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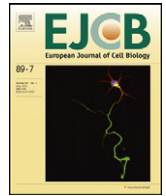
The carboxyl tail of Cx43 augments p38 mediated cell migration in a gap junction-independent manner

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ABSTRACT

The expression of connexin 43 (Cx43) has been shown to correlate with an enhanced migration of several cell types such as glioma or neural crest cells, but the mechanism remains unclear. We studied whether Cx43 also affects migration in non-neural cells and whether or not this is related to gap junction formation. Therefore, we analysed the migratory activity of HeLa cells under conditions of controlled connexin (Cx) expression. The expression of Cx43 enhanced their migration significantly as compared to Cx deficient wild-type cells. Expression of only the carboxyl tail of Cx43 (Cx43CT, AA 257-382) without channel forming capacity enhanced migration similarly as the full length protein. In contrast, the expression of the N-terminal part of Cx43 (Cx43NT, AA 1-257), which partially retained the gap junction channel function of Cx43, did not increase migration. The enhanced cell migration of HeLa cells expressing either full length Cx43 or the Cx43CT was associated with an increased activation of the p38 MAP kinase. The additional incubation with a specific inhibitor of p38 activation diminished the migration of HeLa-Cx43 cells to levels of control transfected cells.

As a proof of concept, we studied whether Cx43 also modulates the migration of endothelial progenitor cells (EPC) which play an important role in angiogenesis. In these cells, which expressed Cx43 as the only connexin, the downregulation of Cx43 by siRNA resulted in a significantly decreased migration.

These results demonstrate that expression of Cx43 augments migration via modulation of p38 MAP kinase activity. The carboxyl tail of Cx43 plays an essential role in this signalling pathway which is independent of gap junction function.

Introduction

Connexins constitute a large family of proteins known to be primarily involved in the formation of gap junctions. One of the most ubiquitously found connexins is Cx43 being amply expressed e.g. in certain structures of the gastrointestinal tract (Wang and Daniel, 2001) and the heart and blood vessels (Laird, 2006; Rodriguez-Sinovas et al., 2007), where a functional coupling of cells by electrical or small molecule signal conduction results in the formation of functional syncytia, which is important for coordination of cell behaviour. However, functions of Cx43 are not restricted to the formation of gap junctions. Several studies using deletion mutants or connexins incompetent for channel forming exert biological effects. For example, the growth-inhibiting effects of Cx43 on C6 glioma, Neuro2a, or HeLa cells (Dang et al., 2003; Moorby and Patel, 2001; Olbina and Eckhart, 2003), as well as tumors

(Huang et al., 1998b; McLachlan et al., 2006; Qin et al., 2002), the modulation of skeletal muscle differentiation (Squecco et al., 2006), or the modification of transcription and of cell cycle control (Giepmans, 2004; Iacobas et al., 2003) do not seem to require intact cell to cell communication. Such effects should particularly play an important role when connexin-expressing cells have to operate predominantly in an isolated state, as it can be the case during cell migration. Cell migration is an important process not only during development but also in wound healing, in tumor metastasis, and in vascular growth and angiogenesis (Risau, 1990). Indeed, Cx43 is highly expressed in migrating neural crest cells during embryogenesis (Huang et al., 1998a; Lo et al., 1999). Likewise, the expression of Cx43 has modulating effects on the migration of cardiac neural crest cells during development. These cells show in vitro poor motile function with low directionality when Cx43 is knocked out (Huang et al., 1998a; Lo et al., 1999). Conversely, overexpression of Cx43 in these cells enhanced cell motility with high directionality and increased velocity (Xu et al., 2006). Reduced motility associated with downregulation or knock out of Cx43 has also been described in other cell types, e.g. in NIH3T3 cells (Wei et al., 2005). In glioma cells, the expression of Cx43 also corre-

lated with cell migration, and accordingly, downregulation of Cx43 decreased cell motility and invasion (Bates et al., 2007). The mechanisms by which Cx43 can modulate cell migration are still unclear. However, in C6-glioma cells, increased cell motility was only seen when a full length Cx43 was expressed while cells expressing a C-terminal truncated Cx43 did not show it, implying an important role for the carboxyl tail of Cx43 in the regulation of glia cell migration (Bates et al., 2007; Moorby, 2000). Similar results were obtained in migrating neurons during brain development (Cina et al., 2009). However, Elias et al. reported that the carboxyl tail of Cx43 was not essential for neuronal migration in developing rat neocortex, since a C-terminal truncated Cx43 was able to rescue the neuronal migration defect after siRNA knockdown of endogenous Cx43 (Elias et al., 2007). Therefore, the potential regulatory role of the carboxyl tail of Cx43 for the migration process remains to be elucidated. It also remains unclear whether the potential influence of the carboxyl tail is a specific feature of neuronal and glia cells, respectively, or whether this is a general mechanism that can be found in non-tumor and non-neural cells as well. Therefore, we tested whether Cx43 dependent migration requires gap junctional communication or reflects a carboxyl tail related, gap junction-independent process in HeLa cells and endothelial progenitor cells. We also hypothesized that an augmenting effect of the carboxyl tail of Cx43 could affect activation of p38 MAP kinase, which has been shown to be involved in the control of cell migration (Huang et al., 2004).

To clarify the role of the carboxyl tail on migration and p38 activation, we studied the effects of an isolated expression of the carboxyl tail of Cx43 in HeLa cells, which allows for controlled expression of connexins. Wild-type HeLa cells do not express any connexin, but can be stably transfected with Cx43 or Cx43-mutants (Elfgang et al., 1995). This is a clear prerequisite for this kind of study since in cells expressing several connexins the knockdown of one connexin has been reported to affect the expression of other connexins in the same cell (Kruger et al., 2002). Moreover, as a proof of concept we also aimed to study the effects of a knockdown of Cx43 in a vascular cell type. Of particular interest is the understanding of effects of Cx43 on migration of endothelial progenitor cells which need to migrate under conditions of vasculogenesis, angiogenesis and vascular repair.

Materials and methods

Cells and culture conditions

HeLa wild-type cells (HeLa-wt) and HeLa cells expressing rat Cx43 (HeLa-Cx43) were a kind gift from Dr. Klaus Willecke (University of Bonn, Germany). HeLa-wt cells transfected with the empty vector pBEHpac18 (CTL; kindly provided by Dr. Willecke, Bonn) using SuperFect (Qiagen) served as controls. HeLa-wt cells were cultivated in Dulbecco's modified Eagle medium (DMEM, Invitrogen), supplemented with 10% new born calf serum (NBCS, Biochrom), penicillin (100 U/ml, Sigma) and streptomycin (100 μg/ml, Sigma). HeLa-Cx43 and empty vector-transfected cells (HeLa-CTL) were grown in the same medium but additionally supplemented with 1 μg/ml puromycin (Sigma, Germany). HeLa cells transfected with the empty vector pcDNA4/myc-His B (HeLa-CL), pcDNA4-GFP, pcDNA4-Cx43NT-GFP and pcDNA4-Cx43CT-GFP were maintained in culture medium for HeLa-wt supplemented with 200 μg/ml Zeocin, due to a Zeocin resistance encoded on the vector pcDNA4/myc-His B (Invitrogen). Mouse embryonic EPC were a kind gift from Dr. Antonis Hatzopoulos (Division of Cardiovascular Medicine, Vanderbilt University Medical Center, USA). EPC were cultivated in DMEM supplemented with 20% fetal calf serum (FCS, Biochrom), 1 mM MEM non essential amino acids (Invitrogen), penicillin (100 U/ml, Sigma) and streptomycin

(100 μg/ml, Sigma), 2 mM L-glutamine (Invitrogen), 0.1 mM β-mercaptoethanol (Applichem) and 2 mM Hepes pH 7.5. All cells were maintained at 37 °C and 5% CO_2.

Plasmid constructs

The GFP plasmid pcDNA4-GFP was generated by amplification and subcloning of the open reading frame of the green fluorescent protein (GFP; variant sg143GFP) by PCR from the plasmid pFred143 (Ludwig et al., 1999; Stauber et al., 1998) in pcDNA4/myc-His B (Invitrogen). The PCR primers encoded a unique restriction site (underlined) in front of the ATG start codon in the 5′primer and after the stop codon in the 3′primer for cloning. Sequence of 5′primer: 5′-GACGATCTAGAGATGGCTAGTTCCGGA-3′ and 3′primer sequence: 5′-CGAGGTTTAAACTCAACCGTACAGCTCGTC-3′. The cDNA fragment encoding the N-terminal part of Cx43 (Cx43NT; AA 1-257) was synthesized by PCR using ratCx43 cDNA as a template and cloned into the pcDNA4-GFP plasmid. Sequence of 5′primer Cx43NT: 5′-CTCGGATCCATGGGTGACTGGAGT-3′; sequence of 3′primer: 5′-GCTCTCGAGCGTGATGGGCTCAGTGG-3′. The cDNA fragment encoding the carboxyl tail of Cx43 (Cx43CT; AA 257-382) was cloned into the pcDNA3.1-TOPO (Invitrogen) and subcloned in the pcDNA4-GFP. Sequence of 5′primer: 5′-GCCATGTCAA-AAGACTGCGGA-3′, and 3′primer: 5′-AATCTCCAGGTCATCAGGCCG-3′. All constructs were verified by sequencing.

Immunofluorescence

HeLa cells or EPC grown on glass coverslips were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) pH 7.4, for 30 min at room temperature. After rinsing with PBS, cells were permeabilised with 0.1% Triton-X100 in PBS for 4 min. Non-specific binding sites were blocked with 0.5% bovine serum albumin (BSA) in PBS for 1 h. Cells were incubated with rabbit polyclonal anti-Cx43 (Sigma, 1:100) overnight at 4 °C, washed and incubated with a secondary antibody (1:200) coupled to AlexaFluor 546 (Invitrogen) for 1 h at room temperature. Coverslips were mounted and expression and localisation of connexins were analysed using confocal microscopy (Leica).

Western blot analysis

Protein concentrations in cell extracts were determined using the BCA protein assay (Pierce). Samples were boiled in Laemmli buffer (Laemmli, 1970) for 5 min, size-separated by 10% SDS-PAGE, and electrophoretically transferred to a Hybond-P membrane (Amersham) at 0.8 mA/cm² for 1 h. After blocking in 1× Western blocking reagent solution (Roche, Germany) and 0.1% Tween 20 (Sigma) in PBS for 1 h at room temperature, the blots were incubated with rabbit anti-GFP (Abcam, 1:1000) or anti-Cx43 (Sigma, 1:1000) overnight at 4 °C. After incubation, the blots were washed and incubated for 1 h with the according secondary antibodies coupled to horseradish peroxidase (Calbiochem, 1:5000). All antibodies were diluted in 0.5× Western blocking reagent solution (Roche) containing 0.1% Tween 20 (Sigma). Membranes were then washed three times, for 10 min each. Bound antibody was detected by enhanced chemiluminescence (ECL, Applichem). Equal loading was demonstrated by GAPDH detection (1:10,000, Chemicon).

For analysis of p38 activation the cells were seeded in 6-well-plates and synchronized over night in DMEM-0.5% NBCS. After stimulation with DMEM-10% NBCS for 15 and 30 min, cells were lysed in Laemmli buffer and analysed for p38 activation with a polyclonal antibody (rabbit anti-phospho p38 1:1000; Cell Signaling) which detects only phosphorylated (activated) levels of p38 MAP kinase. The secondary antibody (anti-rabbit, Calbiochem) was used at a dilution of 1:1000.

siRNA-mediated knockdown

For siRNA knockdown of Cx43 in EPC, the cells were transfected with synthetic siRNA (Qiagen) at a final concentration of 5 nM using Hiperfect transfection reagent (Qiagen) according to the manufacturer's protocols. The siRNA siCx43-1 was synthesized (Qiagen) containing nucleotides 143-163 (AACAGTCTGCCTTTCGCTGTA) of the mouse Cx43α1 encoding sequence (Wei et al., 2005). As a control (Ctr-si) a non-silencing sequence (AATTCTCCGAACGTGT-CACGT) was used (Qiagen). 24 h after siRNA transfection, migration studies were performed and the expression of Cx43 was analysed by Western blot.

Analysis of cell coupling using fluorescence activated cell sorting (FACS)

To determine cell coupling we used a method based on fluorescence-activated cell sorting. Populations of stably transfected HeLa cells expressing either Cx43, Cx43NT-GFP, Cx43CT-GFP, or empty vector-transfected cells (HeLa-CTL) were seeded on 6-well plates and stained with calcein AM (0.2 μM; Invitrogen) for 30 min at 37 °C and 5% CO_2. Calcein AM is a membrane permeant nonfluorescent molecule. After diffusion into the cells, it is hydrolyzed by esterases and cannot further permeate the cell membrane except when it is transferred by gap junctions. A second cell population (dye acceptor cells), HeLa-Cx43, was stained with PKH26 Red Fluorescent Cell Linker Kit (Sigma) according to the manufacturer's instructions. A cell suspension of 140,000 of these cells/ml was added to the calcein-labelled donor cells. Both cell populations (calcein-labelled donors and PKH 26-labelled acceptors) were co-cultivated for 3 h. Subsequently, loosely attached acceptor cells were removed from each well to tubes and the remaining cells were washed and detached by accutase (PAA Laboratories). After centrifugation at 1200 rpm for 5 min, cells were resuspended and analysed by flow cytometry (10,000 acceptor cells) for double staining, indicating transfer of the calcein dye into the PKH 26-labelled cells. Fluorescence was measured (FAC-Scan, BD) at an excitation wavelength of 488 nm. Emission light of calcein and PKH26 was detected at 530 (FL-1) and 575 nm (FL-2), respectively. Cell coupling was determined as percentage of double stained (red and green) cells.

Analysis of cell coupling by transfer of calcium waves between single cells

HeLa-Cx43CT-GFP and HeLa-Cx43NT-GFP cells were seeded together with PKH26-labelled HeLa-Cx43 at a ratio of 1:1 on coverslips. 12–14 h later, the cells were loaded with 4 μM Fura2 (Molecular Probes, USA) for 1 h. Experiments were performed in a heated (37 °C) microscopic chamber mounted on a stage of an inverted microscope (Axiovert, Zeiss, Germany). The Ca^{2+} signal was detected at 505 nm (alternating excitation wavelengths 340 nm and 380 nm) with a camera and using a computerized system (Till Photonics, Gräfelfing, Germany) to control the excitation wavelength and camera settings. Frames at 340 and 380 nm of the analysed areas (300 nm × 300 nm) were stored every 125–250 ms for 75–150 s. The ratio 340/380 (representing intracellular free calcium concentration) was calculated for each frame pair after correction for background signals. A Ca^{2+}_i rise in a single cell was induced by mechanical stimulation using a patch pipette (tip diameter 1 μm) controlled by a step motor driven system (FemtoJet, Eppendorf). Gap junctional calcium transfer between Cx43-expressing HeLa (identified by the PKH26 label) and HeLa-Cx43NT-GFP or HeLa-Cx43CT-GFP was evaluated by studying potential calcium increases in adjacent cells and normalised to the total number of immediately adjacent HeLa-Cx43NT-GFP or HeLa-

Cx43CT-GFP cells. Since mechanical stimulation can also cause ATP release, which may induce calcium increases in adjacent cells in a paracrine, gap junction-independent manner, all experiments were performed in the presence of Apyrase (50 U/ml). At this concentration Apyrase blocked calcium increases to 10 μM exogenous ATP completely.

Migration assay using fluoroblok transwell chambers

For migration assays, transwell chambers (Falcon HTS FluoroBlok Inserts; 8 μm pore size) combined with 24-well cell culture companion plates (Becton Dickinson) were used. As migration stimulus we used serum, in the case of HeLa new born calf serum (NBCS), and for EPC fetal calf serum (FCS), both containing several growth factors of unknown concentrations. Before migration, the cells were serum-starved over night in DMEM containing 0.5% NBCS. The cells were trypsinized, resuspended in DMEM-0.5% NBCS and seeded onto the filter membrane of the insert (HeLa: 5×10^4 cells in 400 μl; EPC: 1×10^5 cells in 400 μl). The bottom compartment of the chambers was filled either with 0.8 ml DMEM-0.5%NBCS only (unstimulated controls) or with 0.8 ml DMEM with serum added as stimulus (migration stimulus: HeLa: DMEM-10%NBCS; EPC: DMEM-20%FCS). Cells were allowed to migrate through the filter membrane as indicated for 8 or 16 h (HeLa) or 22 h (EPC) at 37 °C in a CO_2 incubator. All treatments were performed in triplicate. Following incubation, media were aspirated and the migrated cells, now at the bottom surface of the light-impermeable FluoroBlok filter membranes, were stained with calcein AM (4 μg/ml; Invitrogen) for 90 min at 37 °C in a CO_2 incubator. Migration was then quantified as fluorescence units using a fluorescence reader with bottom reading capability at 485/535 nm (TECAN). Stimulated cell migration was expressed as fold migration (increase) over the control values obtained in unstimulated cells (migrated cells through membrane towards stimulus/migrated cells through membrane without stimulus). Results are represented as mean ± s.e.m. of fold migration as normalised to unstimulated controls.

Migration assay using wound assay chambers

Wound assays were performed using specific wound assay chambers (Ibidi). HeLa cells were trypsinised and resuspended in DMEM-0.5%NBCS. 70 μl of cell suspension (5×10^5 cells/ml) was seeded into each well of the insert. After appropriate cell attachment for 24 h the culture inserts were removed and the cells were incubated with fresh culture medium (DMEM-10% NBCS). The cell migration into the defined cell free gap (500 μm) was observed for 20 or 24 h as indicated under an inverted microscope (Zeiss) with a live cell imaging system (Ibidi). Images were captured with an AxioCam camera (Zeiss). For assay analysis, cells were tracked using the manual tracking software component of the ImageJ programme. After tracking, the cell paths were analysed using the 'Chemotaxis and Migration Tool', a free ImageJ plugin provided by Ibidi to compute center of mass (center of mass of all endpoints) and accumulated distance (mean distance of all cell paths). To study the role of p38 MAP kinase, cells were preincubated 1 h prior to the removal of the insert with 25 μM SB203580 (dissolved in DMSO), with the equal amount of DMSO alone, or left without addition of DMSO or SB203580. Cell migration was studied for 24 h in the continuous presence of SB203580 or sham solution (DMSO) by video imaging as described above.

Data analysis

Data were analysed for statistical differences by the Student's *t*-test for paired and unpaired data after testing for

normal distribution of the data. Because of their non-Gaussian distribution, the data of the calcium transfer were statistically analysed using the Mann–Whitney Rank Sum test. Differences were considered significant at an error probability of $p < 0.05$. Results are expressed as means ± s.e.m. as indicated in the figure legends.

Results

Migration of HeLa cells is significantly increased after expression of Cx43

To study the effect of Cx43 on cell migration, HeLa cells stably transfected with a cDNA encoding rat Cx43 were used. HeLa cells transfected with the empty vector (CTL) or untransfected HeLa cells (HeLa wild-type cells, wt) were used as controls.

Migration of HeLa cells expressing Cx43 as measured by their appearance at the bottom surface of the filter inserted in a modified Boyden chamber (fluoroblok transwell chambers) was significantly higher than migration of Cx43-deficient controls (fold migration: Cx43: 1.88 ± 0.11; wt: 1.37 ± 0.10; CTL: 1.35 ± 0.09; each $n = 4$ independent cell cultures, Fig. 1A). Representative images of unstimulated and stimulated migrated cells (HeLa-Cx43 and HeLa-

wt) from the bottom surface of the membrane are displayed in Fig. 1B.

The presence of Cx43-expressing cells seeded on the bottom of the lower compartment of the Boyden chamber had no enhancing effect on the migration of HeLa-wt cells across the filter membrane (Fig. 1C). Rather, the presence of both, HeLa-wt, or HeLa-Cx43 cells had similar migration-reducing effects on HeLa-wt cells which might be explained by the consumption of growth factors from the stimulating culture medium (DMEM-10%NBCS) by these cells (Fig. 1C).

Likewise, in the wound assay HeLa cells expressing Cx43 migrated over approximately 40% longer distances during a 24 h observation period than HeLa-wt cells and about 66% longer distances than cells transfected with an empty vector (CTL): the accumulated distance of HeLa cells expressing Cx43 was 351 ± 34 μm, that of HeLa-wt cells 251 ± 16 μm and that of HeLa-CTL cells amounted to 211 ± 9 μm ($n = 3$; Fig. 1D, S1). In accordance with a longer migration distance, the center of mass of all endpoints of tracked cells was also shifted significantly further away from the starting point in cells expressing Cx43 (Fig. 1D, S1). The shift of center of mass was 198 ± 24 μm in HeLa-Cx43 and thus 57% higher than observed in HeLa-wt: 126 ± 13 μm and about 80% higher than observed in HeLa-CTL: 108 ± 12 μm (Fig. 1D).
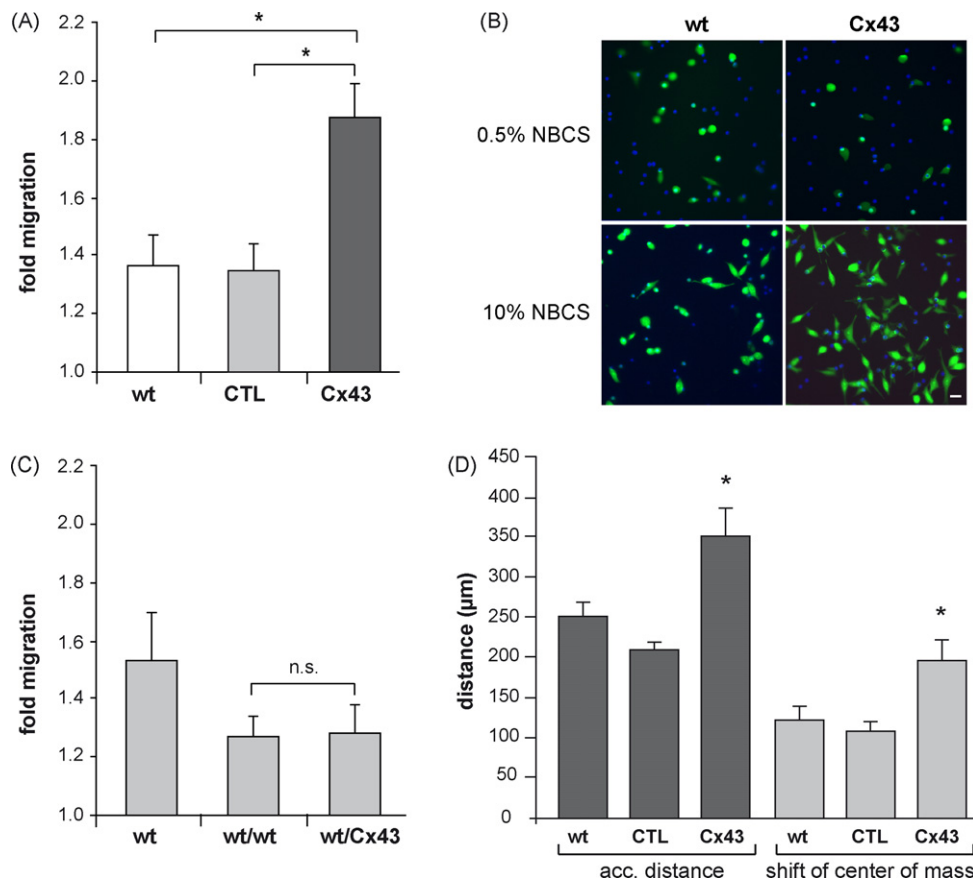

Fig. 1. Enhanced migration of HeLa cells expressing Cx43. (A) Migration analysis using high throughput screening (HTS) fluoroblok transwell chambers. HeLa cells expressing Cx43, empty vector-transfected HeLa (CTL) or wild-type HeLa (wt) were analysed for cell migration in response to 10% NBCS for 8 h. Data are represented as fold migration as normalised to unstimulated controls ($n = 4$ independent cell cultures). Migration of Cx43-expressing HeLa cells is significantly increased as compared to HeLa-CTL or HeLa-wt cells, (*) $p < 0.05$. (B) Migrated cells on the bottom surface of the filter were stained with calcein AM and fixed with methanol. Representative pictures of migrated cells (wt, Cx43) in response to 0.5% NBCS (unstimulated) or 10% NBCS are shown. Scale bar: 20 μm. (C) Migration of HeLa-wt cells is not affected by the presence of Cx43-expressing HeLa seeded on the bottom of the well. Migration was measured using fluoroblok transwell chambers. Cell migration of HeLa-wt in response to 10% NBCS alone (wt) or in response to cultured HeLa-wt (wt/wt) or HeLa-Cx43 (wt/Cx43) was measured after 16 h. The distance from the membrane to the bottom of well is 800 μm. Data are represented as fold migration as normalised to unstimulated controls ($n = 4$ independent cell cultures). Migration of HeLa-wt cells is not affected by HeLa cells expressing Cx43, (n.s.) $p > 0.05$. (D) Migration analysis using wound assay chambers. HeLa cells were seeded into wound assay culture inserts placed in 35 mm Ibidi μ-dishes and observed in a life cell imaging system (Ibidi) for 24 h. The accumulated distance and the shift of center of mass of all tracked cells were significantly higher in HeLa-Cx43 cells compared to HeLa-wt or HeLa-CTL, accumulated distance (*) $p < 0.05$ versus CTL and versus wt and shift of center of mass (*) $p < 0.05$ versus CTL and versus wt. Data are represented as distances ($n = 3$ independent cell cultures).
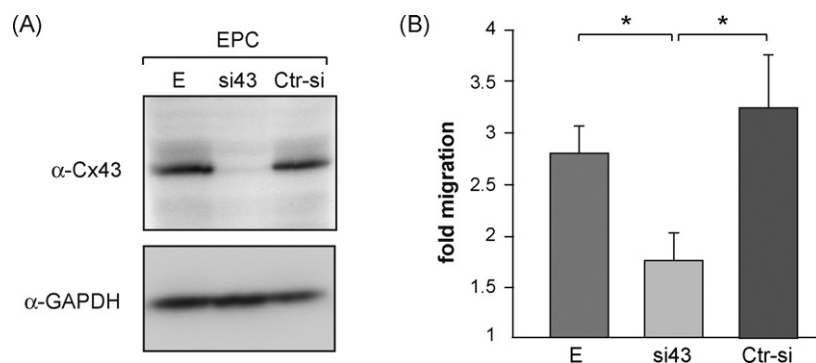
Fig. 2. Silencing of Cx43 by siRNA decreased migration of EPC. (A) Western blot analysis of Cx43 expression in EPC transfected with 5 nM siRNA against Cx43 (si43) or non-silencing control-siRNA (Ctr-si) and untransfected EPC (E), respectively. 24 h after transfection the cells were analysed for Cx43 expression by Western blotting using a monoclonal Cx43-antibody (α-Cx43). Immunostaining for GAPDH was used as loading control. (B) Cell migration of untransfected EPC (E) and EPC transfected with Cx43-siRNA (si43) or control-siRNA (Ctr-si) in response to 20% FCS using HTS-fluoroblok chambers. Data are represented as fold migration (stimulated migration normalised to unstimulated controls; $n = 4$ independent cell cultures). Migration of siCx43-transfected EPC was significantly reduced as compared to untransfected (E) or Ctr-si transfected EPC, (*) $p < 0.05$ versus E and versus Ctr-si.

Downregulation of Cx43 by siRNA resulted in reduced migration of EPC

To study whether the migration-enhancing effects of Cx43 expression could also be observed in a different type of cells we employed mouse embryonic endothelial progenitor cells. Transient transfection of EPC with siRNA specifically targeting mouse Cx43 (si43) markedly reduced Cx43 expression (>80%), whereas transfection with a non-silencing control-siRNA (Ctr-si) for 24 h had no such effect (Fig. 2A). EPC transfected with si43 showed a significantly lower serum-stimulated migration through the membrane of a Boyden chamber as compared to untransfected EPC or EPC transfected with non-silencing siRNA (Ctr-si) which express normal levels of Cx43 (fold migration of EPC: 2.81 ± 0.26; EPC-si43: 1.76 ± 0.27; EPC-Ctr-si: 3.24 ± 0.51; each $n = 4$ independent cell cultures (Fig. 2B)).

Expression of the carboxyl tail of Cx43 is required for enhanced migration

HeLa cells transfected with Cx43NT-GFP or Cx43CT-GFP expressed chimeric proteins of the predicted molecular mass (Cx43NT-GFP ∼55 kDa; Cx43CT-GFP ∼41 kDa) when probed in a Western blot with an antibody to GFP. Expression of full length Cx43 and Cx43CT-GFP was additionally detected with an antibody recognizing an epitope within the carboxyl tail of Cx43 (Fig. 3B). Confocal microscopy showed that Cx43NT-GFP localised to the plasma membrane and mainly in the area of cell–cell contacts whereas Cx43CT-GFP largely localised in the cytoplasmic compartment (Fig. 3C).

FACS analysis revealed that after 3 h of co-culture approximately 60% of the population of full length Cx43-expressing HeLa and 40% of Cx43NT-GFP-expressing HeLa cells had taken up the gap junction permeant dye calcein from coupled donor cells (Fig. 4). In contrast, the amount of HeLa-Cx43 cells displaying green fluorescence after

co-culture with HeLa-Cx43CT-GFP was as low as after co-culture with HeLa-CL which do not express connexins (Fig. 4). The gap junctional communication was further tested by the intercellular propagation of a calcium wave following mechanical stimulation of a single cell. Calcium spreading into neighbouring cells was 84% in Cx43-expressing control cells. In HeLa cells expressing the N-terminal part the amount of coupled cells was approximately 58%. 13% of cells expressing the carboxyl tail only and 12% of HeLa-wt cells also showed calcium increases (Table 1), albeit these occurred slower which is typical for mechanical co-stimulation (Follonier et al., 2008). With regard to their migratory behaviour, both HeLa cells expressing full length Cx43 or Cx43CT-GFP, showed significantly higher migration distances than HeLa-Cx43NT-GFP. Both distances, the accumulated distance and the shift of center of mass were enhanced in HeLa cells expressing Cx43 and Cx43CT-GFP and the accumulated distance was not significantly different between these cell populations. In contrast, the migration distances of Cx43NT-GFP were significantly shorter compared to Cx43 or Cx43CT-GFP and did not differ from the migration distances of empty vector-transfected cells (Fig. 5).

Migration of HeLa cells is reduced by inhibition of p38 MAP kinase

Treatment of cells with a specific inhibitor of p38 (SB203580) resulted in a decreased migration of these cells as assessed in a wound assay (Fig. 6A and B). The migration of Cx43-expressing cells under conditions of p38 inhibition was not different to that observed in untreated or vehicle (DMSO)-treated CTL-transfected cells (Fig. 6A and B).

Serum-induced activation of p38 MAPK is enhanced in HeLa cells expressing the carboxyl tail or full length Cx43

Since migration of HeLa cells was reduced by inhibition of p38 MAP kinase we analysed the status of p38 activation (phosphoryla-

Table 1

Spreading of a calcium signal from a single, mechanically stimulated cell to adjacent cells as a measure of gap junctional coupling.

Co-culture	Cx43/Cx43	Cx43/Cx43NT-GFP	Cx43/Cx43CT-GFP	wt/wt
% coupling				
mean ± SEM	84 ± 9.1	58 ± 7.7[*]	13 ± 6.9	12 ± 4.0
median	100	Median = mean	0[*]	0

Single cells in HeLa co-cultures (Cx43/Cx43NT-GFP or Cx43/Cx43CT-GFP) were stimulated mechanically and the gap junctional calcium transfer to adjacent HeLa-Cx43NT-GFP or HeLa-Cx43CT-GFP was determined. HeLa co-culture of Cx43/Cx43 and wt/wt were used as controls. The number of adjacent cells with a calcium increase was normalised to the total number of adjacent cells (% coupling). Measurements: $n = 15$ (Cx43/Cx43); $n = 14$ (Cx43/Cx43NT-GFP); $n = 10$ (Cx43/Cx43CT-GFP) and $n = 15$ (wt/wt) in 3 different cell cultures.

[*] $p < 0.05$ versus Cx43/Cx43CT-GFP and versus wt/wt.
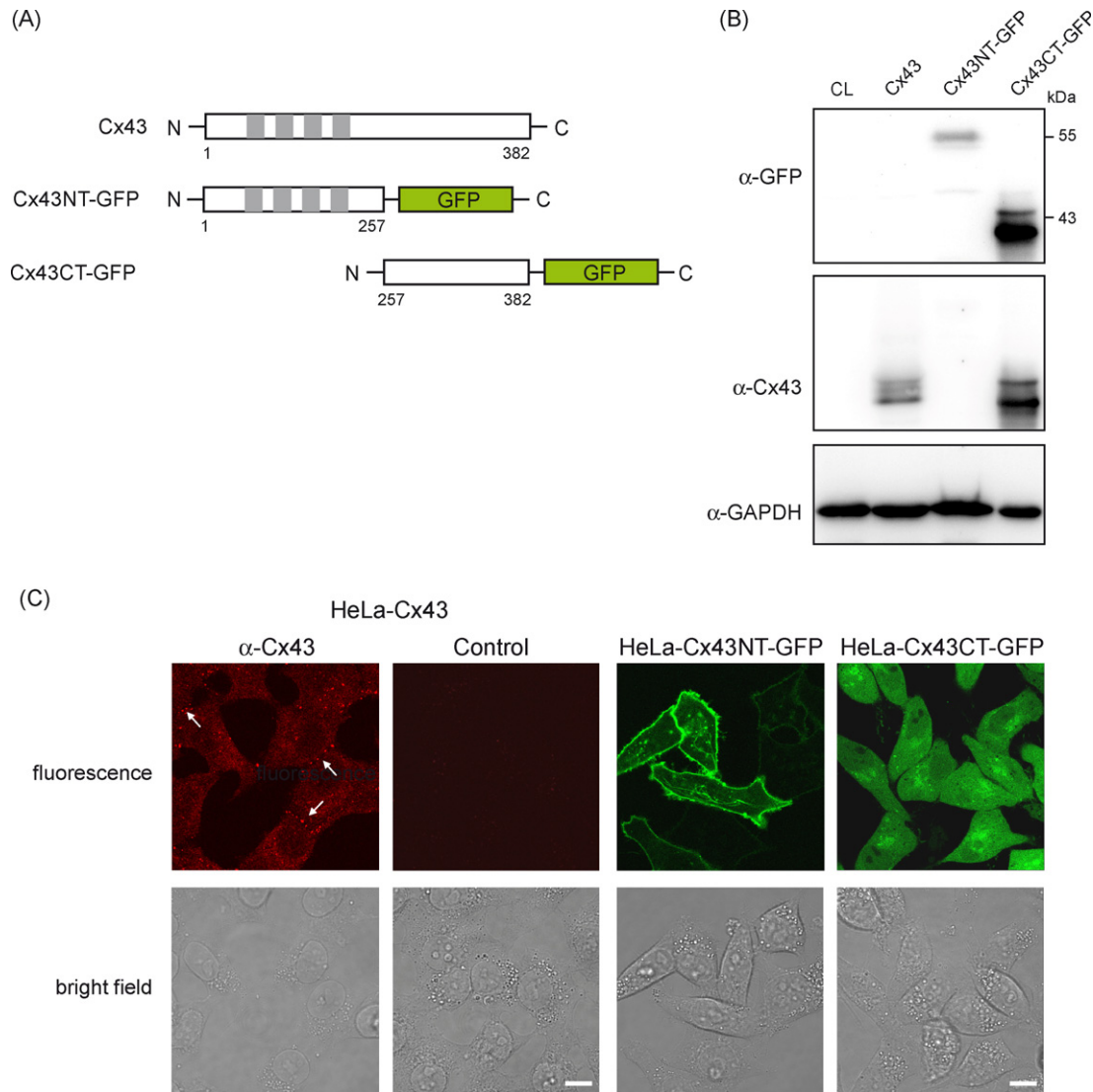
(A)

(B)

(C)

Fig. 3. Expression of truncated Cx43 proteins in HeLa cells. (A) Schematic representation of full length Cx43, the N-terminal part of Cx43 (Cx43NT-GFP; amino acids 1-257) and the carboxyl tail of Cx43 encoding the amino acids 257-382 (Cx43CT-GFP). The truncated Cx43-mutants are covalently coupled to GFP at their C-terminus. The N-terminal part of Cx43 (Cx43NT-GFP) comprises the four transmembranous regions of Cx43 (grey boxes) necessary for its membrane localisation and channel function. (B) Immunoblot detection of full length and truncated proteins of Cx43 in stably transfected HeLa cells. To detect GFP labelled Cx43 truncated proteins, a polyclonal anti-GFP antibody (α-GFP) was used. Subsequently, the membrane was incubated with a polyclonal Cx43-antibody (α-Cx43) which recognises an epitope of the C-terminal region of Cx43 to detect full length Cx43 and Cx43CT-GFP. To confirm equal loading the membrane was reprobed with a monoclonal antibody against GAPDH (α-GAPDH). (C) Left: Immunofluorescence labelling of Cx43 (red) in HeLa-Cx43 cells showed characteristic Cx43 staining in regions of cell–cell contacts (arrows). As a control the permeabilised cells were exposed to secondary antibody alone which displayed background only. Right: GFP-fluorescence: HeLa cells stably transfected with Cx43NT-GFP showed a localisation mainly at the cell periphery with typical spots at cell–cell contacts, whereas the carboxyl tail of Cx43 (Cx43CT-GFP) was found to be localised in the cytoplasm only. Upper row: fluorescence, lower row: bright field. Magnification is identical in all panels. Scale bar: 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

tion) in HeLa cells. Stimulation with 10% NBCS caused a pronounced activation of p38 MAP kinase in all cells which was significantly elevated at all time points studied (Fig. 7A). Activation of p38 in Cx43-expressing HeLa cells was about 49% higher than in CTL-cells, and about 33% higher in HeLa-Cx43CT-GFP compared to CTL-cells, and 35% higher compared to HeLa-Cx43NT-GFP cells ($n = 4$; Fig. 7B).

Discussion

In the present study we have shown that Cx43 enhances serum-induced cell migration in two different cell types, mouse embryonic endothelial progenitor cells (EPC) and HeLa cells. In the latter, expression of truncated Cx43 proteins demonstrated that it is the carboxyl tail of Cx43 which mediates the enhanced cell migration. Moreover, we could show that the carboxyl tail of Cx43 mediates a

more pronounced activation of p38 MAPK. This suggests a specific role of the carboxyl tail of Cx43 in enhancing cell migration via p38 activation which is independent of gap junctional communication.

Cell migration was assessed by two different methods, which in spite of different experimental conditions yielded remarkably similar results. Migration of cells through a membrane towards a stimulus in a modified Boyden chamber was increased by 37% in the presence of Cx43. However, when using this assay it cannot be excluded that enhanced migration was primarily due to less firm cell–cell contacts in the presence of Cx expression. Loosening of cell–cell contacts and disaggregation is required for single cells to migrate through the small pores of the membrane. This possibility could be excluded using the second migration assay which allows direct observation of cell migration by time lapse video microscopy. As in the previous assay, HeLa cells expressing Cx43 migrated over
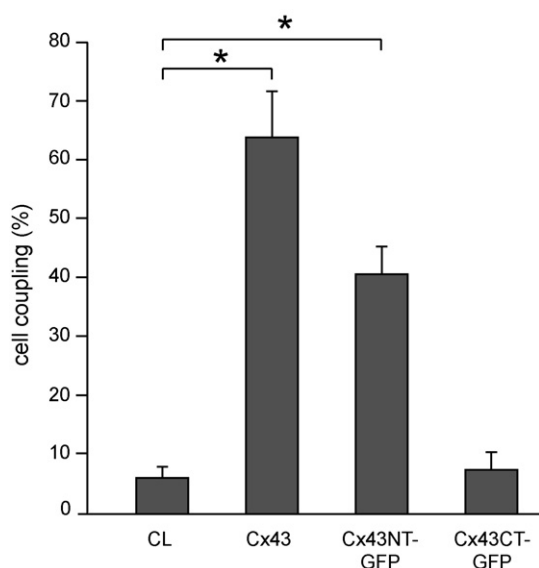
Fig. 4. HeLa cells form functional gap junctions by expression of Cx43 or the N-terminal part of Cx43. HeLa cells expressing Cx43, Cx43CT-GFP, Cx43NT-GFP or empty vector-transfected (CL) cells (donor cells) were stained with $0.2\,\mu M$ calcein (green) for 30 min. After extensive washing, the cells were co-cultivated with PKH-labelled (red) Cx43-expressing HeLa (acceptor cells) for 3 h. The cells were separated by FACS and the amount of green fluorescence was measured in red-labelled Cx43-expressing acceptor cells indicating transfer of dye. Data are represented as percentage of cell coupling, $n = 4$ independent cell cultures, (*) $p < 0.05$.

significantly longer distances than HeLa wild-type cells. Increases in cell proliferation could also be misinterpreted as increases in cell migration when the transmigration through the filter is used. However, in agreement with other groups (Gellhaus et al., 2004; Hirschi et al., 1996; Huang et al., 1998b; Li et al., 2008; Olbina and Eckhart, 2003) we found a reduced proliferation of Cx43-expressing HeLa cells compared to empty vector-transfected HeLa cells (by less than 10%; data not shown). Therefore, we can exclude this possibility in the present experiments.

A pivotal role of Cx43 on cell migration is further underpinned by the fact that downregulation of Cx43 expression in EPC with siRNA indeed decreased migration of these cells. Taken together, these results suggest that Cx43 plays an important modulatory role in control of cell migration. An involvement of Cx43 in cell migration/motility has also been reported in other cell types. The majority of studies analysing the effect of Cx43 expression in cell migration/motility found an increased migration by cells expressing Cx43. For example, in a rat heart cell line (H9c2) an increase in Cx43 expression was related to an augmentation of cell migration (Liu et al., 2007). Similarly, the migration rate of neural crest cells was increased in transgenic mouse embryos overexpressing Cx43 but was reduced in Cx43 knockout embryos (Huang et al., 1998a). Lin and colleagues demonstrated a role for Cx43 in increasing the invasiveness of glial tumor cells which can also probably be explained by enhanced cell migration (Lin et al., 2002). The fact that endothelial cells have been shown to express higher amounts of Cx43 in areas of wound repair (requiring migration of cells) (Kwak et al., 2001) yields additional, albeit circumstantial evidence for an augmenting role of Cx43 in the control of migration. It should be mentioned, however, that some studies found a migration-inhibitory effect of Cx43 (in breast carcinoma cells, in skin lesions), suggesting a cell-type specific role (Qiu et al., 2003; Shao et al., 2005; Simpson et al., 2008). At present, these contradictory results are difficult to explain. However, species or cell differences (inhibitory effects were mainly observed in epithelial cells), or specific differences regarding stimulatory pathways of migration (inhibitory effects were predominantly observed in cell cultures without specific stimulation of cell migration (Shao et al., 2005; Simpson et al., 2008)) could play a role and require further investigation.

Our study shows that Cx43 is not a prerequisite for the induction of migration, since cells lacking Cx43 by silencing the gene as it happens in HeLa-wt cells (epigenetic gene silencing via DNA methylation (King et al., 2000a,b)), or after treatment with siRNA (in EPC), still showed migration. Furthermore, no effect on directionality was observed. However, Cx43 substantially promoted cell migration. There are three possible explanations for this effect:
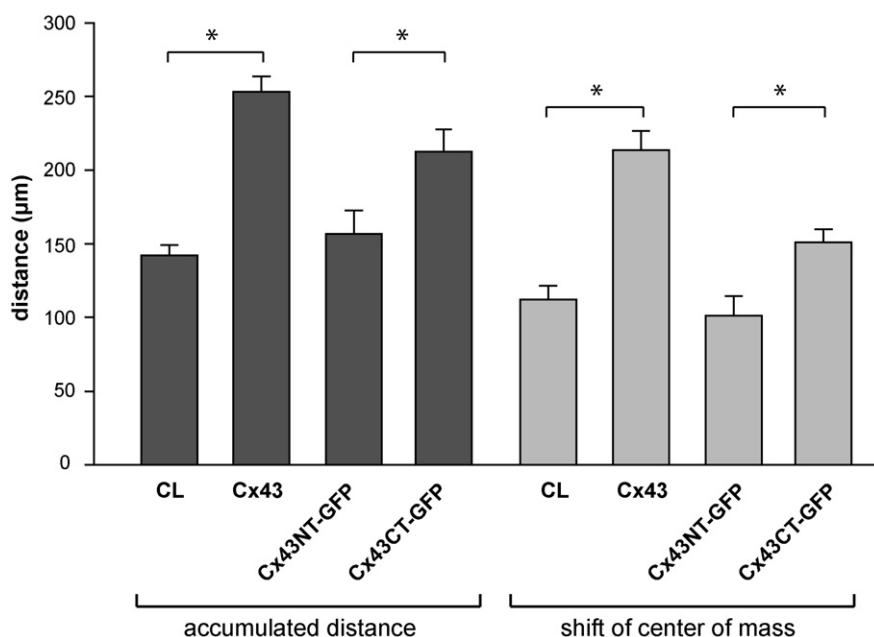

Fig. 5. The carboxyl tail of Cx43 mediates enhanced cell migration of Cx43-expressing cells. Migration analysis of HeLa cells expressing Cx43, Cx43NT-GFP, Cx43CT-GFP or empty vector-transfected HeLa (CL) using wound assay chambers. Cell migration in response to 10% NBCS was analysed for 20 h. HeLa cells expressing full length Cx43 showed increased migration distances compared to empty vector-transfected (HeLa-CL) and HeLa-Cx43CT-GFP showed increased migration distances compared to Cx43NT-GFP-expressing HeLa. Data are represented as distance ($n = 5$ independent cell cultures), (*) $p < 0.05$.
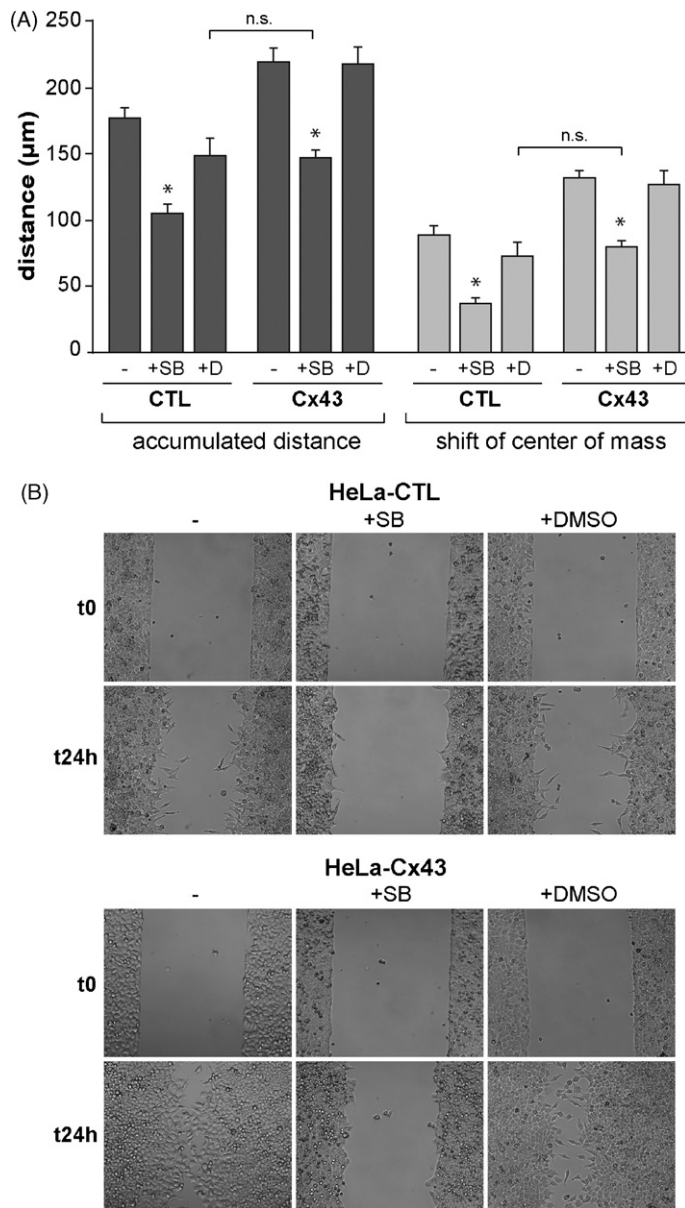
Fig. 6. SB 203580 inhibits migration of HeLa cells. (A) Migration of empty vector-transfected HeLa (CTL) and Cx43-expressing HeLa was analysed with wound assay culture inserts (Ibidi) in the presence of p38 inhibitor SB203580 for 24 h. The cells were pretreated with SB203580 (25 μM), or DMSO for 1 h, or remained untreated and their migration was observed in a live cell imaging system (Ibidi) for 24 h in the continuous presence of 25 μM SB203580 or the solvent DMSO. The accumulated distance and the shift of center of mass of SB203580-treated cells was significantly lower compared to the respective untreated controls (−); (*) $p < 0.05$. Further, the migration distances of SB203580-treated Cx43-expressing cells were comparable to DMSO-treated empty vector-transfected cells (CTL); (n.s.) $p > 0.05$. Data are represented as distances ($n = 5$ independent cell cultures). (B) Representative pictures of the beginning (t0) and the end (t24 h) of the experiments are shown.

(i) Cx43 might form hemichannels that allow the passage of migration-stimulating humoral factors from the cytosol across the cell membrane and/or (ii) expression of Cx43 may lead to synthesis and release of humoral factors that enhance migration or (iii) Cx43 may be part of a signalling pathway that does not include communication across the membrane and would therefore be independent of "channel functions" of Cx43. Hemichannels in the plasma membrane represent connexons (a connexin hexamer) that have not (yet) coupled with its counterpart of an adjacent cell to form a fully functional gap junction channel. Reportedly, they open only under

strictly defined conditions, such as extreme changes of extra- and intracellular Ca^{2+} levels, oxidative stress, or changes of membrane potential (Contreras et al., 2003; De Vuyst et al., 2006; Evans et al., 2006; Ramachandran et al., 2007; Verma et al., 2009). However, our results are not supportive of a role for either hemichannels or humoral factors. Firstly, Cx43-expressing cells, grown in the lower compartment of a Boyden chamber, had no influence on the migration of (non-Cx-expressing) HeLa-wt cells through the filter membrane which makes a role of humoral substances released by Cx43-expressing cells unlikely.

Secondly, and more important, a truncated form of Cx43 without channel forming capacity increased migration in a similar manner as full length Cx43, whereas a truncated form with preserved channel function did not. The channel forming function of the partially truncated but still membrane spanning form (Cx43NT-GFP) was shown by two different methods: (1) transfer of the membrane-impermeable but gap junction-permeable dye calcein measured by FACS analysis and (2) by transmission of Ca^{2+} signals from a single cell to its neighbouring cells. Due to different molecular weights of both gap junction permeable molecules (calcein: 622 g/mol; Ca^{2+}: 40 g/mol) not only different transmission times must be considered but also effects regarding transfer through existing gap junctions (calcium transmission) versus effects of de novo formation of gap junctions (FACS experiments with co-incubation). Newly formed gap junctions, as studied in the FACS assay, show a different regulation than already existing ones which are studied after dye injection. We have described such a behaviour previously (Kameritsch et al., 2003). The gap junctional transfer of Ca^{2+} into adjacent cells, caused by mechanical manipulation of a single cell, which leads to an increase of the free intracellular Ca^{2+} concentration, was measured within minutes whereas the transfer of a larger molecule (AlexaFluor 488) was not observable within 10 min (data not shown). Larger molecules were, however, still able to pass within 3 h as indicated by the FACS experiments though a limited, gap junction-independent transfer of calcein cannot be excluded, considering the relatively high basal value observed in gap junction deficient cells. Such basal levels have also been observed by other groups. For example, Jordan et al. (1999) showed a 15% coupling with Neurobiotin and a 23% coupling with Lucifer Yellow by microinjection in connexin deficient HeLa cells or HeLa cells expressing only GFP. The authors explained these findings by the potential existence of cytoplasmic bridges between incompletely divided daughter cells. In addition, a certain amount of calcein that has not immediately been de-esterified could have been exchanged via intact cell membranes. The capacity of the same or similar but shorter Cx43-mutants to form functional gap junction channels has also been demonstrated by other research groups (Fishman et al., 1991; Gellhaus et al., 2004; Maass et al., 2007; Omori and Yamasaki, 1999). However, we observed lower coupling levels of Cx43NT-GFP-expressing HeLa compared to HeLa-Cx43. Also, Bates et al. described that the expression of a C-terminal truncated mutant of Cx43 (Cx43Δ244-382-GFP) in glioma cells resulted in gap junctional coupling at lower levels than full length Cx43-GFP (Bates et al., 2007). Further, the additional GFP expressed by the truncated Cx43-mutants might also affect the coupling properties as well as the plaque size of gap junctions. The latter seems to be very important for channel opening, even with loss of coupling when plaques become very small (Bukauskas et al., 2000, 2001). It has been further described that the channel gating is changed by fusion of Cx43 with EGFP (Bukauskas et al., 2001). Though we cannot exclude the possibility that these channels have an altered selectivity and may therefore not be able to release a migration supporting factor anymore, this would also be the case during isolated expression of the carboxyl tail of Cx43 without channel function. However, this truncated form expressing the carboxyl tail increased migration considerably. We must therefore conclude that Cx43 has a direct
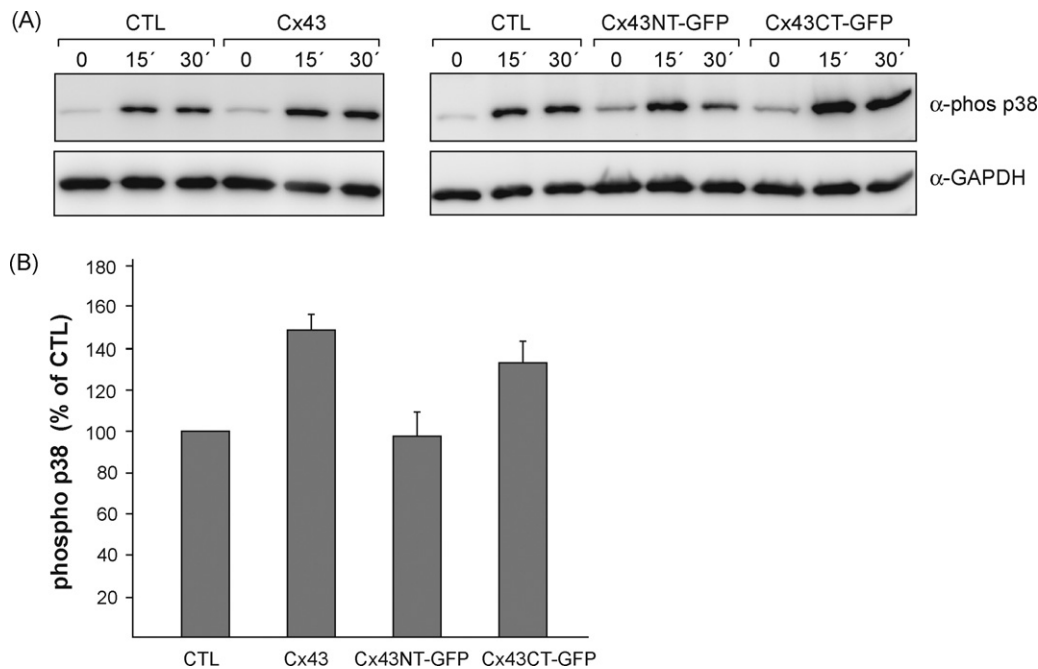
Fig. 7. Enhanced phosphorylation of p38 in HeLa cells expressing the carboxyl tail or full length Cx43. (A) Western blot analysis of phosphorylated (activated) p38 in stably transfected HeLa cells. Cells were serum-starved over night and treated with 10% NBCS for 15 and 30 min. Cell extracts were analysed for p38 phosphorylation with a polyclonal antibody against phospho-p38. Subsequently, the membrane was incubated with a monoclonal antibody against GAPDH (α-GAPDH) to confirm equal loading. (B) Values of phospho-p38 after 15 min of serum incubation were adjusted to total protein content and the results are expressed in terms of percent induction in comparison with the negative control (empty vector-transfected cells; CTL).

role in regulating cell migration and that the carboxyl tail including the amino acids 257–382 mediates this function. This adds to the already existing evidence that some effects induced by Cx43 are independent of gap junction or hemichannel formation. This view is further supported by the observation that a tight association of the carboxyl tail with the plasma membrane as it occurs in gap junction clusters, does not seem to be essential for its modulatory effects on migration since the localisation of this carboxyl tail mutant was found to be mainly cytosolic. Gap junction-independent effects of Cx43 were also observed on cell adhesivity, or cell motility which are subfunctions required for effective migration (Bates et al., 2007; Lin et al., 2002; Zhang et al., 2003). Several studies in HeLa cells, HEK 293 cells, C6-glioma cells, Neuro2a, or in human osteosarcoma U2OS cells described an essential role for the non-channel-forming carboxyl tail in mediating growth inhibition (Dang et al., 2003, 2006; Fu et al., 2004; Moorby and Patel, 2001; Zhang et al., 2003). The most intriguing explanation for such behaviour is that the cytoplasmic tail of Cx43 represents a site of protein–protein interactions that makes it a potential part of a signalosome complex. Of note, protein interactions with a number of cytoskeletal proteins (F-actin, α/β-tubulin, actin-binding proteins like Drebrin and cortactin) and protein kinases (several isoforms of PKC, ERK1/2, Src) as well as phosphatases (PP1, PP2A, RPTP) have been described (Herve et al., 2007; Kardami et al., 2007; Wei et al., 2004). Cx43 also has been reported to co-localise with N-cadherin and N-cadherin-associated proteins at regions of cell–cell contact. Downregulation of either N-cadherin or Cx43 resulted in the loss of these proteins at the cell surface and reduced cell motility in NIH3T3 cells (Wei et al., 2005). Clearly, all these proteins could play an important role in the control of cell migration. In fact, in migrating neural crest cells, Cx43 enhanced motility with high directionality, a phenomenon that was associated with co-localisation of Cx43 and actin-binding proteins (α-actinin, drebrin, IQGAP-1), suggesting an important role for Cx43 in regulating the actin cytoskeleton (Xu et al., 2006).

In order to identify a potential signaling mechanism for the enhanced migration by Cx43, we studied the role of p38 MAPK, which is known to mediate actin organization and growth factor-induced cell migration (Huang et al., 2004; Lamalice et al., 2007; Rousseau et al., 1997). The migration stimulus, serum, induced the activation of p38 MAPK in all HeLa cells, but this activation was specifically enhanced in HeLa cells expressing Cx43 or the carboxyl tail, i.e. in those cells also showing enhanced migration levels. Thus, the expression of full length Cx43 as well as of the carboxyl tail of Cx43 in HeLa cells correlated with an enhanced phosphorylation of p38 in response to the migration stimulus. Moreover, blocking the activity of p38 suppressed cell migration in response to serum in those cells to the basal migration levels observed in untreated HeLa cells lacking Cx43 expression (HeLa-CTL). These results suggest that Cx43, via its carboxyl tail, plays a causal role for enhanced p38 activation in response to serum stimulation, and hence, the augmentation of migration. The mechanism of how the carboxyl tail leads to enhanced p38 phosphorylation was not addressed in the present study. However, it is possible that connexin 43 acts as a scaffold protein to improve phosphorylation of p38 or that it inhibits its dephosphorylation. Further studies are required to address this important question.

In summary, we present evidence for a channel-independent modulator action of connexin 43 on the migratory activity of two different cell types. This modulator action of the carboxyl tail of the protein involves an augmented response of p38 to serum stimulation.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejcb.2010.06.003.

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