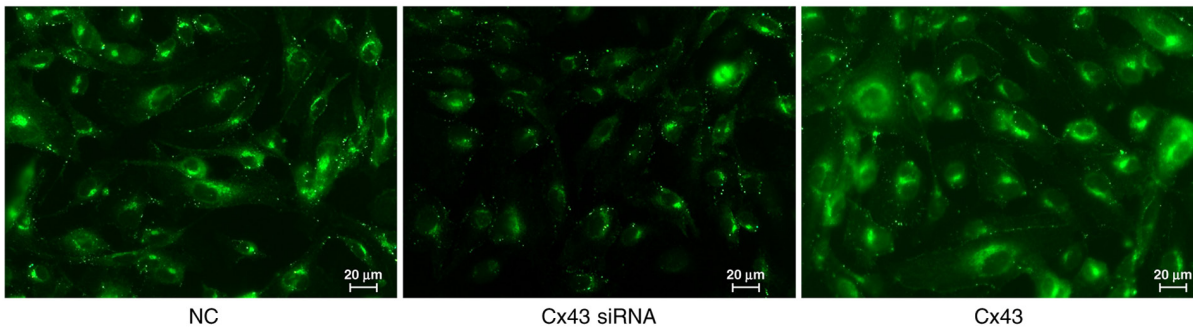


Figure 6. Cell morphology among various groups following immunofluorescence assay (magnification, x400; scale bar, 20 μm). NC, normal control group; Cx43, connexin 43; siRNA, small-interfering RNA.

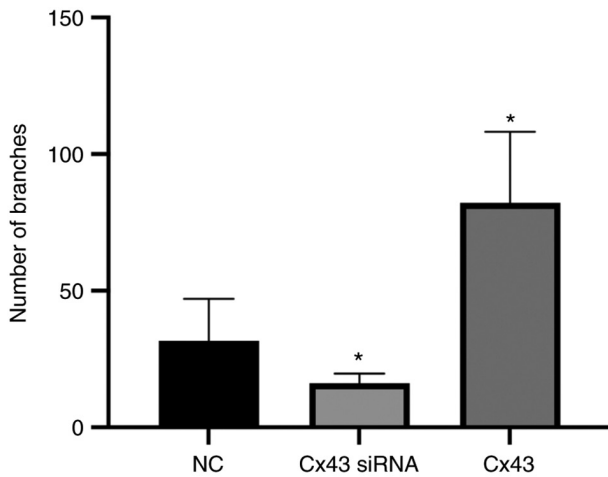
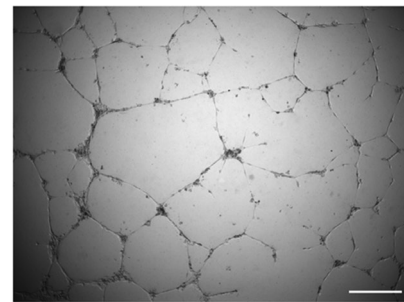
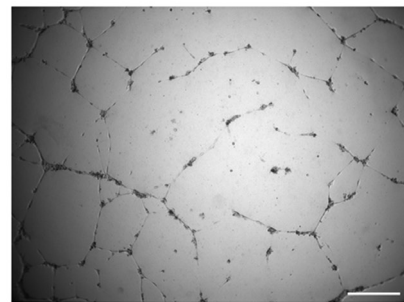


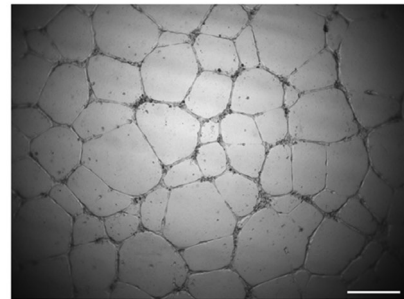
Figure 7. Number of branches in different groups following tube formation assay (the resulting data are presented as the mean \pm SD; * $P < 0.01$ vs. NC; $n = 30$ in each group). NC, normal control group; Cx43, connexin 43; siRNA, small-interfering RNA.



NC



Cx43 siRNA



Cx43

Figure 8. Cell morphology observed via tube formation assay (magnification, x50; scale bar, 250 μm). NC, normal control group; Cx43, connexin 43; siRNA, small-interfering RNA.

that overexpression of Cx43 stimulated the expression of ZO-1, E-cadherin, β -catenin, vWF, and PAI-1, whereas silencing of Cx43 suppressed the expression of these molecules compared with that in the normal control group.

Silencing of Cx43 suppresses and overexpression of Cx43 induces VEGF-associated Ser279 expression in HPMECs. The levels of Ser279 (Fig. 3) and intracellular and membranal levels of Cx43 were significantly decreased in the Cx43-knockdown group, particularly the membranal levels of Cx43 (Fig. 4), whereas the levels were significantly increased in the Cx43 group compared with those in the normal control group ($P < 0.01$) (Figs. 3 and 4). Conversely, the levels of Tyr265, Ser368, and Ser373 in the Cx43-knockdown and overexpression groups were not significantly different from those in the normal control group (Fig. 3). These results suggested that the overexpression of Cx43 stimulated the levels of Ser279 and intracellular and membranal levels of Cx43, particularly the membranal levels of Cx43.

Average fluorescence intensity of different groups. Immunofluorescence results showed that the average fluorescence intensity for the Cx43 overexpression group was higher, whereas for the Cx43-knockdown group it was lower compared

with that for the normal control group (x400; $P < 0.01$). The average fluorescence intensity in the Cx43 overexpression group was increased by 1.4-fold, whereas in the Cx43-knockdown group it was decreased by approximately 0.6-fold compared with that in the normal control group (Figs. 5 and 6).

Cx43 overexpression improves angiogenic potential of HPMECs. Using tube formation assay, the angiogenic potential

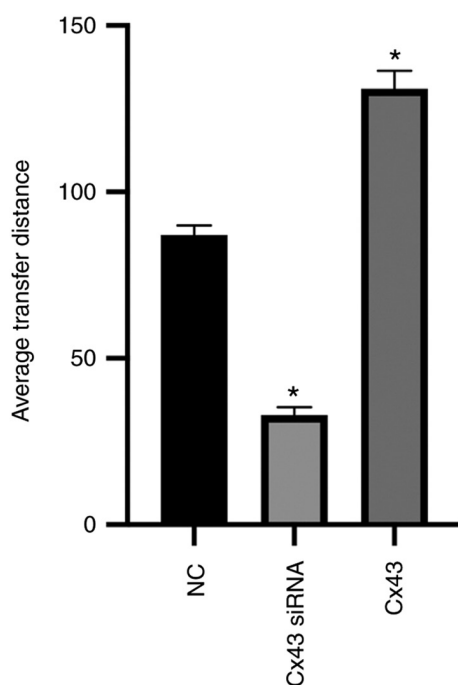


Figure 9. Comparison of the average dye transfer distance among different groups using scrape loading/dye transfer assay (the resulting data are presented as the mean \pm SD; * $P < 0.01$ vs. NC; $n = 8$ in each group). Units are in μm . NC, normal control group; Cx43, connexin 43; siRNA, small-interfering RNA.

(number of segments) was analyzed. The angiogenic potential of HPMECs was increased in the Cx43 overexpression group, whereas it was decreased in the Cx43-knockdown group compared with that in the normal control group ($P < 0.01$). The angiogenic potential in the Cx43 overexpression group was increased by two-fold, whereas that in the Cx43-knockdown group was decreased by approximately 75% compared with that in the normal control group (Figs. 7 and 8).

Cx43 promotes scrape loading dye transfer in HPMECs. Scrape loading/dye transfer assay revealed that the average dye transfer distance of the Cx43 overexpression group was the highest among all groups. It was approximately 1.5-fold higher than that of the normal control group. Conversely, the average dye transfer distance of the Cx43-knockdown group was lower than the other groups; it was approximately 60% lower than that of the normal control group ($P < 0.01$) (Figs. 9 and 10).

Cx43 stimulates proliferation of HPMECs. CCK-8 assay showed that the relative absorbance value (OD value) in the Cx43 overexpression group was increased by approximately 30% compared with that in the normal control group, whereas in the Cx43-knockdown group it was decreased by approximately 25% compared with that in the normal control group ($P < 0.01$) (Fig. 11). These results indicated that overexpression of Cx43 significantly promoted the proliferation of HPMECs, whereas silencing of Cx43 significantly inhibited the proliferation of the cells.

Cx43 stimulates migration of HPMECs. Transwell migration assay revealed that the number of migrating cells in the

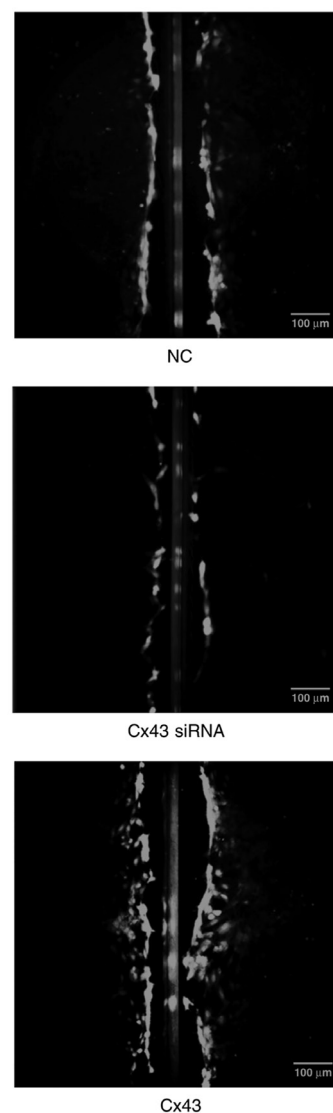


Figure 10. Fluorescence observed in various groups using scrape loading dye transfer assay (magnification, $\times 100$; scale bar, $100 \mu\text{m}$). NC, normal control group; Cx43, connexin 43; siRNA, small-interfering RNA.

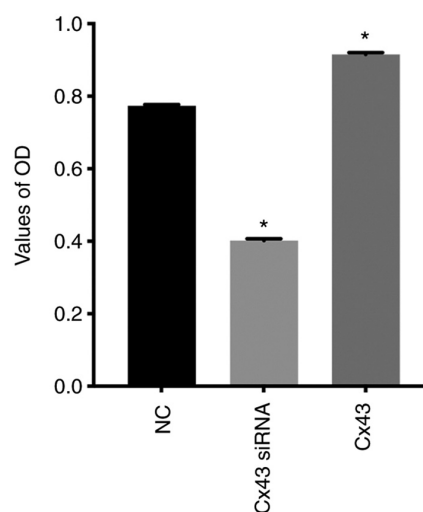


Figure 11. Comparison of the OD value among different groups via CCK-8 assay (the resulting data are presented as the mean \pm SD; * $P < 0.01$ vs. NC; $n = 3$ in each group). OD, optical density; CCK-8, Cell Counting Kit-8; NC, normal control group; Cx43, connexin 43; siRNA, small-interfering RNA.

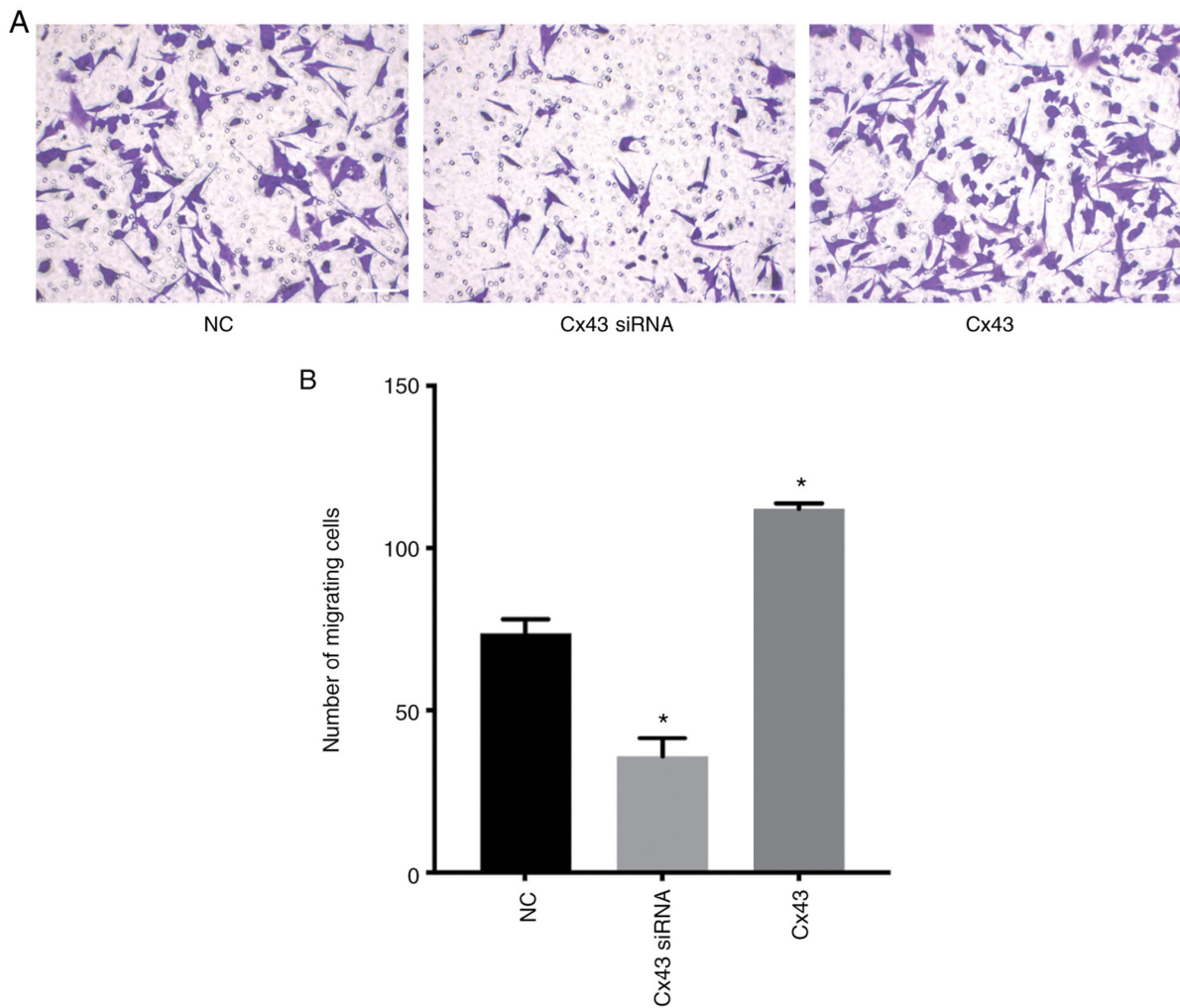


Figure 12. Comparison of the number of migrating cells in the different groups with Transwell migration assay (the resulting data are presented as the mean \pm SD; * $P < 0.01$ vs. NC; $n = 5$ in each group). (A) Representative images of migrating cells (magnification, $\times 100$; scale bar, $25 \mu\text{m}$) and (B) analysis of number of migrating cells. NC, normal control group; Cx43, connexin 43; siRNA, small-interfering RNA.

Cx43 overexpression group was increased, whereas in the Cx43-knockdown group it was decreased compared with that in the normal control group ($P < 0.01$) (Fig. 12). These results indicated that Cx43 overexpression significantly promoted the migration of HPMECs, while silencing of Cx43 significantly inhibited cell migration.

Discussion

Angiogenesis is the process through which blood vessels sprout from pre-existing blood vessels into avascular tissues, following which they undergo reconstruction such that the original irregular blood vessel network is developed into functional branch blood vessels with complex three-dimensional structures (27). It is jointly regulated by growth factors, pro-angiogenic cytokines, and neovascularization inhibitors (28,29). As an indispensable link in the vascularization process, angiogenesis involves proliferation, survival, migration, and differentiation of endothelial cells (30), which play a major role in tumor growth and metastasis (27,31). Moreover, angiogenesis is a major hurdle in the treatment of lung cancer. Therefore, preventing angiogenesis plays an important role

in successfully controlling the progression of lung cancer. Consequently, exploring new methods of anti-angiogenesis therapy is essential for lung cancer treatment.

The phosphorylation site of Cx43 is located at the C-terminus of the cytoplasm, which contains multiple serine, threonine, and tyrosine residues, hence can be phosphorylated by various protein kinases, such as MAPK, PKA, PKC, p34 (Cdc2)/cyclin B kinase, CK1, and pp60 (src) kinase (7,12,22,23). Phosphorylation at specific sites may play a regulatory role in the internalization and degradation of Cx43 channels, thereby affecting gap junctions (10,32). In addition, during normal development and disease processes, protein-protein interactions and modification of the C-terminal of Cx43 are key determinants of gap junction function, size, distribution, and organization (33). Phosphorylation is the primary way to modify the carboxyl end of Cx43. Therefore, phosphorylation of Cx43 may become a new focus of future research.

In the present study it was determined that after mature HPMECs were transfected with the Cx43-overexpressed gene, the expression of adhesion marker proteins ZO-1, E-cadherin, and β -catenin, pro-thrombotic factor vWF, and coagulation factor PAI-1 increased, and the changes in the protein levels

of these molecules were consistent with the changes in their mRNA levels. Following intracellular inhibition of Cx43 using a specific siRNA, the mRNA and protein levels of the aforementioned molecules were significantly reduced. Therefore, overexpression of Cx43 was capable of stimulating the downstream ZO-1, E-cadherin, β -catenin, vWF, and PAI-1 signaling proteins at the molecular and gene levels, which was associated with angiogenesis. Moreover, the proliferation and migration of cells in the overexpression group were significantly increased, whereas those in the silenced group were significantly reduced, suggesting that overexpression of Cx43 can promote the proliferation and migration of HPMECs, which is inconsistent with the results from a study by Koepple *et al*, in which the proliferation level was unchanged (21). It is therefore theorized that the reason for this inconsistency, is the diverse impact of Cx43 on different cell types. However, this hypothesis warrants further investigation. The tube formation experiments indicated that Cx43 can promote angiogenesis while silencing of the Cx43 gene can inhibit the process. This previous study by Koepple *et al* (21) found that using Cx43 mimetic peptide Gap27, after transfection of Cx43 cDNA to block Cx43-dependent gap junctional communication, did not affect angiogenic potential of endothelial cells compared to cells only transfected with Cx43 cDNA, and dye transfer was significantly attenuated in cells treated with Gap27 compared with the solvent control, which demonstrated that the expression of Cx43 stimulated endothelial angiogenesis independently of gap junctional communication *in vitro*. In the present study, it was also determined that the overexpression of Cx43 could improve the dye transfer, while the transfection of Cx43-specific siRNA reduced dye transfer distance distinctly, and these were consistent with their results (21). However, further experiments to verify the specific function of gap junction communication during angiogenesis should be performed. In addition, the expression of Ser279 was increased in the Cx43 overexpression group, and decreased in the silenced group, which demonstrated that Cx43 modulates angiogenesis depending on the activation of its C-terminal phosphate site-Ser279 and the downstream ZO-1, E-cadherin, β -catenin, vWF, and PAI-1 signaling proteins. Following overexpression of Cx43, the intracellular and membranal content of Cx43 was increased; in particular, Cx43 expression in the cell membrane was significantly increased. Conversely, after silencing of Cx43, the Cx43 protein level was significantly decreased, particularly in the cell membrane. These results suggest that the angiogenesis effect of Cx43 may rely on the membranal Cx43 expression level.

A siRNA targeting Cx43 and a recombinant vector that overexpressed the Cx43 gene were successfully designed and constructed. Following transfection of HPMECs, it was revealed that overexpression of the Cx43 gene could induce pulmonary angiogenesis *in vitro*. Cx43 overexpression induced angiogenesis by promoting cell proliferation and migration and may be associated with the activation of adhesion marker proteins ZO-1, E-cadherin, and β -catenin, thrombosis-related factor vWF, and coagulation factor PAI-1. A limitation of the present study is that it only identified proteins ZO-1, E-cadherin, β -catenin, vWF, and PAI-1 as participants in the process of Cx43-associated angiogenesis. However, if these proteins play direct roles or are affected by some other key intermediate proteins of the signaling pathway requires further

research. This pro-angiogenesis process may be activated through activation of intracellular signaling site Ser279 on the C-terminal. In addition, the results demonstrated that silencing of the Cx43 gene using Cx43-siRNA mainly affected the integration of Cx43 and reduced the distribution of Cx43 in the cell membrane, while overexpression of Cx43 had the opposite effect. However, further research should be performed to confirm this finding. In subsequent research, the construction of a Cx43 mutant body (Ser279), protein-protein interactions and other molecular biotechnologies could be used to explore their specific effects.

In summary, the present study demonstrated that activation of the phosphorylation site Ser279 at the C-terminal of Cx43 was an important pathway for Cx43 to regulate angiogenesis *in vitro*. This also promoted angiogenesis by stimulating cell proliferation, migration, and distribution of Cx43 to the cell membrane, possibly via the activation of the downstream ZO-1, E-cadherin, β -catenin, vWF, and PAI-1 signaling proteins. The results indicated that Cx43 and its intracellular signaling site Ser279 on the C-terminal are potential new targets for regulating angiogenesis and exploring new treatment approaches for angiogenesis-associated diseases.

Acknowledgements

Not applicable.

Funding

The present study was supported by the Natural Science Foundation of Shenzhen University General Hospital (grant no. SUGH2019QD007 to ZZ, and grant no. SUGH2019QD014 to XZ) and the Science and Technology Foundation of Nanshan District, Shenzhen (grant no. NS2021167 to ZZ).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ZZ, WC, YiL, YaL, HP, QW and XZ contributed to the study concept, design, experiments, data collection, analysis, and interpretation. ZZ wrote the manuscript. XZ revised the manuscript and gave final approval of the version to be published. ZZ and XZ confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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