ARTICLE
Journal of Cellular Biochemistry 110:589–597 (2010)

Journal of Cellular Biochemistry

The Carboxy-Terminal Tail of Connexin43 Gap Junction Protein Is Sufficient to Mediate Cytoskeleton Changes in Human Glioma Cells

Sophie Crespin,[1] John Bechberger,[2] Marc Mesnil,[1] Christian C. Naus,[2] and Wun-Chey Sin[2]*

[1] *Institut de Physiologie et Biologie Cellulaires, Université de Poitiers, CNRS-UMR 6187, 40 Avenue du Recteur Pineau, 86022 Poitiers Cedex, France*

[2] *Department of Cellular and Physiological Sciences, Life Sciences Institute, The University of British Columbia, Vancouver, British Columbia, Canada*

ABSTRACT

Connexin43 (Cx43) is a ubiquitously expressed member of the gap junction protein family that mediates gap junction intercellular communication (GJIC) by allowing exchange of cytosolic materials. Previous studies have used Cx43 truncated at the cytoplasmic tail (C-tail) to demonstrate that the C-tail is essential to regulate cell growth and motility. Therefore, the aim of our study was to delineate the respective role of the truncated Cx43 and the C-tail in mediating Cx43-dependent signaling. A truncated Cx43 expressing the channel part of the protein (TrCx43, amino acid 1–242) and a construct encompassing only the C-tail from amino acid 243 (243Cx43) were transduced into LN18 human glioma cells. Our results showed that the ability of Cx43 to suppress growth was independent of GJIC as assessed by dye transfer, but was dependent on the presence of a rigid extracellular matrix. We further demonstrated that the C-tail alone is sufficient to promote motility. Surprisingly, Cx43 is also able to increase migration in the absence of the C-tail, suggesting the presence of at least two distinct signaling mechanisms utilized by Cx43 to affect motility. Finally, we used time-lapse imaging to examine the behavior of migrating cells and it was apparent that the C-tail was associated with a lamellipodia-based migration not observed in either mock or TrCx43 expressing LN18 cells. Our study shows for the first time that a free C-tail is sufficient to induce Cx43-dependent changes in cell morphology and that Cx43 signaling is linked to the regulation of the actin cytoskeleton. J. Cell. Biochem. 110: 589–597, 2010.

KEY WORDS: CX43; GAP JUNCTIONS; ACTIN CYTOSKELETON; MIGRATION

The gap junction protein connexin43 (Cx43) is ubiquitously expressed and belongs to a large family of channel proteins consisting of at least 21 members [Simon and Goodenough, 1998]. Like other members of the Connexin (Cx) family, Cx43 is a subunit of a tetra-membrane spanning hexamer with cytosolic N- and C-termini. The C-terminal cytoplasmic tail (C-tail) of Cxs shows the most variability and confers specificity in their actions. The hexamer is also called a hemichannel and two hemichannels between adjacent cells constitute an intercellular channel connecting two cytoplasms. Such channels are gathered as plaques called gap junctions (GJ) which are permeable to ions and small molecules up to 1 kDa, allowing for GJ intercellular communication (GJIC) [Simon and Goodenough, 1998]. GJIC also permits the transmission of growth promoting or suppressive factors, and has been traditionally implicated to be the major route by which gap junctions regulate cell growth [see Loewenstein, 1979; Naus, 2002; Mesnil et al., 2005, for review].

Gliomas are brain cancer that is widely believed to arise from glial-like precursors or differentiated glial cells [Kleihues and Ohgaki, 2000; Singh et al., 2003] that express high levels of Cx43 [Sutor and Hagerty, 2005]. A decrease in Cx43 expression has been linked to increased proliferation of gliomas [Huang et al., 1999; Soroceanu et al., 2001], and Cx43 is therefore suggested to act like a growth suppressor. Accordingly, the re-induction of Cx43 expression in a rat glioma cell line was associated with a decreased ability to divide in vitro [Zhu et al., 1991, 1992]. However, this decreased

Additional Supporting Information may be found in the online version of this article.

Grant sponsor: La Ligue Contre le Cancer (Comités de Charente, Charente Maritime et Vienne); Grant sponsor: Boehringer Ingelheim Fonds; Grant sponsor: Cancéropôle Grand Ouest; Grant sponsor: Canadian Institutes of Health Research; Grant number: MOP-81202.

*Correspondence to: Dr. Wun-Chey Sin, Department of Cellular and Physiological Sciences, Life Sciences Institute, The University of British Columbia, Vancouver, BC, Canada V6T 1Z3. E-mail: wcsin@interchange.ubc.ca

Received 3 September 2009; Accepted 28 January 2010 • DOI 10.1002/jcb.22554 • © 2010 Wiley-Liss, Inc.
Published online 21 April 2010 in Wiley InterScience (www.interscience.wiley.com).

proliferation rate is associated with Cx43 expression in the absence of GJIC restoration [Huang et al., 1998].

Recent studies have revealed a new emerging role of Cx43 in promoting cell migration, such as in normal brain development [Elias et al., 2007; Cina et al., 2009] and in enhancing glioma invasion [Lin et al., 2002; Zhang et al., 2003b; Oliveira et al., 2005; Bates et al., 2007]. Nonetheless, the authors differ on the mechanisms utilized by Cx43 to mediate cell migration. The GJIC, the C-tail of Cx43, or the intercellular adhesion conferred by the extracellular loops of Cx43, have all been suggested to be critical in promoting cell motility. Incidentally, the C-tail of Cx43 has been shown to interact directly with various cytoskeleton proteins implicated in cell motility, including actin, microtubules, actin binding proteins, tight junction and adherens junction proteins [reviewed by Giepmans, 2004; Olk et al., 2009]. In particular, the microtubule-binding domain of Cx43 is required for epicardial cell migration [Rhee et al., 2009]. Abnormal actin organization and reduced directionality have also been observed in neural crest cells derived from Cx43 knockout mice [Xu et al., 2006]. Therefore, it is conceivable that Cx43 modulates cell motility by either directly or indirectly affecting the cytoskeletal network.

A dynamic remodeling of the actin network occurs during cancer cell migration and invasion [Vignjevic and Montagnac, 2008]. In this aspect, the small GTPases signaling cascades are the main regulators of actin reorganization [Van Aelst and D'Souza-Schorey, 1997; Hall, 2005]. Typically, actin filament polymerization induced by either the Cdc42 or Rac-mediated cascades resulted in the emergence of filopodia and lamellipodia at the leading edges, respectively. On the other hand, an alternative contractility based, RhoA-dependent-cell migration mechanism leading to amoeboid movements has also been suggested to occur in glioma cells [Yamazaki et al., 2009].

To decipher the role of the C-tail in mediating glioma cell motility, we designed two truncated constructs of Cx43 lacking either the C-terminal tail or the entire transmembrane domain. Our results showed that GJIC is not required for cell motility, and that the C-tail of Cx43 is sufficient to promote cell motility and mediate Cx43-dependent actin reorganization.

MATERIALS AND METHODS

CELL CULTURE

LN18 cells (from ATCC, CRL-2610) expressing low level of endogenous Cx43 protein were maintained in low glucose Dulbecco's modified Eagle's medium (DMEM) containing L-glutamine (Gibco) and 10% fetal bovine serum (FBS, Biowhittaker) at 37°C with 5% CO_2. A semi-quantitative reverse transcriptase (RT)-PCR showed that Cx45 and Cx46 mRNA were also detected but at a much lower level than Cx43 mRNA (Suppl. Fig. 1). Cx26, Cx30, Cx32, and Cx40 were not present in LN18 cells. HEK293 retroviral packaging cells (a generous gift from Dr. Richard C. Mulligan, Children's Hospital, Boston, MA) were maintained in low glucose DMEM containing L-glutamine, 10% FBS, 1 μg/ml tetracycline, 2 μg/ml puromycin, 0.3 μg/ml G418.

For culturing 3D multicellular spheroids, LN18 cells were plated in 96-well plates in 100 μl of DMEM/F12 containing B27 supplement (Invitrogen), human recombinant EGF (20 ng/ml; Sigma), bFGF (20 ng/ml; Upstate), at a density of 1,000 cells/well.

CONSTRUCTION AND TRANSDUCTION OF RETROVIRAL VECTORS

The coding sequence for human Cx43 was excised from a Bluescript KS+ vector (generous gift from Dr Fishman, Albert Einstein College of medicine, New York, NY). Various cDNAs were obtained by PCR amplification using the following primers: Cx43 (forward: 5′-AAT TGG ATC CAC CAT GGG TGA CTG GAG C-3′, reverse: 5′-AAT TGT TAA CC TAG ATC TCC AGG TCA TCA G-3′), TrCx43 (forward: 5′-AAT TAG ATC TAC CAT GGG TGA CTG GAG CGC C-3′; reverse: 5′-AAT TGT TAA CCT ATC CCT TAA CCC GAT CCT TAA C-3′), 243Cx43 (forward: 5′-AAT TGG ATC CAC CAT GAA GAG CGA CCC TTA CCA TGC-3′; reverse: 5′-AAT TGT TAA CCT AGA TCT CCA GGT CAT CAG-3′). After purification (QIAquick® PCR purification kit, Qiagen), they were inserted into retroviral pMSCVpuro vectors (CLONTECH Laboratories). The resultant plasmids were validated by sequencing (NAPS Unit, Biotechnology Laboratory, University of British Columbia). pMSCVpuro-Cx43, pMSCVpuro-TrCx43, pMSCVpuro-243Cx43 and the empty vector pMSCVpuro plasmids were transfected into HEK293 packaging cells using Lipofectamine™ 2000 reagent (Invitrogen). Serum-free DMEM containing tetracycline was added 6 h following transfection, and the medium was replaced with serum-containing DMEM without tetracycline 24 h post-transfection. The medium was then collected and filtered (0.45 μm filter, BD Biosciences) after 48–72 h. Cell culture media of LN18 cells was replaced with filtered retroviral supernatant for infection. Following two rounds of infection cells were further cultured in selection media containing 800 ng/ml puromycin.

WESTERN ANALYSIS

Upon reaching confluency in 60-mm dishes, cells were rinsed in ice cold phosphate-buffered saline (PBS) and lysed in 800 μl radioimmune precipitation lysis buffer (RIPA) containing phosphatase inhibitors (Sigma) and protease inhibitors (Roche) [Fu et al., 2004]. DNA was sheared by sonication and subsequently samples were centrifuged at 14,000 rpm (10 min, 4°C). The protein-containing fraction was subjected to the colorimetric BCA Protein Assay Kit (Pierce) to determine the protein concentration of the samples. About 30 μg of protein was loaded onto 12% polyacrylamide gels. Electrophoretic separation was conducted using a Mini-Protean 3 electrophoresis system (Biorad) at 100 V. Separated protein was transferred (15 V, 35 min) onto an Immun-Blot® PVDF (polyvinylidene difluoride) membrane (Biorad) using a Transblot SD® semidry transfer cell (Biorad). Following transfer, blots were equilibrated in Tris-buffered saline (TBS; Tris 50 mM, NaCl 150 mM, pH 7.5), incubated in 5% nonfat milk dissolved in TBS containing 0.1% Tween (TBST), and incubated in 1% milk-TBST containing antibodies directed against the protein of interest. Antibodies used included anti-Cx43 (rabbit, Sigma, 1:8,000, or mouse, Upstate, 1:50) and anti-GAPDH (mouse, HyTest Ltd, 1:10,000). Blots were then washed in TBST and incubated in a 1% milk-TBST solution containing appropriate secondary antibody conjugated to horseradish peroxidase (goat anti-rabbit HRP or goat anti-mouse HRP, Cedarlane, 1:10,000). Protein bands were visualized with SuperSignal chemiluminescent substrate (Pierce)

before exposure to X-ray film (Kodak). Densitometry analysis was carried out using ImageJ software [Abramoff et al., 2004] available at (http://rsbweb.nih.gov/ij/) to determine relative changes in protein levels.

IMMUNOCYTOCHEMISTRY

Cells (40,000) were plated on 12-mm glass coverslips in 12-wells plate and fixed in 4% paraformaldehyde (8 min, room temperature) at 90% confluency, permeabilized in 0.1% Triton X-100 (10 min, room temperature), washed twice with PBS, and incubated in blocking solution (bovine serum albumin 4%) prior to incubation in blocking solution containing primary antibody against Cx43 (Sigma, rabbit polyclonal, 1:1,000 or Upstate, mouse monoclonal antibody, 1:20). Coverslips were then washed prior to incubation in blocking solution containing secondary antibody (Molecular Probes, goat anti-rabbit IgG, 1:400 or goat anti-mouse IgG, 1:400). Actin cytoskeleton was stained by incubation with Alexa 488-conjugated phalloidin (Molecular Probes, 1:400). Coverslips were washed and mounted onto microscope slides with Prolong Gold® containing 4′,6-diamidino-2-phenylindole (DAPI, Molecular Probes). Fluorescent images were acquired with Olympus Fluoview 1.6 using a confocal microscope (Olympus).

PRELOADING/DYE COUPLING

GJIC was evaluated according to the established preloading method previously described [Goldberg et al., 1995]. Briefly, 250,000 cells were plated in duplicate in 60-mm plates. When the cell reached confluency after 2 days, donor cells were bathed for 20 min at 37°C in a dye solution (5 μM calcein-AM [Molecular Probes] and 10 μM DiI [Sigma-Aldrich] in an isotonic [0.3 M] glucose solution), rinsed several times with isotonic glucose, trypsinized, seeded onto confluent unlabeled recipient sister cultures at a ratio of 1:500, and maintained for 4 h in the cell culture incubator (37°C). After 4 h, diffusion of calcein-AM via the GJIC was observed. DiI is not cell permeable and therefore identified the donor cells. Cells were examined by epifluorescence microscopy (Axioplan 2) and GJIC was assessed and quantified by the number of cells that received passage of calcein dye from the donor cells. All donor cells identified were included in the analysis.

PROLIFERATION ASSAY

Cells (40,000) were plated in 12-well plates in triplicate. The rate of cell proliferation was determined on days 1, 4, 5, 6, 7, and 8 post-plating using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay, Promega). Evaluation of the number of cells, characterized by their mitochondrial activity, was performed with a Mithras microplate reader (Berthold Technologies) at 490 nm.

SOFT AGAR ASSAY

A two-layer soft agar system was used in this assay. Briefly, 2 ml of medium containing 0.6% Noble agar (Sigma) was cast as a bottom layer for each 60-mm plate. Cells were then seeded in triplicates at 10,000 per plate in 2 ml of 0.3% agar medium above the bottom layer. Following 14 days of incubation with fresh medium added every 7 days, samples were stained overnight with 0.01% crystal violet. Colonies were viewed under a microscope (Axioplan 2) and 30 images were captured for each dish using Axiovision. The area of each colony was measured and colonies with a size $>500\ \mu m^2$ were scored as positive. The average area of the colonies was calculated by summing the area of all measured colonies and dividing by the total number of colonies measured.

WOUND HEALING MOTILITY ASSAY

Cells (0.25×10^6) were grown to confluency in 35-mm dishes (3 days). The confluent monolayer was scraped using a blue tip (Costar). Cells were rinsed several times in PBS to remove loose and detached cells and images were acquired immediately and 24 h after the scrape. Migration distances were measured using Axiovision software (Zeiss).

TIME-LAPSE LIVE IMAGING

Cells (0.25×10^6) were grown to confluency in 35-mm dishes (3 days). The confluent monolayer was scraped using a blue tip. Cells were rinsed several times in PBS to remove loose and detached cells and images were acquired 24 h after the scrape. The migration behavior of LN18 cells expressing Cx43, TrCx43, or 243Cx43 was followed for 1 h at 40× magnification using a confocal microscope (Olympus) with a heating block to maintain the temperature at 37°C. Four images per minute were acquired with Olympus Fluoview 1.6 software.

STATISTICAL ANALYSIS

Quantitative data were subjected to statistical analysis using InStat® software. Results are expressed as mean ± SEM. Student's *t*-test analysis was used for statistical comparisons with $P < 0.05$ considered significant. Each experiment was repeated three or more times with similar results.

RESULTS

TRUNCATED CX43 LACKING THE CARBOXY-TERMINAL TAIL IS DEFECTIVE IN INTERCELLULAR COMMUNICATION IN LN18 HUMAN GLIOMA CELLS

We first sought to determine whether the C-tail of Cx43 is sufficient to mediate Cx43-dependent growth control by overexpressing two truncated forms of Cx43 in LN18 cells, a human glioma cell line derived from a grade IV glioblastoma expressing low levels of endogenous Cx43. The first construct (243Cx43) contains only the entire C-tail from amino acid 243 (Fig. 1A). The expression of this construct was previously linked to a decreased proliferation rate of cardiomyocytes and HeLa cells [Dang et al., 2006]. The second construct (TrCx43) is truncated at amino acid 242 (Fig. 1A) which is shorter than the K258stop construct commonly used in the majority of the previous studies, where a stop codon was introduced at amino acid 258 [Kretz et al., 2004; Gong et al., 2006; Maass et al., 2007]. Our construct was made with the intention to avoid the potential regulatory effects due to phosphorylation of Ser255 by the MAP kinases [Cameron et al., 2003].

LN18 cells were transduced with either the full-length Cx43 or the truncated Cx43 constructs (Fig. 1A). Western analysis confirmed the

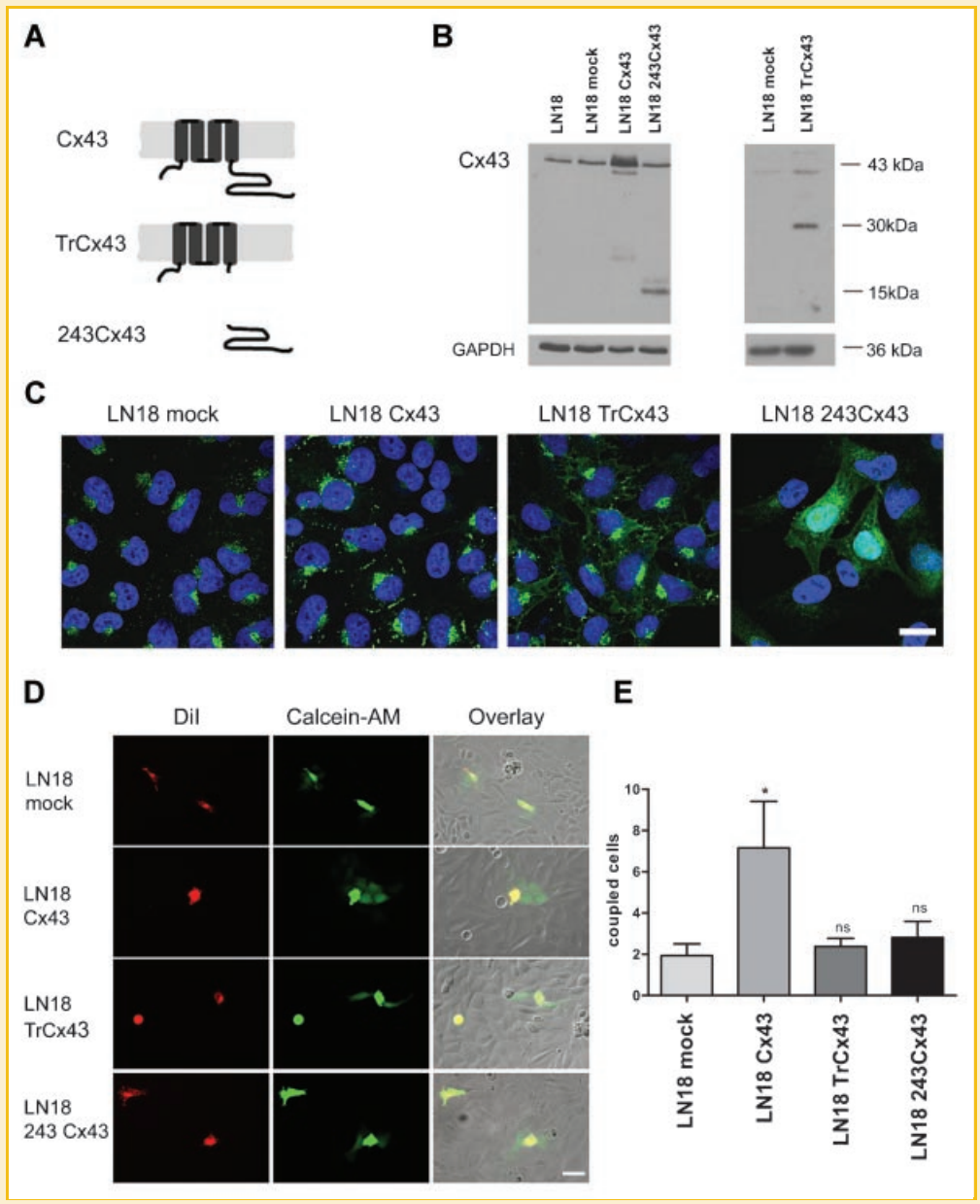

Fig. 1. Characterization of LN18 glioma cells expressing Cx43 mutants. A: Schematic representation of different Cx43 constructs expressed in LN18 cell line. Cx43: Full-length human Cx43. TrCx43: Truncated at 242 amino acids of the human Cx43. 243Cx43: Carboxy-tail of human Cx43 starting at amino acid 243. B: Detection of Cx43, TrCx43 and 243Cx43 by western blotting. The different forms were detected at the expected size, respectively at 43, 30, and 15 kDa. GAPDH was used as a loading control. C: Immunostaining of Cx43 in LN18 mock, LN18 Cx43, LN18 TrCx43, and LN18 243Cx43 cells. Nuclei are stained by TO-PRO®-3. Bar: 20 μm. D: Communication assay on LN18 cell line expressing Cx43, TrCx43, or 243Cx43. Donor cells were preloaded with DiI (red) and calcein-AM (green). Bar: 50 μm. The number of coupled cells was estimated by counting the number of cells stained by calcein-AM (green). Only the full-length Cx43 appeared to be able to restore GJIC. E: Quantification of coupling data shown in D. Student's *t*-test: $^{*}P < 0.05$.

recombinant Cx43 proteins were correctly expressed with the expected size of 43 kDa for the full-length Cx43, 30 kDa for TrCx43, and 15 kDa for 243Cx43 (Fig. 1B). A semi-quantification using Image J software normalized to the reference protein GAPDH indicated that overexpression of full-length Cx43 increased the level of total Cx43 in LN18 by a factor of 1.7. In contrast, TrCx43 and 243Cx43 did not increase the level of endogenous Cx43 (Fig. 1B). We also confirmed that full-length Cx43 was localized to punctuate staining at the plasma membrane of cell–cell contact areas in LN18 Cx43 cells (Fig. 1C) while the parental cell line LN18 transduced by empty retroviral particles (LN18 mock) expressed a low amount of endogenous Cx43 in the cytoplasm. However, LN18 TrCx43 cells exhibited a limited punctuate membrane staining and a diffuse cytoplasmic signal (Fig. 1C). In contrast, 243Cx43 cells exhibited a strong but diffuse cytoplasmic staining only (Fig. 1C).

We next investigated whether the GJIC between LN18 cells was altered by the expression of these Cx43 constructs. Although LN18 cells expressed low levels of Cx43, the parental line coupled very

poorly (Fig. 1D). GJIC investigated by preloading assay showed a significant increase in the number of coupled cells only in LN18 Cx43 cells (7.2 ± 2.3 coupled cells vs. 1.9 ± 0.6 for LN18 mock) but not in LN18 TrCx43 cells (2.4 ± 0.4 coupled cells), indicating that the C-tail of Cx43 is needed for restoration of GJIC in these cells (Fig. 1D). As expected, the expression of the C-tail of Cx43 alone did not affect the intercellular coupling of LN18 cells (1.9 ± 0.6).

THE CARBOXY-TERMINAL TAIL OF CX43 IS SUFFICIENT TO REDUCE ANCHORAGE-INDEPENDENT GROWTH

The restoration of Cx43 expression in gliomas has been linked to decreased proliferation in rat and human glioma cell lines [Zhu et al., 1991, 1992; Huang et al., 1998]. Furthermore, this effect does not appear to require functional GJIC in human glioma cells [Huang et al., 1998]. To determine whether GJIC was similarly required for the growth effect mediated by Cx43 in LN18 cells, we investigated the proliferation rate of LN18 cells with two 3-dimensional growth assays. We first measured the proliferation rate of LN18 multicellular spheroids [Kelm et al., 2003] cultured in the absence of both serum and an adhesive substratum, and we did not observe any significant difference in the growth rate among LN18 cells expressing different Cx43 variants (Fig. 2A). This unexpected result is in contrast to previous studies [Zhu et al., 1991, 1992; Cirenei et al., 1998; Huang et al., 1998] and the discrepancy could be due to the level of overexpression [Naus et al., 1992] or culture conditions. In our case, retroviral transduction led to lower but a more physiological level of overexpression (about 2.4-fold over the endogenous Cx43) (Fig. 1A, see also Suppl. Fig. 1A) when compared to the use of strong expression promoters in previous studies [Naus et al., 1992; Zhu et al., 1992; Cirenei et al., 1998; Huang et al., 1998]. In addition, our earlier result also demonstrated that the level of Cx43 overexpression is correlated with its ability to suppress growth [Sin et al., 2008]. To determine the growth characteristics of the cells independent of their migration ability, we examined the growth of LN18 cells in the presence of a basic matrix of agar as a substratum that provided matrix rigidity to the cultured cells. In contrast to our previous results with free floating spheroids (Fig. 2A), we observed a reduction in growth, as measured by the sizes of the colonies formed by all three LN18 cell lines expressing various forms of Cx43 (Fig. 2B). LN18 mock cells formed larger colonies than LN18 Cx43, LN18 TrCx43, and LN18 243Cx43 cells. However, the total number of colonies resulting from the same initial cell seeding did not differ significantly among the different LN18 lines, suggesting Cx43 affects growth but not the ability of LN18 cells to form cell colonies in an anchorage-independent manner. Our results reveal that Cx43-dependent growth control synergized with the extracellular environment, and expression of the C-tail of Cx43 alone is sufficient to reduce growth.

THE CARBOXY-TERMINAL TAIL OF CX43 INCREASES GLIOMA MIGRATION

Recent evidence has confirmed the pivotal role of Cx43 in neuronal cell migration [Elias et al., 2007; Cina et al., 2009] and glioma invasion [Oliveira et al., 2005; Bates et al., 2007]. We therefore investigated whether the C-tail of Cx43 is sufficient to affect cell motility, in addition to its growth suppressive effect shown above.

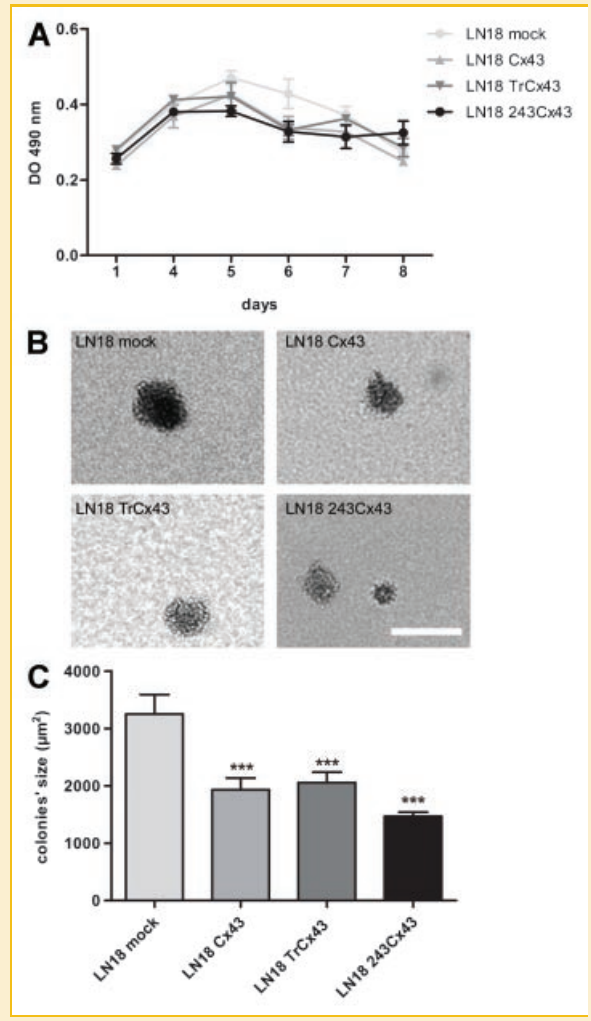

Fig. 2. Expression of Cx43 mutants reduced anchorage-independent growth of LN18 cells. A: Proliferation assay of LN18 multicellular spheroids showing no significant difference in growth rate of LN18 cells expressing different constructs of Cx43. The number of cells was evaluated with a color reaction measuring the optical density at 490 nm as described in the Materials and Methods Section. B: Anchorage independent soft agar proliferation assay showed reduction of growth in LN18 cells expressing Cx43, TrCx43, or 243Cx43. Colonies' size was estimated after 2 weeks in 30 randomized fields. Bar: 100 μm. C: Quantification of the colonies' size. Student's *t*-test: $^{***}P < 0.001$.

Here, the migration rate was investigated by a standard wound healing assay (Fig. 3A). Surprisingly, all three Cx43 constructs were equally effective in increasing migration rate of LN18 cells as assessed by wound healing assays. The LN18 mock cells covered a distance of 151.2 ± 5.9 μm, versus 213.4 ± 10.3 μm (+41%) for LN18 Cx43 cells, 206.2 ± 6.7 μm (+36%) for LN18 TrCx43 cells, and 226.5 ± 5.4 μm (+51%) for LN18 243Cx43 cells in 24 h (Fig. 3B). Taken together, our results suggest that at least two different mechanisms could be utilized by Cx43 in mediating growth and motility, the extracellular loop that is present in both full-length Cx43 and TrCx43 affecting cell adhesion, and the interaction of carboxy-terminal tail with intracellular signaling components.

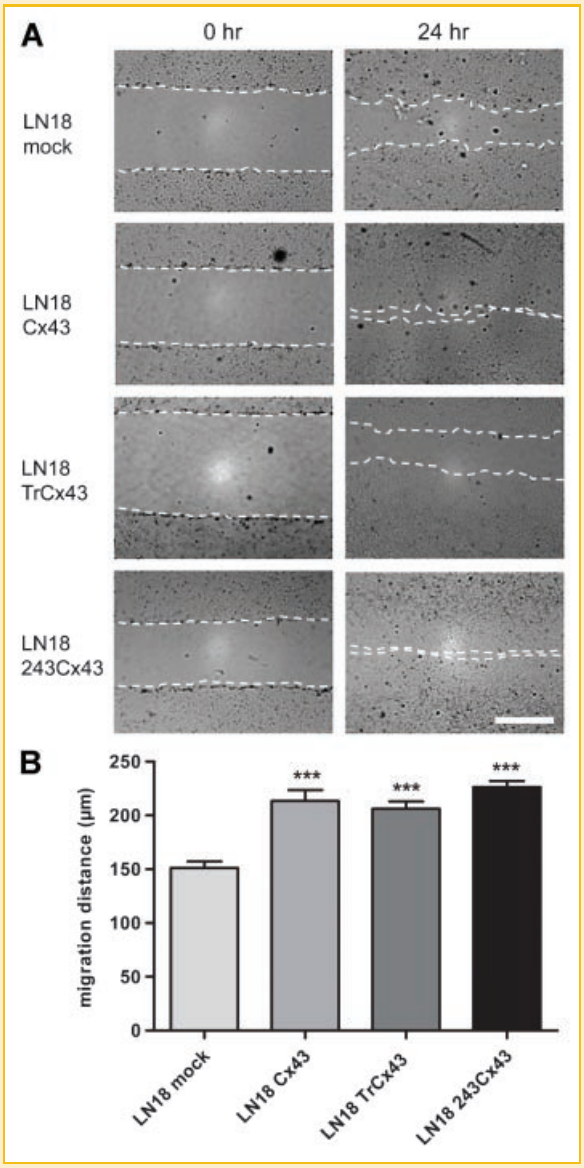

Fig. 3. Increased migration of LN18 cells expressing Cx43, TrCx43, and 243Cx43 as determined by wound healing assay. Dashed lines indicate the migrating front. A: Representative pictures of LN18 cells at 0 h (when a wound was made), and 24 h later. B: The migration ability was estimated quantitatively by measuring the remaining area between the two edges of the wound at 24 h. The covered distance was estimated in eight different areas of the culture, in three independent experiments. Student's *t*-test: $^{***}P < 0.001$.

THE CARBOXY-TERMINAL TAIL OF CX43 IS SUFFICIENT TO INDUCE ACTIN CYTOSKELETON REORGANIZATION

In an attempt to distinguish the possible mechanisms utilized by Cx43 in mediating cell motility, we examined the behavior of migrating LN18 cells following a wound. Time-lapse live imaging revealed protrusions all around the perimeter of the migrating LN18 mock cells (Fig. 4A). These "bleb-like" protrusions were also observed in LN18 cells expressing the truncated Cx43. However, the protrusions in LN18 TrCx43 cells were polarized to the migrating front (Fig. 4A).

On the contrary, LN18 Cx43 and LN18 243Cx43 cells showed a flatten morphology characterized by active lamellipodial protrusions during migration (Fig. 4A). When we stained the migrating cells along the wound with phalloidin to visualize the actin cytoskeleton, we observed numerous filopodia present in LN18 Cx43 and LN18 243Cx43 cells that were absent in LN18 mock and LN18 TrC43 cells (Fig. 4B). Thus, it appeared that increased polarity manifested by these Cx43 expressing cells, whether via "bleb-like" protrusions or lamellipodia formation, may help to explain their increased migration (Fig. 3). The close relationship between Cx43 and the actin cytoskeleton is further reinforced by the co-staining of full-length Cx43 with the actin-rich protrusions in migrating LN18 Cx43 cells (Fig. 4C).

DISCUSSION

Previous screening of primary human glioma tissues indicated that Cx43 is mostly downregulated in high-grade glioma tumors [Huang et al., 1999; Soroceanu et al., 2001; Pu et al., 2004]. Accordingly, restoration of Cx43 expression in glioma cells has been associated with a reduction of proliferation rate [Zhu et al., 1991, 1992; Huang et al., 1998]. While it was initially proposed that the growth suppression was mediated by a GJIC-dependent pathway, it has become increasingly clear that Cx43 can mediate growth control primarily independent of GJIC restoration [Huang et al., 1998; Qin et al., 2003; Zhang et al., 2003b]. In this aspect, the C-tail of Cx43 is often proposed to play a pivotal role in growth suppression since its deletion will abrogate Cx43-mediated growth control [Moorby and Patel, 2001; Zhang et al., 2003b]. The importance of C-tail has been attributed to its multiple interacting sites with various signaling proteins [reviewed by Giepmans, 2004; Olk et al., 2009; Laird, 2010], including N-cadherin [Xu et al., 2001], and the matricellular protein CCN3 [Fu et al., 2004; Gellhaus et al., 2004]. Since a number of these Cx43-interacting proteins are associated with the cytoskeleton, it is perhaps not surprising that Cx43 has also been repeatedly shown to be necessary for cell migration, for example, in brain development and glioma invasion [Xu et al., 2001; Zhang et al., 2003a; Bates et al., 2007; Elias et al., 2007; Cina et al., 2009].

Until now, it is still unclear whether the C-tail of Cx43 is essential for optimal GJIC. While the K258stop mutant has been shown to form a functional channel by patch clamp analysis [Kretz et al., 2004; Gong et al., 2006; Maass et al., 2007], the same mutant showed attenuated coupling as determined by dye coupling [Kozoriz et al., 2010]. In agreement with our results in LN18 cells, the expression of a Cx43 mutant truncated at amino acid 251 did not show restoration of GJIC in HeLa cells when investigated by dye coupling [Martinez et al., 2003]. However, a rat Cx43 truncated at amino acid 244 showed partial GJIC restoration in C6 glioma cells [Bates et al., 2007], indicating that GJIC by truncated Cx43 may be dependent on several factors, such as the functional state of endogenous Cx43 and the molecular environment of each cell type. To confirm this initial observation is not due to an artifact, we also transduced TrCx43 in two other human glioma cell lines—U87 expressing higher levels of endogenous Cx43, and U251 expressing comparable levels of Cx43

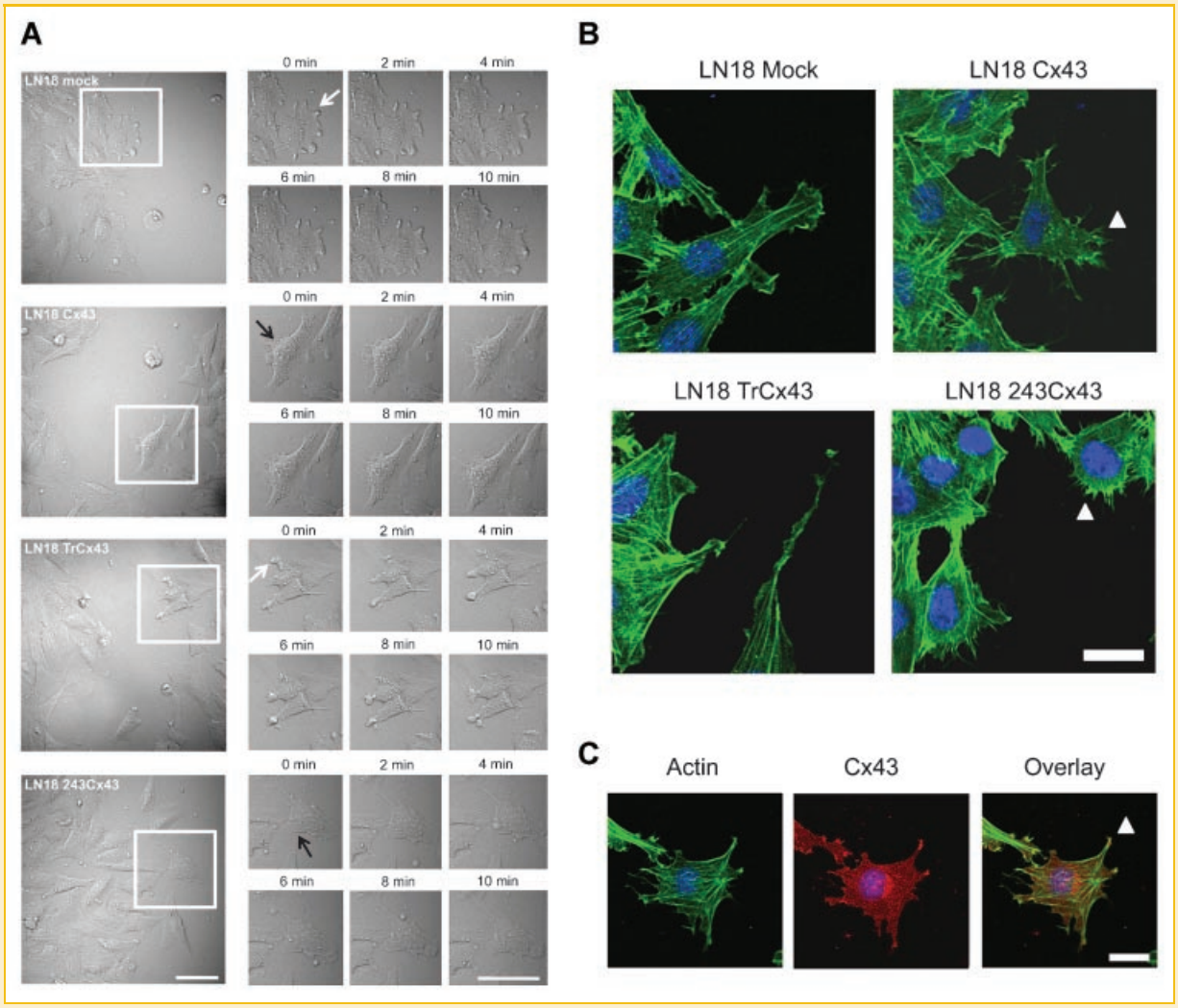

Fig. 4. The C-terminal tail is sufficient to mediate Cx43-dependent cytoskeletal effects. A: Time-lapse live imaging of LN18 mock and LN18 cells expressing Cx43, TrCx43, and 243Cx43 at the edge of a wound. Multiple "bleb-like" protrusions (white arrows) were observed for LN18 mock and Ln18-TrCx43 cells only. LN18 Cx43 and LN18-243Cx43 cells showed predominantly lamellipodia-like protrusions (black arrows). Bar: 50 μm. B: Immunostaining of LN18 cells expressing mock, Cx43, TrCx43, or 243Cx43 with Alexa 488-conjugated phalloidin (green) to visualize the actin cytoskeleton. LN18 Cx43 and LN18 243Cx43 exhibited numerous actin-rich filopodia (arrowheads) not observed in LN18 mock and LN18 TrCx43 cells. Bar: 20 μm. C: Co-staining of full-length Cx43 with the actin-rich filopodia (arrowhead) in migrating LN18 cells at the wound. Bar: 10 μm.

to LN18; TrCx43 did not induce GJIC as determined by dye transfer in either cell line (Suppl. Fig. 2A,B).

Surprisingly, we observed a similar reduction in the proliferation rate of TrCx43, 243Cx43, and full-length Cx43 expressed in LN18, U251 and U87 glioma cells, respectively (Fig. 2, Suppl. Fig. 2C). Therefore, restoration of GJIC, as determined by dye transfer, is not necessary for Cx43 to mediate growth suppression in these glioma lines. Our result reinforces previous studies showing that Cx43 suppresses proliferation independently of GJIC [Huang et al., 1998; Qin et al., 2002; Zhang et al., 2003a]. In addition, we only noticed a reduction of proliferation rate when the glioma cells were cultured in the presence of a semi-solid agar matrix, suggesting that the extracellular environment is critical in Cx43-mediated growth control.

The effect of TrCx43 and 243Cx43 on cell motility is less clear cut. Expression of full-length Cx43 in LN18 cells increased cell migration, which has also been observed by others [Lin et al., 2002; Zhang et al., 2003a; Oliveira et al., 2005]. In LN18 cells, both TrCx43 and 243Cx43 increased migration similarly, which is in contrast to our previous study showing the lack of the C-tail in TrCx43 is associated with the absence of enhanced migration [Bates et al., 2007]. However, Cx43-mediated cell motility appears to show variations in different cell lines, as the same TrCx43 enhanced migration in U87 cells but not in U251 cells (Supp. Fig. 2D). Taken together, our results do not support a major role of GJIC in cell migration. This is manifested by the lack of enhanced migration in cells expressing GJIC-restored full-length Cx43. We also showed that the carboxyl tail is not the only domain that Cx43 can utilize for its effects on cell migration. Although we cannot exclude GJIC may facilitate growth suppression and migration due to the presence of low endogenous gap junction-mediated coupling in parental LN18 cells [Robe et al., 2005] (Fig. 1), the fact that the cytoplasmic

243Cx43 induced the same morphological changes as the full-length Cx43 suggests the uncoupling of channel function with cytoskeletal reorganization. Furthermore, several recent studies have indicated that GJIC is not a prerequisite for a role of connexins in cell migration [Xu et al., 2006; Elias et al., 2007].

A closer examination on the architecture of the actin cytoskeleton in TrCx43, 243Cx43, and full-length Cx43 expressing LN18 cells revealed differences suggesting that even though all three constructs are capable to increase cell motility, they might utilize distinct signaling pathways. Time-lapse imaging showed that both Cx43- and 243Cx43-expressing cells not only exhibited a flat morphology but also migrated in a lamellipodia/filopodia-based fashion. While overexpression of Cx43 has been shown to flatten the morphology of cancer cells such as the hepatocarcinoma cells [Ionta et al., 2009], human glioma cells [Huang et al., 1998] and rat C6 glioma cells [Zhu et al., 1991], this is the first report that the C-tail by itself is capable of altering cell morphology. Indeed, TrCx43, lacking a C-tail, did not substantially alter the cell morphology when overexpressed in LN18 cells. LN18 mock contained multiple ''bleb-like'' protrusions that became more ''polarized'' and ''consolidated'' in TrCx43 expressing cells. It is not clear whether the presence of the microtubule-binding domain in our TrCx43 mutant may contribute to its increased cell motility as a recent finding has suggested the tubulin-binding domain is necessary for cell migration [Rhee et al., 2009]. Cellular protrusions manifested by membrane blebbing, observed in both mock and TrCx43 LN18 cells is a more recently investigated migration behavior that appears to be mediated by RhoA instead of the classical Rac/Cdc42 pathway [Charras and Paluch, 2008; Fackler and Grosse, 2008]. These membrane protrusions are proposed to be driven by cytosolic pressure instead of the classical route by which the protruding force is derived from polymerization of actin that results in the formation of lamellipodia and filopodia, the classical phenotype of the small GTPases Rac1 and Cdc42, respectively [Van Aelst and D'Souza-Schorey, 1997; Hall, 2005].

Our results clearly indicated the presence of the C-tail is essential for the formation of lamellipodia and filopodia, suggesting that Cx43 organizes the actin cytoskeleton via the Rac and Cdc42 pathway. This is not the first time that Cx43 has been linked to the small GTPases. The C-tail of Cx43 interacts with several cytoskeletal proteins [see Olk et al., 2009, for review]. In particular, p120 catenin and N-cadherin have been shown to localize with Cx43 [Xu et al., 2001]. Overexpression of p120 catenin induces the activation of Rac and Cdc42 leading to an increase in cell motility [Noren et al., 2000]. In addition, our recent data also shown that CCN3, a Cx43 binding protein, activates Rac1 directly [Sin et al., 2009]. It is therefore conceivable that Cx43 mediates cell motility via activation of the small GTPases pathway. Interestingly, Cx43 has recently been reported to interact with cortactin [Squecco et al., 2006], an actin-associated protein that promotes motility by enhancing lamellipodia formation [Bryce et al., 2005]. On the other hand, in the absence of the C-tail, TrCx43 appears to utilize an alternative pathway to modulate cell motility. RhoA has recently been shown to be as important as Rac in regulating glioma invasion [Yamazaki et al., 2009]. It is therefore conceivable that the RhoA-dependent ''blebbing'' motility may become more prominent in the absence of Rac and Cdc42 activation.

Taken together, our results showed for the first time that the carboxyl tail of Cx43 is sufficient to mediate Cx43-dependent actin reorganization. While it is still unclear how the C-tail affects the cytoskeletal organization, the C-tail has been recently shown to affect gene expression pattern [as reviewed by Kardami et al., 2007]. Our finding confirms that Cx43 is more than just a channel protein, and that it may regulate the actin cytoskeleton directly either via its C-tail or through a discrete region present in the truncated Cx43.

ACKNOWLEDGMENTS

The work was supported by ''La Ligue Contre le Cancer'', the Boehringer Ingelheim Fonds, ''Cancéropôle Grand Ouest,'' and the Canadian Institutes of Health Research (grant reference: MOP-81202 to WCS and CCN). We thank C. Lai for his assistance in soft agar and transwell assays.

REFERENCES

Abramoff MD, Magelhaes PJ, Ram SJ. 2004. Image processing with ImageJ. Biophotonics Int 11:36–42.

Bates DC, Sin WC, Aftab Q, Naus CC. 2007. Connexin43 enhances glioma invasion by a mechanism involving the carboxy terminus. Glia 55:1554–1564.

Bryce NS, Clark ES, Leysath JL, Currie JD, Webb DJ, Weaver AM. 2005. Cortactin promotes cell motility by enhancing lamellipodial persistence. Curr Biol 15:1276–1285.

Cameron SJ, Malik S, Akaike M, Lerner-Marmarosh N, Yan C, Lee JD, Abe J, Yang J. 2003. Regulation of epidermal growth factor-induced connexin 43 gap junction communication by big mitogen-activated protein kinase1/ERK5 but not ERK1/2 kinase activation. J Biol Chem 278:18682–18688.

Charras G, Paluch E. 2008. Blebs lead the way: How to migrate without lamellipodia. Nat Rev Mol Cell Biol 9:730–736.

Cina C, Maass K, Theis M, Willecke K, Bechberger JF, Naus CC. 2009. Involvement of the cytoplasmic C-terminal domain of connexin43 in neuronal migration. J Neurosci 29:2009–2021.

Cirenei N, Colombo BM, Mesnil M, Benedetti S, Yamasaki H, Finocchiaro G. 1998. In vitro and in vivo effects of retrovirus-mediated transfer of the connexin 43 gene in malignant gliomas: Consequences for HSVtk/GCV anticancer gene therapy. Gene Ther 5:1221–1226.

Dang X, Jeyaraman M, Kardami E. 2006. Regulation of connexin-43-mediated growth inhibition by a phosphorylatable amino-acid is independent of gap junction-forming ability. Mol Cell Biochem 289:201–207.

Elias LA, Wang DD, Kriegstein AR. 2007. Gap junction adhesion is necessary for radial migration in the neocortex. Nature 448:901–907.

Fackler OT, Grosse R. 2008. Cell motility through plasma membrane blebbing. J Cell Biol 181:879–884.

Fu CT, Bechberger JF, Ozog MA, Perbal B, Naus CC. 2004. CCN3 (NOV) interacts with connexin43 in C6 glioma cells: Possible mechanism of connexin-mediated growth suppression. J Biol Chem 279:36943–36950.

Gellhaus A, Dong X, Propson S, Maass K, Klein-Hitpass L, Kibschull M, Traub O, Willecke K, Perbal B, Lye SJ, Winterhager E. 2004. Connexin43 interacts with NOV: A possible mechanism for negative regulation of cell growth in choriocarcinoma cells. J Biol Chem 279:36931–36942.

Giepmans BN. 2004. Gap junctions and connexin-interacting proteins. Cardiovasc Res 62:233–245.

Goldberg GS, Bechberger JF, Naus CC. 1995. A pre-loading method of evaluating gap junctional communication by fluorescent dye transfer. Biotechniques 18(3):490–497.

Gong XQ, Shao Q, Lounsbury CS, Bai D, Laird DW. 2006. Functional characterization of a GJA1 frameshift mutation causing oculodentodigital dysplasia and palmoplantar keratoderma. J Biol Chem 281:31801–31811.

Hall A. 2005. Rho GTPases and the control of cell behaviour. Biochem Soc Trans 33:891–895.

Huang RP, Fan Y, Hossain MZ, Peng A, Zeng ZL, Boynton AL. 1998. Reversion of the neoplastic phenotype of human glioblastoma cells by connexin 43 (cx43). Cancer Res 58:5089–5096.

Huang RP, Hossain MZ, Sehgal A, Boynton AL. 1999. Reduced connexin43 expression in high-grade human brain glioma cells. J Surg Oncol 70:21–24.

Ionta M, Sant'ana Ferreira RA, Pfister SC, Machado-Santelli GM. 2009. Exogenous Cx43 expression decrease cell proliferation rate in rat hepatocarcinoma cells independently of functional gap junction. Cancer Cell Int 9:22.

Kardami E, Dang X, Iacobas DA, Nickel BE, Jeyaraman M, Srisakuldee W, Makazan J, Tanguy S, Spray DC. 2007. The role of connexins in controlling cell growth and gene expression. Prog Biophys Mol Biol 94:245–264.

Kelm JM, Timmins NE, Brown CJ, Fussenegger M, Nielsen LK. 2003. Method for generation of homogeneous multicellular tumor spheroids applicable to a wide variety of cell types. Biotechnol Bioeng 83:173–180.

Kleihues P, Ohgaki H. 2000. Phenotype vs genotype in the evolution of astrocytic brain tumors. Toxicol Pathol 28:164–170.

Kozoriz MG, Bechberger JF, Bechberger GR, Suen MWH, Moreno AP, Maass K, Willecke K, Naus CC. 2010. The connexin43 C-terminal region mediates neuroprotection during stroke. J Neuropathol Exp Neurol 69:196–206.

Kretz M, Maass K, Willecke K. 2004. Expression and function of connexins in the epidermis, analyzed with transgenic mouse mutants. Eur J Cell Biol 83:647–654.

Laird DW. 2010. The gap junction proteome and its relationship to disease. Trends Cell Biol 20:92–101.

Lin JH, Takano T, Cotrina ML, Arcuino G, Kang J, Liu S, Gao Q, Jiang L, Li F, Lichtenberg-Frate H, Haubrich S, Willecke K, Goldman SA, Nedergaard M. 2002. Connexin 43 enhances the adhesivity and mediates the invasion of malignant glioma cells. J Neurosci 22:4302–4311.

Loewenstein WR. 1979. Junctional intercellular communication and the control of growth. Biochim Biophys Acta 560:1–65.

Maass K, Shibayama J, Chase SE, Willecke K, Delmar M. 2007. C-terminal truncation of connexin43 changes number, size, and localization of cardiac gap junction plaques. Circ Res 101:1283–1291.

Martinez AD, Hayrapetyan V, Moreno AP, Beyer EC. 2003. A carboxyl terminal domain of connexin43 is critical for gap junction plaque formation but not for homo- or hetero-oligomerization. Cell Commun Adhes 10:323–328.

Mesnil M, Crespin S, Avanzo JL, Zaidan-Dagli ML. 2005. Defective gap junctional intercellular communication in the carcinogenic process. Biochim Biophys Acta 1719:125–145.

Moorby C, Patel M. 2001. Dual functions for connexins: Cx43 regulates growth independently of gap junction formation. Exp Cell Res 271:238–248.

Naus CC. 2002. Gap junctions and tumour progression. Can J Physiol Pharmacol 80:136–141.

Naus CC, Elisevich K, Zhu D, Belliveau DJ, Del Maestro RF. 1992. In vivo growth of C6 glioma cells transfected with connexin43 cDNA. Cancer Res 52:4208–4213.

Noren NK, Liu BP, Burridge K, Kreft B. 2000. p120 catenin regulates the actin cytoskeleton via Rho family GTPases. J Cell Biol 150:567–580.

Oliveira R, Christov C, Guillamo JS, de Bouard S, Palfi S, Venance L, Tardy M, Peschanski M. 2005. Contribution of gap junctional communication between tumor cells and astroglia to the invasion of the brain parenchyma by human glioblastomas. BMC Cell Biol 6:7.

Olk S, Zoidl G, Dermietzel R. 2009. Connexins, cell motility, and the cytoskeleton. Cell Motil Cytoskeleton. 66:1000–1016.

Pu P, Xia Z, Yu S, Huang Q. 2004. Altered expression of Cx43 in astrocytic tumors. Clin Neurol Neurosurg 107:49–54.

Qin H, Shao Q, Curtis H, Galipeau J, Belliveau DJ, Wang T, Alaoui-Jamali MA, Laird DW. 2002. Retroviral delivery of connexin genes to human breast tumor cells inhibits in vivo tumor growth by a mechanism that is independent of significant gap junctional intercellular communication. J Biol Chem 277:29132–29138.

Qin H, Shao Q, Thomas T, Kalra J, Alaoui-Jamali MA, Laird DW. 2003. Connexin26 regulates the expression of angiogenesis-related genes in human breast tumor cells by both GJIC-dependent and -independent mechanisms. Cell Commun Adhes 10:387–393.

Rhee DY, Zhao XQ, Francis RJ, Huang GY, Mably JD, Lo CW. 2009. Connexin 43 regulates epicardial cell polarity and migration in coronary vascular development. Development 136:3185–3193.

Robe PA, Nguyen-Khac M, Jolois O, Rogister B, Merville MP, Bours V. 2005. Dexamethasone inhibits the HSV-tk/ganciclovir bystander effect in malignant glioma cells. BMC Cancer 5:32.

Simon AM, Goodenough DA. 1998. Diverse functions of vertebrate gap junctions. Trends Cell Biol 8:477–483.

Sin WC, Bechberger JF, Rushlow WJ, Naus CC. 2008. Dose-dependent differential upregulation of CCN1/Cyr61 and CCN3/NOV by the gap junction protein Connexin43 in glioma cells. J Cell Biochem 103:1772–1782.

Sin WC, Tse M, Planque N, Perbal B, Lampe PD, Naus CC. 2009. Matricellular protein CCN3(NOV) regulates actin cytoskeletal reorganization. J Biol Chem 284:29935–29944.

Singh SK, Clarke ID, Terasaki M, Bonn VE, Hawkins C, Squire J, Dirks PB. 2003. Identification of a cancer stem cell in human brain tumors. Cancer Res 63:5821–5828.

Soroceanu L, Manning TJ, Jr., Sontheimer H. 2001. Reduced expression of connexin-43 and functional gap junction coupling in human gliomas. Glia 33:107–117.

Squecco R, Sassoli C, Nuti F, Martinesi M, Chellini F, Nosi D, Zecchi-Orlandini S, Francini F, Formigli L, Meacci E. 2006. Sphingosine 1-phosphate induces myoblast differentiation through Cx43 protein expression: A role for a gap junction-dependent and -independent function. Mol Biol Cell 17:4896–4910.

Sutor B, Hagerty T. 2005. Involvement of gap junctions in the development of the neocortex. Biochim Biophys Acta 1719:59–68.

Van Aelst L, D'Souza-Schorey C. 1997. Rho GTPases and signaling networks. Genes Dev 11:2295–2322.

Vignjevic D, Montagnac G. 2008. Reorganisation of the dendritic actin network during cancer cell migration and invasion. Semin Cancer Biol 18:12–22.

Xu X, Li WE, Huang GY, Meyer R, Chen T, Luo Y, Thomas MP, Radice GL, Lo CW. 2001. Modulation of mouse neural crest cell motility by N-cadherin and connexin 43 gap junctions. J Cell Biol 154:217–230.

Xu X, Francis R, Wei CJ, Linask KL, Lo CW. 2006. Connexin 43-mediated modulation of polarized cell movement and the directional migration of cardiac neural crest cells. Development 133:3629–3639.

Yamazaki D, Kurisu S, Takenawa T. 2009. Involvement of Rac and Rho signaling in cancer cell motility in 3D substrates. Oncogene 28:1570–1583.

Zhang W, Nwagwu C, Le DM, YongVW, Song H, Couldwell WT. 2003a. Increased invasive capacity of connexin43-overexpressing malignant glioma cells. J Neurosurg 99:1039–1046.

Zhang YW, Kaneda M, Morita I. 2003b. The gap junction-independent tumor-suppressing effect of connexin 43. J Biol Chem 278:44852–44856.

Zhu D, Caveney S, Kidder GM, Naus CC. 1991. Transfection of C6 glioma cells with connexin 43 cDNA: Analysis of expression, intercellular coupling, and cell proliferation. Proc Natl Acad Sci USA 88:1883–1887.

Zhu D, Kidder GM, Caveney S, Naus CC. 1992. Growth retardation in glioma cells cocultured with cells overexpressing a gap junction protein. Proc Natl Acad Sci USA 89:10218–10221.