

# Inhibition of Cx43 attenuates ERK1/2 activation, enhances the expression of Cav-1 and suppresses cell proliferation

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Received January 7, 2018; Accepted July 31, 2018

DOI: 10.3892/ijmm.2018.3828

**Abstract.** In addition to being an important component of the gap junction, connexin 43 (Cx43) has been shown to regulate other cellular functions, including cell proliferation. This regulatory role of Cx43 may be important in therapeutic situations, including wound healing or ischemic injuries. Caveolin-1 (Cav-1) has been shown to regulate angiogenesis. The aim of the present study was to analyze whether Cx43 counter-regulates Cav-1 in controlling the proliferation and migration of endothelial cells. The inhibition of Cx43 with niflumic acid, flufenamic acid and 18- $\alpha$ -glycyrrhetic acid in cultured human umbilical vein endothelial cells resulted in decreased phosphorylation of extracellular signal-regulated kinase (ERK)1/2 and increased expression of Cav-1, as shown by western blot analysis. Furthermore, the inhibition of Cx43 resulted in a 50 $\pm$ 7% decrease in cell proliferation, determined using a crystal violet assay, a 48 $\pm$ 5% decrease in migration, determined using a migration assay, and a 49 $\pm$ 6% decrease in endothelial tube formation, determined using a Matrigel assay, compared with the control. Similar results were obtained following specific inhibition of Cx43 by mimetic peptides (Gap26 and Gap27). Inhibition of the mitogen-activated protein kinase kinase/ERK pathway with PD-98059 resulted in an increased expression of Cav-1 and a reduction in the expression of Cx43. Furthermore, cell proliferation, migration and tube formation in endothelial cells were impaired. By contrast, downregulation of the protein expression of Cav-1 by small interference RNA resulted in increased expression of Cx43 and phosphorylation of ERK1/2. Accordingly, the number of cells in the Cav-1 treated-group increased by 35 $\pm$ 5% compared with the controls. The data of the present study showed that Cav-1 suppressed cell proliferation by inhibiting the activity of Cx43,

which is upstream of ERK1/2. The downregulation of Cav-1 protein resulted in loss of the inhibitory activity of Cav-1 on cell proliferation and led to increased cell proliferation. This counter-regulatory effect of Cx43 may be of importance in therapeutic angiogenesis.

## Introduction

Angiogenesis, a multistep process by which new blood vessels are formed from pre-existing vessels, is important in different physiological and pathophysiological situations, including wound healing, cancer, and ischemic diseases (1). During angiogenesis, endothelial cells proliferate and migrate as 'tube-like sprouts' from pre-existing vessels (2).

Cell proliferation is a key step in angiogenesis. Gap junctions have been demonstrated to be important not only in the regulation of cell proliferation and growth but also in the differentiation and development of blood vessels (3-5). The gap junctions serve as critical gatekeepers in cell proliferation and several other cellular processes by regulating the exchange of ions, amino acids, secondary messengers, including ATP, and different growth regulators (6-9). The gap junctions are composed of connexins, which are a family of transmembrane proteins that form channels connecting the cytoplasm of adjacent cells (10-13). The hexameric arrangement of connexins form single-membrane channels, hemichannels or connexons, which align their counterparts in adjacent cell membranes to constitute a complete intercellular channel (14,15). Several connexins, including connexin (Cx)37, Cx40, Cx43 and Cx45, are expressed in different cell types (16,17); however, in endothelial cells, Cx43 is predominantly expressed (18).

In addition to being an important component of the gap junction, Cx43 has been shown to regulate different cellular processes, including cell proliferation and migration (19-22). Notably, a reduction in the expression of Cx43 in endothelial cells by nicotine treatment impaired angiogenesis (23). In addition, mitogen-activated protein kinases (MAPKs) may regulate the expression of Cx43 in endothelial cells (24). These findings suggest that Cx43 is involved in different steps of angiogenesis.

The expression pattern of Cx43 has been shown to be altered in cell proliferation and cell migration during ischemic

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*Key words:* connexin 43, caveolin-1, angiogenesis

injury (25,26), cancer (27,28), atherosclerosis (29,30) or wound healing (31,32). Similarly, the overexpression or loss of caveolin-1 (Cav-1) alters different cellular functions, including cell proliferation and permeability under different conditions (33,34).

Several studies have shown that Cx43 co-localizes with other proteins, including fibroblast growth factor receptors (22), cytoplasmic proteins (35) and cytoskeleton proteins (36). In addition, published literature has demonstrated that Cx43 interacts with caveolin proteins in different cell types (37,38). However, its interaction with Cav-1 protein appears to be cell type-specific and its functional importance remains to be fully elucidated. Cav-1, the structural protein of caveolae, is involved in intracellular communication in gap junctions and also in cell cycle control (33). It is also well documented that the overexpression of Cav-1 inhibits mitogenic signaling in a number of cell lines (39,40).

The aim of the present study was to investigate the mutual effect of Cx43 and Cav-1 proteins in different steps of angiogenesis, including cell proliferation, migration and tube formation, in endothelial cells. Whether Cx43 counter-regulates Cav-1 in controlling angiogenesis is of interest as the two proteins work differently under different pathophysiological conditions.

## Materials and methods

**Materials.** Cell culture dishes and serum reduced Matrigel™ were purchased from BD Biosciences (San Jose, CA, USA). Anti-Cx43 (cat. no. 610062) and anti-Cav-1 (cat. no. 610057) antibodies were from BD Biosciences. Cav-1 small interference (si)RNA (cat. no. sc-29241) was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Endothelial cell basal medium plus supplement pack was from PromoCell (Heidelberg, Germany). Different pharmacological inhibitors of Cx43, including 18  $\alpha$ -glycyrrhizinic acid ( $\alpha$ -GA) and niflumic acid (NA) and flufenamic acid (FA) were from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). Connexin mimetic peptides, Gap26 (G-26; cat. no. CX2605-P-1) was from Alpha Diagnostic International, Inc. (San Antonio, TX, USA), and Gap27 (G-27; cat. no. G794) were from Sigma; Merck KGaA (Darmstadt, Germany). Fetal calf serum (FCS) and penicillin-streptomycin were from Gibco®; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Hank's balanced salt solution (HBSS), collagenase and trypsin-EDTA solutions were from PAA Laboratories (Pasching, Austria). The jetSI™-ENDO transfection reagent was purchased from (Polyplus-transfection SA, Illkirch, France). The negative siRNA (cat. no. SI03650318) was from Qiagen GmbH (Hilden, Germany), and mitogen-activated protein kinase kinase (MEK)1 inhibitor, PD-98059, was from Calbiochem; Merck KGaA. Anti-extracellular signal-regulated kinase (ERK)1/2 (cat. no. 9102) and anti-phosphorylated (p)ERK1/2 (cat. no. 9101) were purchased from Cell Signalling Technology, Inc. (Danvers, MA, USA). Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (cat. no. NA9340), HRP-conjugated anti-mouse (cat. no. NA931) antibodies and anti-vinculin antibody (cat. no. V9131) were purchased from Sigma-Aldrich; Merck KGaA. Pierce™ ECL solution was from Thermo Fisher Scientific Inc. All other chemicals were of the highest available quality.

**Cell culture.** Human umbilical veins were maintained at 4°C in a sterile container and obtained once a week from the Department of Gynaecology, University Hospital Giessen and Marburg (Giessen, Germany) between August 2009 and August 2013. Human umbilical vein endothelial cells (HUVECs) were isolated and cultured as previously described (41). Briefly, untraumatized human umbilical veins were cannulated and perfused with HBSS to remove traces of blood. Following this, the lumen of the vein was filled with 0.025% collagenase solution (w/v) and incubated for 30 min at 37°C. Collagenase solution containing endothelial cells was subsequently removed via perfusion of the vein with 30 ml of HBSS containing 3% (v/v) FCS to terminate the reaction. The effluent was then collected in a 50 ml falcon tube and centrifuged for 5 min at 250 x g at room temperature. The supernatant was then removed and the cell pellet was resuspended in endothelial cell culture medium containing 0.1% (v/v) gentamycin. Following this, cells were seeded to 3-4 primary culture dishes. Following a total of 2 h of incubation at 37°C in 5% CO<sub>2</sub>, cells were extensively washed with HBSS to remove non-endothelial cells and cell debris. Adherent cells were then incubated in 15-20 ml of endothelial cell culture medium containing 2% (v/v) penicillin/streptomycin at 37°C and 5% CO<sub>2</sub>. Following 24 h, cell culture medium was replaced with fresh endothelial cell culture medium. For experiments, HUVECs were cultured in endothelial cell culture medium supplemented with 10% (v/v) FCS, 0.4% (v/v) endothelial growth supplement with heparin, 0.1 ng/ml human epithelial growth factor, 0.1  $\mu$ g/ml hydrocortisone, 1 ng/ml human basic fibroblast growth factor, 100 IU/ml penicillin G and 100  $\mu$ g/ml streptomycin. Cells were seeded either on 12-well plates or 96-well plates. The experiments were performed when cells in the monolayers reached 100% confluence. For the present study, informed consent was obtained from patients, and ethical approval was obtained from the Ethics Committee of Justus-Liebig University of Giessen (Giessen, Germany; no. AZ132/09). The study confirms the principles outlined in the 'Declaration of Helsinki' (42).

**Determination of cell proliferation.** The endothelial cells were seeded in endothelial cell basal medium at a density of  $2.5 \times 10^3$  in a 96-well plates cultured in a humidified atmosphere at 37°C and 5% CO<sub>2</sub>. After 24 h, the endothelial cells were treated at 37°C in a 5% CO<sub>2</sub> atmosphere with Cx43 pharmacological inhibitors, including NA (50  $\mu$ M), FA (50  $\mu$ M), and  $\alpha$ -GA (25  $\mu$ M) or specific mimetic peptides G-26 and G-27 (0.25  $\mu$ g/ml) and MEK1 inhibitor, PD-98059 (20  $\mu$ M). Subsequently, the endothelial cells were incubated for 48 h. Analysis of cell proliferation was determined by crystal violet staining. At the end of the experiments, the cells were briefly fixed for 30 min with 5.5% gluteraldehyde at room temperature. Following three washes with water, the cells were dried for 1 h. The cell nuclei were then stained with 1% crystal violet solution (pH 5.4) for 20 min. Following another washing step, the cells were dried and treated with 10% acetic acid, and the absorbance of the crystal violet staining was measured at 595 nm using a microplate reader (Sunrise™; Tecan Group, Inc., Männedorf, Switzerland).

**Migration assays.** For migration assays, a self-made section of silicone rubber (length, 3 mm) was fixed in the center

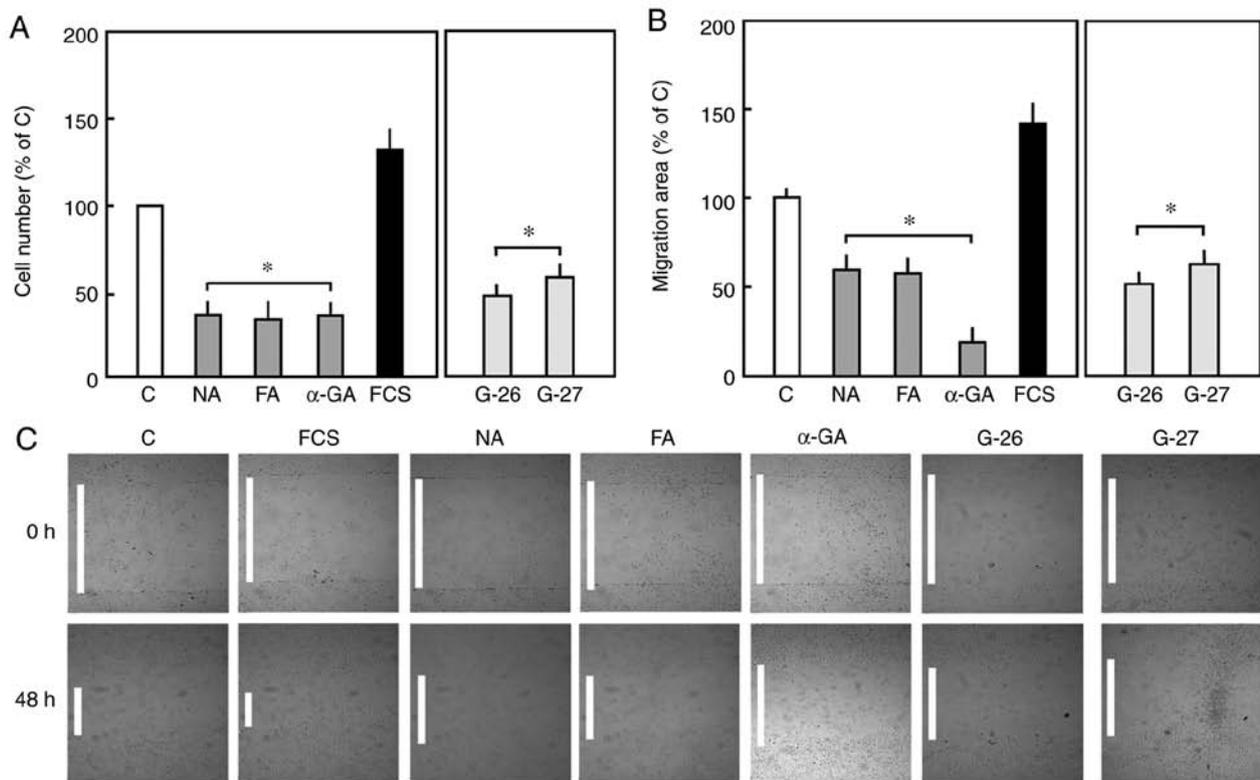


Figure 1. (A) Effect of Cx43 inhibition on the proliferation of endothelial cells. Endothelial cells were treated with Cx43 inhibitors (NA, 50  $\mu$ M), (FA, 50  $\mu$ M) or ( $\alpha$ -GA, 25  $\mu$ M), or specific mimetic peptides (G-26 and G-27, 0.25  $\mu$ g/ml) for 48 h. For the positive control, the cells were incubated in 20% serum. Subsequently, cell proliferation was determined by crystal violet staining. The number of cells in the control group was set to 100%. (B) For the migration assay, the cells were seeded, and removal of a rubber section was considered the initial time point (0 h). The cells were treated with the same concentration of Cx43 inhibitors or mimetic peptides against Cx43, incubated for 48 h (considered the endpoint), and the area of migration was measured. The area of migration for control cells was considered as 100%. (C) Images of migration (magnification,  $\times 2$ ). Data are shown as the mean  $\pm$  standard error of the mean of 3-5 experiments of independent cell preparations \* $P < 0.05$ , vs. C. Cx43, connexin 43; NA, niflumic acid; FA, flufenamic acid;  $\alpha$ -GA, 18  $\alpha$ -glycyrrhizinic acid; C, control; FCS, fetal calf serum.

of a 12-well plate, and endothelial cells were seeded at a density of  $1 \times 10^5$  per well. The removal of the silicone rubber from the culture plates was considered as the initial time point (0 h) and the endothelial cells were treated with Cx43 inhibitors or PD-98059. The cells were incubated for 48 h, which was considered the endpoint for the migration assay. At the end of experiments, images of the migrated cells were captured with an inverted microscope (magnification,  $\times 2$ ) using a CCD camera, and the migration area was quantified using Cell D software version 5.1 (Olympus Corporation, Tokyo, Japan).

**Tube formation assay.** Matrigel (200  $\mu$ l) was deposited into wells in a 24-well plate and allowed to solidify for 30 min at 37°C. The endothelial cells ( $4 \times 10^4$  cells) were added to each well. Following of incubation at 37°C for 5 h, the cells were treated with Cx43 inhibitors or with PD-98059. The cells were incubated on Matrigel for a further 24 h at 37°C and images were captured by phase contrast microscopy (Olympus). Random fields of view/well were examined for image capture. The total number of tubes from the nodes of endothelial cells was quantified using Cell D software (Olympus).

**Transfection of endothelial cells.** The endothelial cells were seeded according to the experiments being performed. Subconfluent monolayers of endothelial cells were transfected

for 48 h with a specific siRNA against Cav-1 (50 nM) or with a negative siRNA (50 nM) using jetSI™-ENDO transfection reagent according to the manufacturer's protocol.

**SDS-PAGE and western blot analysis.** For immunoblot analyses, the experimental incubation of cultures was terminated by rapid removal of medium and the addition of 2X SDS sample buffer [250 mM Tris/HCl (pH 6.8), 20% glycerol, 2% (w/v) SDS, 0.001% (w/v) bromophenol blue, 10% (v/v) 2-mercaptoethanol, 1 mM DTT (added fresh prior to use) and 10  $\mu$ l Benzonase (250 U) in 200 mM  $MgSO_4$ ]. Protein concentration was determined by Pierce™ BCA protein assay kit (Pierce; Thermo Fisher Scientific, Inc.; cat. no. 23227), and equal amounts of protein (30  $\mu$ g) in lysis buffer were resolved by 12% SDS-PAGE and transferred onto nitrocellulose membranes. Subsequently, the membranes were blocked for 1 h at room temperature under constant shaking in either 1X TBST [5% (w/v) non-fat milk powder] or TBST containing 3% bovine serum albumin (BSA; w/v; Sigma-Aldrich; Merck KGaA) when phospho-specific antibodies were applied. The primary antibodies were diluted in TBST. The membranes were incubated with the appropriate primary antibodies (anti-Connexin43, anti-Caveolin-1, anti-ERK1/2, anti-pERK1/2 and anti-Vinculin; all used at a 1:1,000 dilution in 3% BSA) with gentle shaking overnight at 4°C. The membranes were washed three times with

TBST and incubated with the appropriate secondary antibody (HRP-conjugated anti-rabbit IgG or HRP-conjugated anti-mouse IgG; both used at a dilution of 1:2,000 in 3% BSA) for 1 h at room temperature. Following washing, the protein bands were visualized by enhanced chemiluminescence and detected with a CCD camera using the Bio-Rad ChemiDoc system (Bio-Rad Laboratories, Inc. Hercules, CA, USA) according to the manufacturer's protocol.

**Statistical analysis.** Statistical analyses were performed using Graphpad Prism software version 5 (GraphPad Software, Inc., La Jolla, CA, USA). Data are shown as the mean  $\pm$  standard error of the mean of 3-6 experiments using independent cell preparations. The comparison of means between groups was performed by one-way analysis of variance followed by a Bonferroni post hoc test.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Inhibition of Cx43 impairs cell proliferation, migration and tube formation of endothelial cells.** The pharmacological inhibition of Cx43 using NA (50  $\mu$ M), FA (50  $\mu$ M), and  $\alpha$ -GA (25  $\mu$ M) resulted in significant reductions in cell proliferation and migration of endothelial cells. Similarly, the specific inhibition of Cx43 using mimetic peptides, Gap-26 or Gap-27, also suppressed the proliferation and migration of endothelial cells compared with respective control cells (Fig. 1A-C).

To investigate the effect of Cx43 inhibition on angiogenesis, endothelial cells were seeded on Matrigel to perform a tube formation assay. The treatment of endothelial cells with Cx43 inhibitors, NA, FA and  $\alpha$ -GA, for 24 h markedly reduced tube formation (Fig. 2A and B).

**Effect of Cx43 inhibition on the expression of Cav-1 and activation of ERK1/2.** The pharmacological inhibition of Cx43 resulted in a significant decrease in the expression of Cav-1, as shown in Fig. 3A. However, the long-term treatment of endothelial cells with Cx43 inhibitors enhanced the expression of Cav-1 compared with that in the control cells (Fig. 3B). Furthermore, Cx43 inhibitors significantly reduced the phosphorylation of ERK1/2 compared with untreated cells (Fig. 3C). Taken together, these data suggested that the expression of Cx43 and Cav-1 is critical in the proliferation and migration of endothelial cells.

**ERK1/2 is involved in the expression of Cx43 and Cav-1.** As ERK1/2 is known to be involved in the regulation of Cx43 and Cav-1 protein expression in different cell types (43,44), the present study examined whether ERK1/2 affects the protein expression of Cx43 and Cav-1 in endothelial cells. PD-98059 (20  $\mu$ M), an MEK inhibitor, was used to assess the expression of these proteins. The pre-treatment of endothelial cells with PD-98059 significantly reduced the expression of Cx43. By contrast, the inhibition of ERK1/2 by PD-98059 enhanced the expression of Cav-1, which is a protein that is known to be associated with reduced cell proliferation (Fig. 4A). Therefore, the effect of PD-98059 on the proliferation and migration of endothelial cells was examined. The pharmacological inhibition of ERK1/2 resulted in a significant reduction in the proliferation

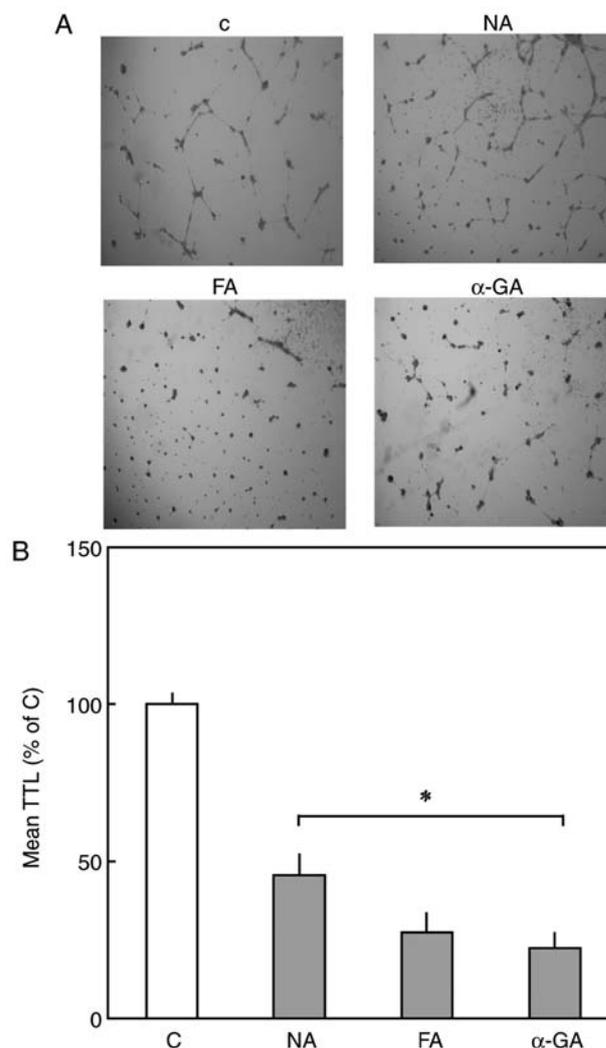


Figure 2. For the tube formation assay,  $4.2 \times 10^4$  cells were seeded onto Matrigel coated-24-well plates. After 3 h, cells were treated with Cx43 inhibitors and incubated for 24 h. (A) Images of cells (magnification,  $\times 2$ ) were captured and (B) TTL was determined. Data are shown as the mean  $\pm$  standard error of the mean of 3-5 experiments of independent cell preparations \* $P < 0.05$ , vs. C. Cx43, connexin 43; NA, niflumic acid; FA, flufenamic acid;  $\alpha$ -GA, 18  $\alpha$ -glycyrrhizinic acid; C, control; TTL, total tube length.

and migration in endothelial cells (Fig. 4B), suggesting the involvement of Cx43 and Cav-1 via the MEK/ERK pathway.

In the above data, the inhibition of Cx43 increased the expression of Cav-1, and the MEK/ERK pathway was found to serve as an intermediate signalling element. Limited data in the literature show the interaction of these two proteins (37,38), however, it may be that an intermediate signalling element has not been identified. In the present study, it was identified that the MEK/ERK pathway serves as an intermediate signaling element.

**siRNA mediated-downregulation of Cav-1 increases the expression of Cx43 and promotes ERK1/2 activation.** A siRNA transfection strategy was used to selectively down-regulate the protein expression of Cav-1. The immunoblot probed for Cav-1 in endothelial cells, 48 h following transfection with specific Cav-1 siRNA (Cav-1 siRNA) is shown in Fig. 5A, and quantification is shown in Fig. 5B. The expression

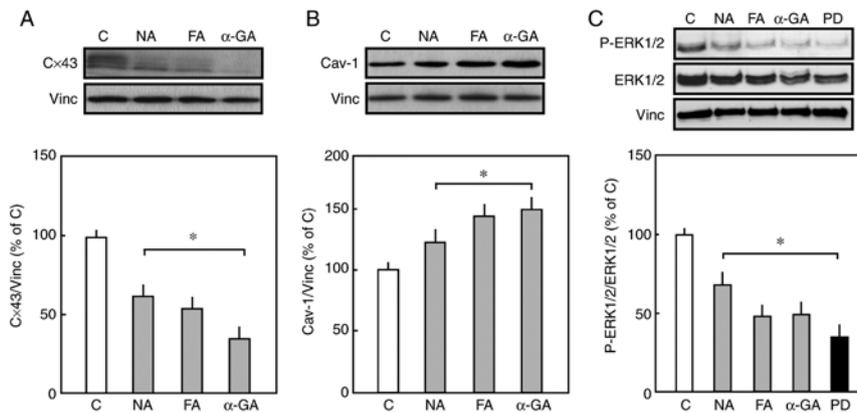


Figure 3. (A) Effect of Cx43 inhibitors on expression levels of Cx43 and Cav-1. Representative western blots of total Cx43 and Vinc as an internal control. The endothelial cells were treated with Cx43 inhibitors (NA, FA, or  $\alpha$ -GA) for 48 h. Densitometric analysis of the expression of Cx43 is shown. The mean total expression of Cx43 in control cells in the absence of inhibitors was set to 100%. (B) Representative western blots of total Cav-1 and Vinc as an internal control. Densitometric analysis of total expression of Cav-1 is shown. The mean total expression of Cav-1 in the control cells in the absence of inhibitors was set to 100%. (C) Representative western blots of p-ERK1/2, total ERK1/2, and Vinc as endogenous control. Densitometric analysis of p-ERK1/2 is shown. The mean expression of p-ERK1/2 in the control was set to 100%. Data are presented as the mean  $\pm$  standard error of the mean of three separate experiments of independent cell preparations, \* $P < 0.05$ , vs. C. Cx43, connexin 43; Cav-1, caveolin 1; Vinc, vinculin; ERK, extracellular signal-regulated kinase; p-ERK, phosphorylated ERK; NA, niflumic acid; FA, flufenamic acid;  $\alpha$ -GA, 18  $\alpha$ -glycyrrhizinic acid; PD, PD-98059; C, control.

of Cav-1 was efficiently downregulated with Cav-1 siRNA, whereas the expression of Cav-1 remained unchanged in the untransfected cells or in the cells that were transfected with negative siRNA. Furthermore, the specific downregulation of Cav-1 protein significantly enhanced the expression of Cx43 by  $25 \pm 5\%$  compared with that in the control. The downregulation of Cav-1 resulted in increased ERK1/2 phosphorylation compared with that in the control.

An increased expression of Cx43 is linked to cell proliferation, migration and the angiogenic activity of endothelial cells (18). The downregulation of Cav-1 increased the expression of Cx43 and consequently increased the proliferation and migration of endothelial cells (Fig. 5C and D). Tube formation was also markedly increased in the Cav-1-downregulated cells compared with that in the control (Fig. 5E and F).

## Discussion

The major findings of the present study were that the inhibition of Cx43 impaired the proliferation, migration and angiogenic activity of endothelial cells. In addition, the inhibition of Cx43 led to increased expression of Cav-1. Targeted downregulation of the Cav-1 protein increased the expression of Cx43 and promoted the activation of ERK1/2. The MEK/ERK pathway was found to be an intermediate signalling element.

The pharmacological inhibition of Cx43 significantly reduced the proliferation, migration and tube formation in endothelial cells. These findings are consistent with previous studies, which reported that the expression of Cx43 regulated cell proliferation (18). However, the role of the expression of Cx43 remains to be fully elucidated. Previous studies described that change in the expression of Cx43 affects cellular processes in a cell-type-dependent manner (18,19,23). Inhibition of the expression of Cx43 in keratinocytes led to increased cell proliferation at wound sites (38), whereas its effect was found to be the opposite in endothelial cells. Furthermore, a reduction in the expression of Cx43 inhibited the proliferation of endothelial cells by enhancing the expression of co-regulatory

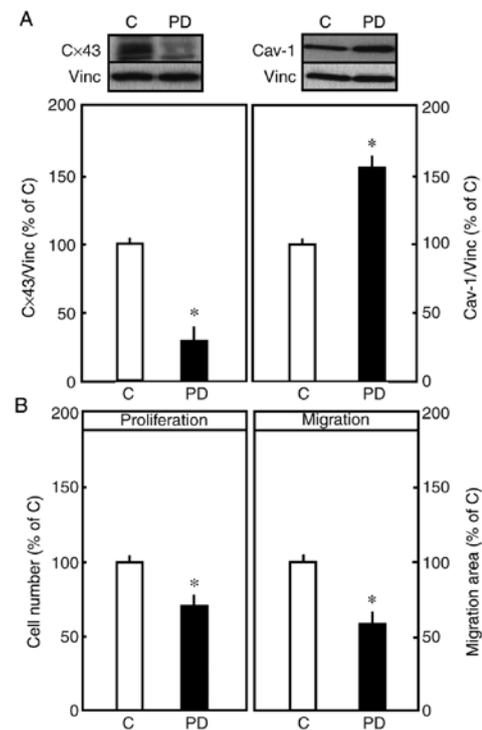


Figure 4. (A) Effect of PD-98059 (MEK1 inhibitor) on the expression of Cx43 and Cav-1. Representative western blots of total Cav-1 and Vinc (internal control). The endothelial cells were treated with PD-98059 ( $20 \mu\text{M}$ ) for 48 h. Densitometric analysis of total expression of Cx43 is shown. The mean of total expression of Cx43 in the control cells in the absence of inhibitors was set to 100%. Representative western blots of total Cav-1 and Vinc (internal control). Densitometric analysis of total expression of Cav-1 is shown. The mean total expression of Cav-1 in the control cells in the absence of inhibitors was set to 100%. (B) Endothelial cells were treated with MEK1 inhibitor (PD-98059,  $20 \mu\text{M}$ ) for 48 h. Cell proliferation was determined by crystal violet staining. The number of cells in the control was set to 100%. In the migration assay, the cells were seeded at a density of  $1 \times 10^5$  in 12-well plates. After 48 h, the cells were treated with PD-98059 and incubated for 48 h, and the area of migration was measured. The area of migration of control cells was considered as 100%. Data are shown as the mean  $\pm$  standard error of the mean of three separate experiments of independent cell preparations, \* $P < 0.05$ , vs. C. Cx43, connexin 43; Cav-1, caveolin 1; Vinc, vinculin; MEK1, mitogen-activated protein kinase kinase 1; C, control; PD, PD-98059.

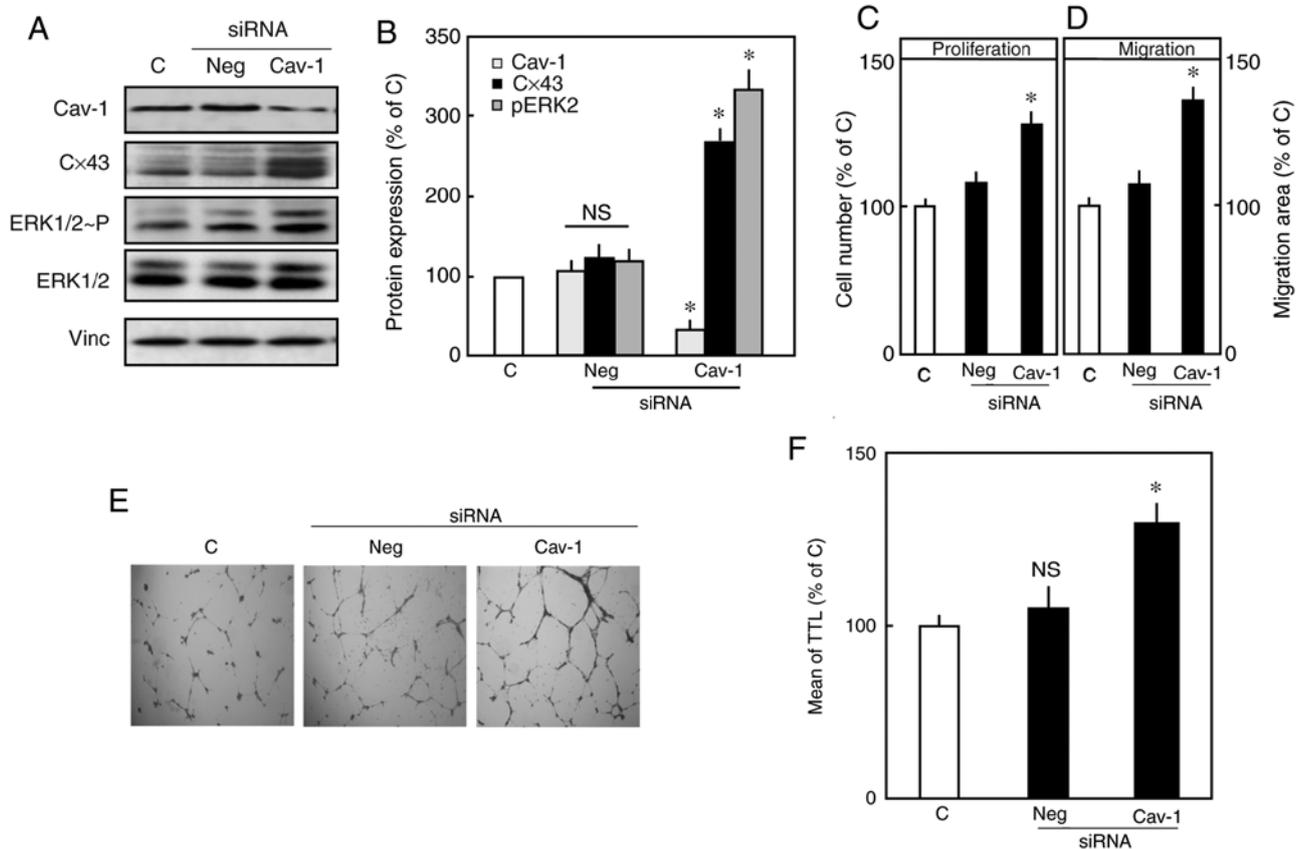


Figure 5. siRNA mediated-downregulation of Cav-1 increases the expression of Cx43 and promotes ERK1/2 activation. Endothelial cells were transfected with either specific siRNA against Cav-1 or negative siRNA (50 nm), or left untreated as a control, for 48 h. Following transfection, the cells were harvested and lysed, and proteins levels were analyzed in immunoblots probed with Cav-1, Cx43 and p-ERK1/2 antibodies. Vinc was probed as an internal control. (A) Representative western blots of total Cav-1, Cx43 and P-ERK1/2, total ERK1/2, and Vinc. (B) Densitometric analysis of total Cav-1, Cx43 and P-ERK1/2. The mean total expression in the control was set to 100%. Data are presented as the mean  $\pm$  standard error of the mean of three separate experiments of independent cell preparations, \* $P < 0.05$ , vs. C. (C) Effect of downregulating Cav-1 on the proliferation of endothelial cells. Following transfection, cell proliferation was determined, and the number of control cells was set to 100%. (D) Results of the migration assay. Following transfection, the area of migration was measured, with the area of migration of the control cells set as 100%. (E) Images (magnification, x2) and (F) quantification of the tube formation assay. Following 48 h transfection, the cells were trypsinized and seeded onto a Matrigel coated-24-well plate. After 24 h, the TTL was determined. Data are shown as the mean  $\pm$  standard error of the mean of 3-5 experiments of independent cell preparations, \* $P < 0.05$ , vs. C. Cx43, connexin 43; Cav-1, caveolin 1; Vinc, vinculin; ERK, extracellular signal-regulated kinase; P-ERK, phosphorylated ERK; siRNA, small interference RNA; Neg, negative control; C, control; TTL, total tube length; n.s. non-significant.

factors, plasminogen activator inhibitor-1 and von Willebrand factor. The c-jun N-terminal kinase pathway is reported to be involved in the regulation of this process (18,23). Taken together, these data are in line with previous findings that the expression of Cx43 is involved in the angiogenic activity of endothelial cells (23).

The role of MAPKs in regulating the expression of Cx43 remains controversial and may, to a certain extent, be dependent on the cell type. Polontchouk *et al* (24) demonstrated that the phosphorylation of ERK1/2 enhanced the expression of Cx43 in cardiomyocytes. Polontchouk *et al* showed that endothelin-1 enhanced the expression of Cx43 via the ETA receptor by activating ERK1/2. In the present study, it was observed that inhibition of the phosphorylation of ERK1/2 led to decreased expression of Cx43, however, this mechanism requires further elucidation. The present study demonstrated that the pharmacological inhibition of Cx43 reduced the phosphorylation of ERK1/2 but not the expression of ERK1/2.

It has been shown previously that Cx43 mediates signaling to adjacent cells by releasing secondary messengers into

the extracellular environment. A reduction of Cx43 function in osteoblast cells by pharmacological inhibitors markedly reduces the activation of ERK1/2 (7). In the literature, it has been shown that ATP is released across the Cx43 channels, and the disruption of gap junctions by pharmacological treatment impairs the release of ATP from Cx43 channels (8,9,45,46). ATP stimulates cell proliferation via purinergic receptor, which is dependent on the ERK1/2 activation (47). However, Cx43 dependent-ATP release and its role in paracrine intercellular signaling remain to be fully elucidated. The data obtained in the present study suggest that the expression of Cx43 and activation of ERK1/2 are interdependent, and ATP release across Cx43 may be involved.

Previous studies have shown that Cav-1 controls the cell cycle (33,40). Schubert *et al* (37) first reported that Cx43 interacts and co-localizes with Cav-1. Cx43 directly interacts and binds with the scaffolding domain (residues 82-101) and C-terminal domain (135-178) of Cav-1 protein (37). The present study found that the inhibition of Cx43 enhanced the expression of Cav-1, and data shows

that the overexpression of Cav-1 protein inhibits mitogenic signaling and acts as a cell cycle suppressor protein by inhibiting ERK1/2 activity (39). From these findings, it is likely that the suppression in ERK1/2 activity results in the reduced expression of Cx43, which was shown in the results from the present study.

The pharmacological inhibition of either Cx43 or ERK1/2 enhanced the expression of Cav-1. Data from previous studies demonstrates that Cav-1 may interact and regulate the activities of several signaling molecules [33-35]. The targeted down-regulation of Cav-1 has been shown to selectively activate the MEK/ERK pathway (48-50). It is also well established that the MEK/ERK pathway is important in cell proliferation (51). To further characterize the role of the expression of Cx43 in angiogenesis, an siRNA transfection strategy was used to downregulate the expression of Cav-1. The targeted down-regulation of Cav-1 not only increased the expression of Cx43 but also the phosphorylation of ERK1/2. Accordingly, the cell proliferation, migration and tube formation of endothelial cells were increased.

Therefore, based on the above findings, a mechanism is suggested where the inhibition of Cx43 attenuated the activation of ERK1/2 and enhanced the expression of Cav-1, leading to suppressed cell proliferation. By contrast, the siRNA mediated-downregulation of Cav-1 rescued the expression of Cx43 by activating ERK1/2 and consequently increasing cell proliferation.

In the present study, the inhibition of Cx43 increased the expression of Cav-1, but reduced cell proliferation and angiogenesis. As Cx43 and Cav-1 are membranous proteins, this observation led to investigation of the combined effect of Cx43 and Cav-1 in the control of angiogenesis. It was observed that the inhibition of one molecule affected the expression of the other molecule and vice versa. Therefore, it may be that one protein counter-regulates the effect of other protein. Of note, it was found that the MEK/ERK pathway served as an intermediate signaling element in this context.

In conclusion, the data obtained in the present study support the hypothesis that Cx43 and Cav-1 are important in angiogenesis. The MEK/ERK pathway was identified to be an intermediate signaling element. The expression of Cx43 regulated cell proliferation, migration and tube formation in endothelial cells. Cx43 counter-regulated Cav-1, and the two proteins converged on the MEK/ERK pathway. Therefore, the present study suggested that pharmacological interventions may improve endothelial cell functions in different physiological and pathophysiological situations, including ischemic injuries, wound healing, diabetes, atherosclerosis, or cancer therapy.

### Acknowledgements

The authors would like to thank Mrs H. Thomas, Mrs D. Reiz, Mrs G. Albohn, and Mrs S. Schäfer (Department of Cardiology and Angiology, University Hospital Giessen and Marburg, Giessen Germany) for their technical support. Authors wish to thank Dr M. Aslam (Department of Cardiology and Angiology, University Hospital Giessen and Marburg) for editing the manuscript.

### Funding

This study was supported by an Anschubsfinanzierung and Von-Behring-Röntgen-Stiftung (2011) grant from The Justus-Liebig University (Giessen, Germany) and (grant no. SFB547; Projects A3 and A4) Deutsche Forschungsgemeinschaft.

### Availability of data and materials

Datasets used during the current study are available from the corresponding author on a reasonable request.

### Authors' contributions

MA performed the majority of the experiments, analysed the data and wrote the manuscript. MA and CC performed the cell proliferation experiments. MAR contributed to the siRNA transfection experiments, evaluated the data and proof read the manuscript. TN and DG designed, supervised the study and approved the final manuscript. DG provided the financial support.

### Ethics approval and consent to participate

The study was approved by the Ethics Committee of the Justus-Liebig University (Giessen, Germany).

### Patient consent for publication

Patient consent was obtained for the current study.

### Competing interests

The authors declare that they have no competing interests.

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