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Chloride intracellular channel 4 is involved in endothelial proliferation and morphogenesis in vitro

Jennifer J. Tung,

Department of Obstetrics/Gynecology and Pathology, Herbert Irving Comprehensive Cancer Center, Columbia University Medical Center, 1130 St. Nicholas Ave, 923, New York, NY 10032, USA

Oliver Hobert,

Biochemistry and Molecular Biophysics, Howard Hughes Medical Institute, Columbia University Medical Center, New York, NY 10032, USA

Mark Berryman, and

Department of Biomedical Sciences, Ohio University College of Osteopathic Medicine, Athens, OH 45701, USA

Jan Kitajewski

Department of Obstetrics/Gynecology and Pathology, Herbert Irving Comprehensive Cancer Center, Columbia University Medical Center, 1130 St. Nicholas Ave, 923, New York, NY 10032, USA

Jan Kitajewski: jkk9@columbia.edu

Abstract

New capillaries are formed through angiogenesis and an integral step in this process is endothelial tubulogenesis. The molecular mechanisms driving tube formation during angiogenesis are not yet delineated. Recently, the chloride intracellular channel 4 (CLIC4)-orthologue EXC-4 was found to be necessary for proper development and maintenance of the *Caenorhabditis elegans* excretory canal, implicating CLIC4 as a regulator of tubulogenesis. Here, we studied the role of CLIC4 in angiogenesis and endothelial tubulogenesis. We report the effects of inhibiting or inducing CLIC4 expression on distinct aspects of endothelial cell behavior in vitro. Our experiments utilized RNA interference to establish cultured human endothelial cell lines with significant reduction of CLIC4 expression, and a CLIC4-expressing lentiviral plasmid was used to establish CLIC4 overexpression in endothelial cells. We observed no effect on cell migration and a modest effect on cell survival. Reduced CLIC4 expression decreased cell proliferation, capillary network formation, capillary-like sprouting, and lumen formation. This suggests that normal endogenous CLIC4 expression is required for angiogenesis and tubulogenesis. Accordingly, increased CLIC4 expression promoted proliferation, network formation, capillary-like sprouting, and lumen formation. We conclude that CLIC4 functions to promote endothelial cell proliferation and to regulate endothelial morphogenesis, and is thus involved in multiple steps of in vitro angiogenesis.

Keywords

Angiogenesis; CLIC4; Endothelial cells; Proliferation; Tubulogenesis

Introduction

Angiogenesis entails the formation of new blood vessels from the sprouting, branching, and pruning of pre-existing vessels and is essential for embryonic development, proper organ development, tissue growth, and repair. Deviations in the regulation of angiogenesis are important factors in pathological processes such as atherosclerosis, diabetic retinopathy, and malignant tumor growth. The basic cellular mechanisms of angiogenesis begin with proangiogenic factors stimulating endothelial cells to degrade the local basement membrane surrounding an existing vessel followed by endothelial cell rearrangement, proliferation, and migration into the surrounding stroma [1]. A neo-vessel is generated when new sprouts form lumens in a process termed tubulogenesis. After the formation of lumens, anastomosis of tubes occurs, which involves branching out and reconnecting to form networks of vessels. Pruning of elementary sprouts follows and the basement membrane is reformed. Finally, accessory cells such as pericytes or smooth muscle cells are recruited to help stabilize the vessel and provide control of luminal diameter.

In endothelial biology, the critical underlying process of endothelial tube formation and tube maintenance is still poorly understood. There are several current models for mammalian vascular tubulogenesis. One model involves a chain of endothelial cells forming vesicles that enlarge, merge, and fuse intra- and inter-cellularly to form contiguous lumens [2]. This process involves the acquisition of cell apical-basal polarity, cytoskeletal reorganization, assembly of intercellular junctions, and membrane domain specification [3]. The formation of the *Caenorhabditis elegans* excretory canal provides an example of this process [4].

Recently, members of the glutathione *S*-transferase-related chloride intracellular channel (CLIC) protein family have been implicated in tubulogenesis. The CLIC family, composed of seven family members in mammals, is defined by a roughly 230 conserved amino acid core sequence at the C-terminus [5]. CLIC family proteins are widely expressed in multicellular organisms and are involved in a variety of processes including tube formation, secretion, cell division, apoptosis, and cell motility [6]. Several CLIC family proteins have been shown to form intracellular ion channels that auto-assemble and auto-insert into specific cellular membranes [6]. In addition to their association with membranes, CLIC proteins can also exist as soluble cytosolic and nuclear proteins, suggesting that CLICs may have alternate, non-channel functions [7]. Currently, only CLIC1 and CLIC4 are reported to be expressed in endothelial cells [5, 8, 9].

Among CLIC proteins, CLIC4 has been studied most extensively. CLIC4 has been shown to be associated with cytoskeletal proteins such as dynamin I, actin, and tubulin suggesting a role in cellular morphology as well as cell signaling [10, 11]. A recent study found that CLIC4-orthologue EXC-4 in *C. elegans* plays a critical role in both the proper development and the maintenance of the *C. elegans* excretory canal [4]. Lumenal cysts and other morphological defects were observed at the surface of the canal in *exc-4* mutants, providing the first evidence that CLICs function in tubulogenesis. It was also demonstrated that EXC-4 was continuously needed for normal tube size maintenance [4].

This study investigates human CLIC4 as a regulator of angiogenic processes by using in vitro techniques that mimic multiple distinct steps of angiogenesis. Based on the EXC-4 precedent, we hypothesized that reducing CLIC4 expression in endothelial cells would negatively affect angiogenesis, while increasing CLIC4 expression would promote endothelial angiogenesis. Human primary endothelial cells were used to represent their in vivo counterpart and are suggested to be better suited for in vitro studies than immortalized endothelial lines based upon assessment of gene expression patterns [12]. We indeed found that reduced CLIC4 expression levels in endothelial cells inhibited network formation and

cell proliferation. CLIC4 knockdown affected capillary-like network formation and lumen formation, and reduced CLIC4 expression had no effect on cell migration, but led to a modest effect on cell survival. CLIC4 overexpression promoted proliferation, network formation, capillary-like sprouting, and lumen formation in vitro. Together, these results indicate that CLIC4 expression is required for multiple steps of angiogenesis.

Materials and methods

Ethics statement

No human or animal subjects were used in this study, and the collection of human umbilical venous endothelial cells (HUVECs) from umbilical cords was approved by the Columbia University IRB (IRB-AAAB9642).

Cells and culture

Human umbilical venous endothelial cells were isolated from human umbilical veins as previously described [13] and cultured on dishes coated with type I rat tail collagen (VWR, West Chester, PA) in EGM-2 BulletKit medium (Lonza, Basel, Switzerland). Detroit 551 fibroblasts and 293T fibroblasts were purchased from ATCC (Manassas, VA) and cultured in Eagle's Minimum Essential Medium (EMEM) (ATCC, Manassas, VA) and Iscove's Modified Dulbecco's Medium (Invitrogen, Carlsbad, CA), respectively. Both media were supplemented with 10% FBS (Invitrogen, Carlsbad, CA), and 0.01% Pen-Strep (Invitrogen, Carlsbad, CA). Unless otherwise noted, cells were cultured under standard conditions in a humidified incubator at 37°C, 5% CO₂.

CLIC4 gene silencing and overexpression

Human CLIC4 shRNA-containing constructs were purchased from Sigma-Aldrich (St. Louis, MO) and screened for significant *CLIC4* knockdown in HUVECs by qRT-PCR and immunoblotting as described below. Oligos were provided in lentiviral vector pLKO.1-puro, which carries puromycin resistance allowing for selection of shRNA-expressing cells. Preparation of stably infected HUVEC lentiviral lines were performed as described below. Two shRNA were selected with the target sequences of 5'-GCATATAGTGATGTAGCCAAA-3' and 5'-GCCGTAA TGTGTAACAGAATT-3' denoted as constructs shRNA3 and shRNA5, respectively. pLKO.1-puro, denoted as plko sc, expressing a scrambled shRNA insert that does not target any known genes, served as a control. A full-length human *CLIC4* cDNA was prepared by RT-PCR using mRNA from HMVEC (human dermal microvascular endothelial cells) with the following primers: 5'-ATGGATCCGCCACCATGGCGTTGTCGATGCCGCTGAATG-3' forward and 5'-ATGTCGACTTACTTGGTGAGTCTTTTGG-3' reverse. The sequence of the cloned CLIC4 was determined and found to be identical with that of human CLIC4 (AF097330) except for a G to A nucleotide change in position 291, which did not affect the coding sequence. The full-length CLIC4 cDNA was cloned into lentiviral pCCL.pkg.wpre and screened for significant *CLIC4* overexpression by qRT-PCR and immunoblotting. pCCL.pkg.wpre-expressing GFP, denoted as pccl, served as a control for this overexpressing line.

Lentivirus-mediated stable expression of shRNA and CLIC4 in HUVEC

For lentiviral gene transfer, the lentiviral vector pLKO.1 was used for shRNA knockdown lines while pCCL was used for the overexpressing HUVEC line. About 2.5×10^6 293T packaging cells were seeded and transfected with 3 µg pVSVG, 5 µg pMDLg/pRRE, 2.5 µg pRSV-Rev, and either 10 µg pLKO.1-CLIC4 shRNA (CLIC4 knockdown), pLKO.1-scrambled shRNA (knockdown control), pCCL-CLIC4 (CLIC4 overexpression), or pCCL-GFP (overexpression control). Lentivirus-containing supernatants were collected 48 and 56

h after transfection, passed through a 0.45 μm filter, and added to 1×10^6 low-passage HUVECs. About 48 h after infection, pLKO.1-expressing HUVECs were selected with puromycin (3 $\mu\text{g/ml}$) for 72 h and maintained with puromycin at 1.5 $\mu\text{g/ml}$.

CLIC4 immunoblotting

HUVEC lysates were prepared in TENT lysis buffer (50 mM Tris pH 8.0, 2 mM EDTA, 150 mM NaCl, and 1% Triton X-100) containing protease inhibitors (2 μM PMSF and 1 $\mu\text{g/ml}$ each of leupeptin, pepstatin, and aprotinin). Lysates were boiled after addition of SDS sample buffer in the presence of 2-mercaptoethanol for 5 min. Protein concentrations were determined using the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA), and sample volumes were adjusted to load equivalent amounts of total protein on SDS-PAGE. After SDS-PAGE and electro-blotting onto PVDF membrane, blocking in 5% milk and incubation with primary polyclonal rabbit anti-human CLIC4 (B134) or human CLIC1 (B121) antisera [14] was performed at a dilution of 1:2500 in 2.5% milk. Peroxidase-conjugated goat anti-rabbit antibody (Sigma, St. Louis, MO) was used at 1:5000 in 2.5% milk for detection. Protein bands were visualized by Enhanced Chemiluminescence (GE Healthcare Bio-Sciences Corp., Piscataway, NJ).

Proliferation and survival analysis

Low-passage HUVECs were seeded on collagen-coated 24-well plates at 10,000 cells/well for proliferation assays and 30,000 cells/well for survival assays. For proliferation assays, cells were cultured in serum free medium (SFM) supplemented with 20 ng/ml EGF (Invitrogen, Carlsbad, CA) and 20 ng/ml recombinant human VEGF-A165 (Research Diagnostics, Inc., Concord, MA). After 96 h, cell numbers were assessed with the Cell Counting Kit-8 WST-8 assay (Dojindo Molecular Technologies, Gaithersburg, MD), and a calibration curve was generated from known numbers of cells according to the manufacturer's protocol. Survival assays were performed similarly with cells cultured either in SFM or SFM supplemented with 20 ng/ml EGF (Invitrogen, Carlsbad, CA). For survival assays, cell numbers were assessed after 24 and 48 h with the Cell Counting Kit-8 (Dojindo Molecular Technologies, Gaithersburg, MD). All assays were performed in triplicate and repeated at least three times.

Migration analysis

To determine changes in directed cell migration, cells were seeded to confluence on collagen-coated 12-well plates at 1×10^6 cells/well in EGM-2 medium (Lonza, Basel, Switzerland). After 24 h, cell monolayers were bisected by scraping away cells across the midline of each well with a pipette tip. The dislodged cells were then removed with three PBS washes, the medium was replaced, and cells were incubated under standard conditions. Migration into the scraped area was photographed at 0, 3, 6, 9, and 12 h time points. Experiments were performed in triplicate and repeated at least two times.

Network formation assay

HUVEC lines cultured in SFM supplemented with 20 ng/ml EGF (Invitrogen, Carlsbad, CA) and 20 ng/ml rhVEGF-A165 (Research Diagnostics, Inc., Concord, MA) were evaluated for the ability to form networks of cords between two layers of porcine collagen gel (Wako USA, Richmond, VA) as previously described [15]. Cells were seeded between the two layers of porcine collagen at 100,000 cells/well. Network formation and branching were documented by photography after 96 h. Experiments were performed in triplicate and replicated at least three times. Quantification for surface area was done by assessing collagen gel sandwiches 96 h post-seeding by applying MTT (Dojindo Molecular Technologies, Gaithersburg, MD) for 3 h, photographing the networks, and using Image-Pro

Plus (Media Cybernetics, Bethesda, MD) software to calculate the surface area sum occupied by colored objects, which in this case would be the MTT-treated HUVECs. Branchpoints were quantified by counting the number of branchpoints per field of vision at 10× objective for knockdown cell lines and 5× objective for overexpression lines. One branchpoint was considered any focal intersection of two or more cords. For experiments involving mitomycin C treatment, cells were exposed to mitomycin C (Sigma–Aldrich, St. Louis, MO) at 25 µg/ml for 45 min prior to seeding. After 45 min of exposure to mitomycin C, cells were used immediately for downstream applications. Mitomycin C was dissolved in dH₂O and kept at 4°C as a 1 mg/ml stock solution. Accordingly, cells without mitomycin C treatment received treatment with dH₂O.

Capillary sprouting assay

The capillary sprouting assay was performed as previously described [16] with two modifications: thrombin concentration and Detroit 551 fibroblast seeding. Briefly, HUVECs and Detroit 551 fibroblasts (ATCC, Manassas, VA) were cultured in M199 with 10% FBS and 0.01% Pen-Strep (Invitrogen, Carlsbad, CA). HUVECs were attached to dextran-coated Cytodex 3 microcarrier beads (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) at ~400 HUVECs/bead. HUVEC-coated beads were then embedded at ~250 beads/well of a 24-well plate in a fibrin clot consisting of 2 mg/ml fibrinogen (Sigma–Aldrich, St. Louis, MO), 0.15 U/ml aprotinin (Sigma–Aldrich, St. Louis, MO), and 0.0625 U/ml thrombin (Sigma–Aldrich, St. Louis, MO). After 24 h, Detroit 551 fibroblasts were seeded on top of the fibrin gel at 7.5×10^4 and 1.5×10^5 cells/well. Experiments were performed in triplicate and replicated at least three times.

Statistics

Independent two-tailed Student's *t*-tests were performed to test for significant differences in means between one cell line and its respective control for all statistical analyses shown in graphs.

Results

Altered CLIC4 expression does not significantly affect endothelial cell migration

To assess the function of CLIC4 in individual steps of angiogenesis in vitro, we generated HUVEC lines stably infected with lentivirus-based vectors to reduce or increase expression of CLIC4. CLIC4 knockdown and overexpression were confirmed at the protein level by immunoblotting (Fig. 1a, b). Immunoblotting for CLIC1 showed that CLIC4 shRNA did not target CLIC1, which is also expressed in endothelial cells, indicating that the shRNA treatments were specific for CLIC4 (Fig. 1a).

Established knockdown cell lines were referred to as shRNA3 and shRNA5 with plko sc (pLKO.1-puro vector with scrambled shRNA insert) as the control. The CLIC4 overexpression cell line was named CLIC4 with pccl (pCCL.pkg.wpre) containing GFP being its respective control. The cellular morphology of knockdown and overexpression HUVEC lines was qualitatively assessed. For this analysis, equivalent numbers of cells from each of the lines were seeded and cell cultures were photographed 48 h later, when cells were still subconfluent (Fig. 1c, d). We detected no major changes in cellular morphology with the exception of HUVEC shRNA5, which appeared more rounded relative to control cells (Fig. 1c). We noted that both HUVEC shRNA3 and HUVEC shRNA5 lines appeared more sparse than control or overexpressing cells, indicating that CLIC4 knockdown may reduce cell growth.

Previous studies have documented that CLIC4 is associated with cytoskeletal proteins in a variety of cells [10, 11, 17] and that CLIC4 expression levels affect cell motility in some cell types [18]. To determine whether endothelial cell migration is affected by reduced or increased CLIC4 expression, we utilized a migration assay to assess effects of CLIC4 on directed cell migration. For this assay, we scraped a line through confluent monolayers of the HUVEC CLIC4 knockdown and overexpression cultures grown on type I collagen. Endothelial cell migration into the cell-free area was then monitored at various time points over a 12 h period. CLIC4 knockdown or overexpression resulted in no observable effect on the migration rates of HUVECs when compared with control lines. Representative data at 3–6 h time points are shown in Fig. 2.

CLIC4 expression promotes endothelial cell proliferation

Chloride intracellular channel 4 has been documented to play a role in cell proliferation of squamous cancer cell lines and to affect survival of keratinocytes, squamous cancer cell lines, and human osteosarcoma lines [19, 20]. To explore the role of CLIC4 in endothelial cell proliferation and survival, we utilized WST-8-based survival and proliferation assays on our CLIC4 knockdown and over-expressing cell lines. Proliferation assays were performed by scoring cells after seeding equal numbers of HUVECs on type I collagen-coated plates and culturing in SFM supplemented with EGF and VEGF-A for 96 h. We found that reduction of CLIC4 expression led to significant inhibition of endothelial cell proliferation (Fig. 3a), while overexpression of CLIC4 resulted in a significant promotion of endothelial cell proliferation (Fig. 3b). These results indicate that expression of CLIC4 promotes endothelial cell proliferation.

For survival assays, equivalent numbers of knockdown, overexpression, or control HUVECs were seeded on type I collagen-coated plates and cultured for 24 or 48 h in SFM alone or SFM supplemented with the survival signal EGF. We found that in the absence of EGF, CLIC4 knockdown had no significant effect on endothelial cell survival at 24 h when compared with control lines (Fig. 4). At 48 h, knockdown lines display a modest but significant increase in cell survival (Fig. 4a). CLIC4 overexpression significantly decreased endothelial cell survival at 48 h, but not at 24 h (Fig. 4b). Thus, in contrast to the pronounced results for proliferation, CLIC4 expression may play a limited role in decreasing endothelial cell survival.

CLIC4 promotes endothelial capillary-like network formation

Since CLIC4 has been suggested to function in tubulogenesis, we assessed the effect of CLIC4 expression on endothelial cell morphogenesis by determining the ability of the various CLIC4 knockdown or overexpressing cell lines to organize into capillary-like networks.

To assess capillary-like network formation, HUVECs were seeded between two porcine collagen gel layers and cultured in SFM containing VEGF-A and EGF for 96 h. As shown in Fig. 5a, CLIC4 knockdown resulted in a dramatic reduction in cord formation, network formation, and branching, whereas overexpression resulted in a modest but significant increase in capillary-like network formation. Quantification of surface area occupied by network structures confirmed that CLIC4 knockdown results in a significant decrease in surface area occupation by endothelial cells: HUVEC shRNA3 and HUVEC shRNA5 covered only 72.7 and 70.2% of the surface area relative to HUVEC plko sc set at 100% (Fig. 5b). By comparison, HUVEC CLIC4 overexpression covered 112.4% of the surface area relative to HUVEC pccl set at 100% indicating a significant increase in endothelial surface area occupation by endothelial networks.

Results from quantifying the number of branchpoints per field of view also indicate that CLIC4 expression affects endothelial network formation. One branchpoint was considered any focal intersection of two or more cords. Knockdown cell lines exhibited a significant reduction in the number of branchpoints per field of vision when compared with plko sc control (Fig. 5c). Consistently, a significant increase in branchpoint numbers was found in CLIC4-overexpressing lines compared with control pccl (Fig. 5d). These results indicate that CLIC4 expression promotes endothelial network formation and branching.

It has been reported that the network formation assay we utilize here is dependent to some extent on cell proliferation [15]. Thus, we included an additional control group to help distinguish potential effects of CLIC4 knockdown on proliferation versus network formation. This control group consisted of plko sc cells treated with mitomycin C (mmc; 25 μ g/ml for 45 min), which blocks cell division. We found that CLIC4 knockdown resulted in significantly more severe inhibition of network formation when compared with mitomycin C-treated control cells (Fig. 6a). The inhibitory effect of mitomycin C on cell proliferation was confirmed using the proliferation assay done with endothelial cells grown as a monolayer for 120 h, the duration of the network formation assay (Fig. 6b). In fact, mitomycin C-treated control HUVECs showed greater inhibition of proliferation than HUVECs shRNA3.

Quantification of network surface area and number of branchpoints (Fig. 6c, d) confirmed that CLIC4 knockdown cells formed a less-extensive capillary-like network than mitomycin C-treated control cells. HUVEC network surface area occupation analysis indicates that HUVEC shRNA3 and shRNA5 covered only 74.1 and 63.9% of the surface area occupied by mitomycin C-treated control, respectively. Branchpoint number analysis at 5 $\times$ objective also indicates that CLIC4 knockdown lines significantly inhibit network branching when compared with mitomycin C-treated control. We conclude that reducing CLIC4 expression has effects on in vitro endothelial network formation independent from the effects of reduced CLIC4 expression on proliferation.

Altered CLIC4 expression affects capillary-like sprouting and lumen formation in vitro

Since the network structures in the previous assay are formed from cords without lumens, we assessed the effect of CLIC4 expression on in vitro capillary-like tube formation in a fibrin bead assay. HUVECs were adhered to dextran-coated Cytodex-3 beads and embedded in a fibrin clot. D551 fibroblasts were then seeded and cultured in EGM-2 medium for 10 days as a monolayer on top of the fibrin clot to provide secreted factors [21]. Photographic documentation occurred on or between days 3 and 11, with sprout formation assessment beginning on day 3; sprout extension, branching, and lumen formation assessed from days 4 to 11; and anastomosis assessed from days 7 to 11. The sprouts resulting from this assay were multicellular, lumen-containing processes, consistent with previously published results [16].

Chloride intracellular channel 4 knockdown HUVEC lines exhibited stunted sprouting and formed fewer sprouts (Fig. 7a, b). Sprouts that formed from CLIC4 knockdown HUVECs showed undersized sprouting indicating a defect in sprout elongation into the surrounding environment. Although HUVEC shRNA3 cells formed only shortened sprouts, these sprouts still contained lumen. In contrast, HUVEC shRNA5 cells, which have less CLIC4 expression than HUVEC shRNA3, showed a reduction in lumen-containing structures. Quantification of HUVEC shRNA5 results revealed that this lumen-forming defect was significant when compared with the number of lumen-containing sprouts control cells formed (Fig. 7c). Disjointed lumen formation was also observed within CLIC4 knockdown cell line shRNA5 (Fig. 7a, b). CLIC4 overexpression resulted in increased and more rapid sprouting when compared with pccl control cells (Fig. 7d). Quantification of these results

indicated that CLIC4 overexpression led to an increase in lumen-containing sprouts (Fig. 7e). Together, these data demonstrate the necessity for appropriate CLIC4 expression to allow for in vitro capillary sprouting. Disrupting CLIC4 function was found to impair HUVEC morphogenesis and lumen formation.

Discussion

Chloride channels are known to function in several cellular processes such as maintaining membrane potentials, controlling cell volume, and regulating cellular pH levels. To date, at least five independent chloride channel families have been documented in mammals [20]. To further emphasize the importance of intracellular chloride regulation, defects of intracellular chloride channels are associated with numerous diseases including those that affect the human neuromuscular, renal, skeletal, and respiratory systems [22]. As the most recently discovered intracellular chloride channel family, our knowledge of CLIC functions is still evolving, and this study sought to determine if intracellular chloride channels also play a role in angiogenic processes and pathologies.

Currently, there are seven identified mammalian CLICs defined by a C-terminal sequence of roughly 230 amino acids that displays GST-like structure and function [5]. CLICs are highly homologous to each other and are conserved between vertebrate and invertebrate species [23]. Unlike other mammalian ion channels, CLICs are present in cells as both soluble cytoplasmic proteins and integral membrane proteins [5]. CLIC proteins have been shown to localize to intracellular membranes such as those of the Golgi, endoplasmic reticulum, mitochondria, and other membrane types [4, 20, 23]. CLIC4 localization can vary in different cell types and this seems to be specific to its function [20]. For example, in pro-apoptotic keratinocytes, CLIC4 translocates from the mitochondria to the nucleus, with the level of translocation being a factor in stress response regulation [24, 25]. Overexpression of CLIC4 in cells was also shown to promote membrane localization where CLIC4 exhibits anion channel activity [26].

In general, the activities of CLIC proteins are not well-documented and their precise functions in cellular processes are poorly understood. Even less is known about CLIC4 function in endothelial cells, and CLIC1 and CLIC4 are the only members of the CLIC family confirmed to be expressed in endothelial cells to date [5, 8, 9]. However, recent research identified a *C. elegans* homologue of CLIC4, EXC-4, as having tubulogenic functions, indicating a potential role for CLIC4 in endothelial angiogenesis [4]. Another study found CLIC4 to be among 120 proteins affected during VEGF-A driven endothelial tubulogenesis [9].

We hypothesized that reduced CLIC4 expression in primary endothelial cells would negatively affect angiogenic processes, while increasing CLIC4 expression would lead to promoted endothelial angiogenesis. To test this hypothesis, RNAi was used to generate stable cultured primary HUVEC lines exhibiting CLIC4 knockdown expression. CLIC4-overexpressing HUVEC lines were also created using a lentiviral construct expressing full-length CLIC4. To analyze the effects of CLIC4 expression on various steps of in vitro angiogenesis, we utilized in vitro assays assessing endothelial cell survival, proliferation, migration, network formation, tube formation, and capillary sprouting. We present here a comprehensive analysis of the potential angiogenic functions of CLIC4 in vitro.

We found that CLIC4 is involved in several steps of in vitro angiogenesis after observing aberrant network formation, capillary sprouting, and lumen formation due to altered CLIC4 expression (Figs. 5, 6 and 7). We also found that reduced CLIC4 expression has a modest effect on endothelial cell survival (Fig. 4) and a significant effect on cell proliferation (Fig.

3). With these results, we conclude that CLIC4 expression is required for proper endothelial cell proliferation and morphogenesis in vitro. Thus, we have defined a novel angiogenic signaling pathway and present CLIC4 as a potential target for anti-angiogenic therapies.

Using transient transfection of human telomerase-immortalized microvascular endothelial (TIME) cells with CLIC4 antisense constructs, a previous study [9] documented decreased in vitro angiogenesis with an indication of reduced tube-like structures. In contrast to this report, our study documented several steps in angiogenesis dependent on proper CLIC4 expression, including cellular proliferation, endothelial network formation, and lumen formation, providing a deeper understanding of the role of CLIC4 in angiogenesis. This is the first study on CLIC4 function in primary endothelial cells (HUVEC), demonstrating a role for CLIC4 in endothelial proliferation and branching morphogenesis.

The mechanism by which CLIC4 functions to regulate in vitro angiogenesis is still unclear. A current model for endothelial tubulogenesis is based on vesicular fusion and cord hollowing [2, 3]. This process is known to involve cell apical–basal polarity [2, 3], but the factors and molecular mechanisms that regulate this process are still not well-understood. CLIC4 has previously been localized to intracellular vesicles in proliferating endothelial cells [9]. In *C. elegans*, EXC-4 is reported to localize to the apical luminal membrane of the excretory cell [4]. It is possible that the chloride ion channel function of CLIC4 plays a role in regulating vacuole formation, enlargement, and fusion since chloride channels are known to regulate water transport between a cell and its environment. As an ion channel, another plausible mechanism for CLIC4 involvement in angiogenesis would involve regulating electric potentials generated by the acidification required for vesicular fusion [27]. The resolution of the precise mechanisms of CLIC4 function in angiogenesis is an important future goal to further understand this novel angiogenic regulator.

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Fig. 1.

Establishing CLIC4 knockdown and overexpression in endothelial cells. **a** Immunoblotting with polyclonal rabbit anti-CLIC4 antibody confirms knockdown of CLIC4 protein expression in shRNA3 and shRNA5 endothelial cell lines compared with plko sc control line. Immunoblotting with anti-CLIC1 antibody confirms that knockdown by shRNA is specific to CLIC4 and does not interfere with CLIC1 expression. **b** Immunoblotting with the anti-CLIC4 antibody confirms CLIC4 overexpression in the CLIC4 endothelial cell line compared with pccl control. Alpha-tubulin served as a loading control for all immunoblots. **c** Established HUVEC shRNA3 knockdown cells exhibited no major change in endothelial cell morphology when compared with plko sc control cells. HUVEC shRNA5 appear to be more rounded when compared with plko sc. HUVEC refer to endothelial cells that have not been infected with any constructs. **d** No major change was observed in endothelial cell morphology between pccl control cells and CLIC4-overexpressing cells

Fig. 2. CLIC4 expression has no effect on endothelial cell migration. **a** CLIC4 knockdown cell lines shRNA3 and shRNA5 exhibited no noticeable change in directed endothelial cell migration 6 h after scraping in a migration assay when compared with control plko sc. **b** Likewise, CLIC4-overexpressing cells exhibited no noticeable change in endothelial cell migration 6 h after scraping when compared with control pccl

Fig. 3. CLIC4 expression promotes endothelial cell proliferation. **a** Inhibition of CLIC4 expression by shRNA resulted in decreased endothelial cell proliferation when compared with plko sc control. **b** CLIC4 overexpression resulted in a significant increase in endothelial cell proliferation when compared with control pccl. (* $P < 0.05$; ** $P < 0.01$)

Fig. 4. Altering CLIC4 expression affects endothelial cell survival. **a** CLIC4 knockdown lines significantly increased cell survival at 48 h, but not 24 h post-seeding. **b** CLIC4 overexpression resulted in significantly decreased endothelial cell survival at 48 h, but not 24 h post-seeding. (* $P < 0.05$; ** $P < 0.01$)

Fig. 5.

CLIC4 expression promotes in vitro endothelial network formation. **a** Microscopy of CLIC4 knockdown or overexpressing cells in a collagen network formation assay shows decreased network formation with CLIC4 knockdowns, while CLIC4 overexpression resulted in increased network formation when compared with respective controls. **b** Quantification confirms that CLIC4 knockdown results in decreased network formation by showing that reduced CLIC4 expression leads to a significant reduction in surface area coverage by endothelial cells. CLIC4 overexpression is shown to lead to increased network formation with a significant increase in surface area occupied by endothelial cells. Control standards were set at 100% surface area occupation. **c** Quantification of branchpoints for each knockdown cell line further confirms CLIC4 knockdown inhibition on endothelial network formation. Knockdown cell lines form significantly fewer branchpoints than control plko sc. Quantification was done by counting visible branchpoints per field of view at 10× objective. **d** CLIC4-overexpressing cells form significantly more branchpoints than control pccl. Quantification was done by counting visible branchpoints per field of view at 5× objective. One branchpoint was considered any focal intersection of two or more cords. (* $P < 0.05$; ** $P < 0.01$)

Fig. 6.

Reduced CLIC4 expression inhibits endothelial network formation independent of its proliferative defect. **a** Comparison of network formation by knockdown cell lines and control cells treated with mitomycin C indicates that there is inhibition of network formation independent from reduced CLIC4 inhibition of proliferation. **b** A proliferation assay confirms mitomycin C growth arrest of plko sc control cells. **c** Quantification of surface area occupied by endothelial cells confirms an additional defect associated with reduced CLIC4 expression independent of reduced CLIC4 proliferation inhibition. Mitomycin C-treated control standards were set at 100% surface area occupation. **d** Quantification of branchpoints further confirms an additional defect associated with reduced CLIC4 expression separate from reduced CLIC4 proliferation inhibition. (* $P < 0.05$ compared with plko sc; ** $P < 0.01$ compared with plko sc; + $P < 0.05$ compared with plko sc + mmc; ++ $P < 0.01$ compared with plko sc + mmc)

Fig. 7.

CLIC4 expression affects capillary-like sprouting in vitro. **a** Microscopy indicates that HUVEC shRNA3 have altered tube morphogenesis in that they tend to form shorter, more stunted tube structures as indicated by a *red arrow*. HUVEC shRNA5 show a decrease in the number of branches per sprout. HUVEC shRNA5 also exhibited stunted sprouts indicated by a *red arrow*. Unlike HUVEC shRNA3 cells, HUVEC shRNA5 cells did not form lumens. *Black arrows* indicate robust, lumen-containing sprouts. **b** Microscopy from a separate trial confirms the phenotypes of HUVEC shRNA3 stunted tube structures and HUVEC shRNA5 stunted sprouts without lumens. **c** Quantification of the number of lumen-containing sprouts shows that HUVEC shRNA5 exhibits a significant decrease in the number of lumen-containing sprouts formed when compared with plko sc control. **d** Microscopy of CLIC4-overexpressing endothelial cells show increased sprouting and lumen formation when compared with plko sc control. Lumen-containing sprouts are indicated by *black arrows*. **e** Quantification for CLIC4-overexpressing endothelial cells indicate a significant increase in the number of lumen-containing sprouts. (* $P < 0.05$; ** $P < 0.01$)