# Close the Gap : a study on the regulation of Connexin43 gap junctional communication

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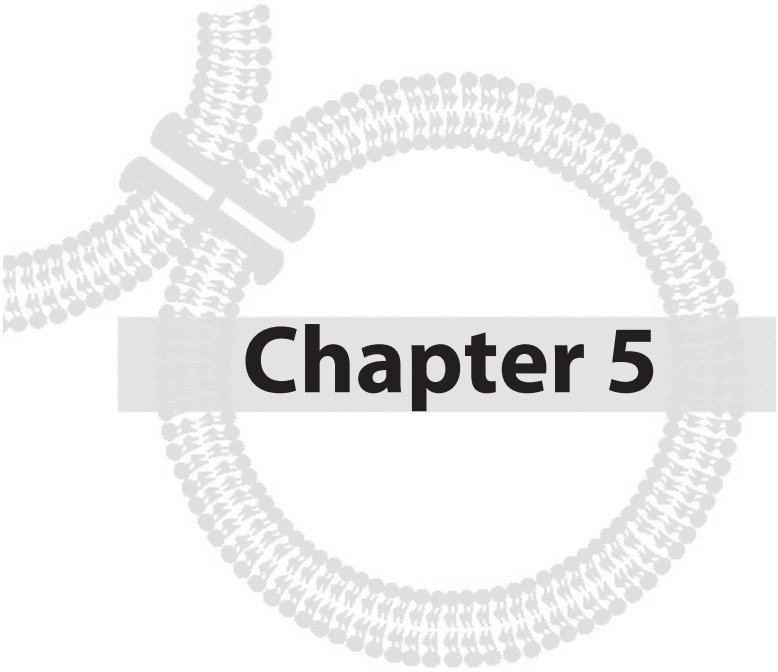

# Chapter 5

## Dependence of cell migration on Connexin43: modulation of N-cadherin expression

Leonie van Zeijl, Trudi Hengeveld , Wouter Moolenaar

**Abstract**

**Direct cell-cell communication through Cx43-based gap junctions is essential for normal cell behaviour. Increasing evidence suggests, however, that Cx43 influences cell proliferation, cell migration and contact inhibition in a communication-independent manner. To investigate the importance of Cx43 for the behaviour of Rat-1 fibroblasts, we knocked down Cx43 by stable expression of Cx43 shRNA. Cx43 knockdown abolished gap junctional communication (GJC), without affecting cell morphology, cell proliferation, contact inhibition or anchorage-independent growth. However, Cx43 knockdown cells showed a ~50% reduced migration speed in an in vitro wound healing assay. Comparing expression levels of various cell-cell contact proteins revealed a strong reduction in N-cadherin expression, both at the mRNA and protein level. However, reconstitution of Cx43 expression and function, did not rescue N-cadherin expression, nor cell migration, indicating that the effect of Cx43 knockdown on migration is GJC independent. In addition, we show that knockdown of N-cadherin alone, is sufficient to reduce the rate of cell migration similar to what is observed in Cx43 knockdown cells. In summary, knockdown of Cx43 inhibits in vitro fibroblast migration, apparently as a result of reduced N-cadherin expression rather than of reduced GJC.**

**Introduction**

The main function of connexin proteins is to form small intercellular channels, called gap junctions. Gap junctions directly connect the cytoplasms of adjacent cells and mediate the diffusion of small molecules, such as second messengers, ions and metabolites[1-3]. Gap junctions are essential for tissue homeostasis and coordinated cell behaviour and loss of gap junctional communication (GJC) is associated with several diseases[4-6]. Most tumours lack functional gap junctions and reconstitution of connexin expression may (partially) invert the tumourigenic phenotype[7-10]. This suggests that restoration of GJC may contribute to contact inhibition. Alternatively, healthy surrounding tissue may control the behaviour of deranged cells, or even induce apoptosis. The transfer of apoptotic signals through gap junctions is known as the "bystander" killing effect[11].

The most abundant and best studied connexin is connexin43 (Cx43). Cx43 is the main connexin in heart cells and Cx43 knockout mice die immediately after birth due to a malformed heart, which is the result of impaired migration of the neural crest cells[12-14]. Cx43 knockout cells from neural crest cell explants show a reduction in polarised cell movement that is comparable to the effect of N-cadherin knockdown on neural crest cell migration[15]. The same authors reported that over-

expression of Cx43 has a similar effect on cell migration, suggesting that a balanced expression of Cx43 is crucial to cell migration[15,16]. Several studies show that Cx43 expression stimulates cell migration, whereas Cx43 knockdown reduces it. In the brain, Cx43 in migrating neurons, which lack adherens junctions, has been reported to increase the adhesive properties of cells, both within one cell type and to neighbouring tissue. In these cells, migration is mediated by Cx43 (or Cx26), in a manner independent of gap junctional communication[17]. It has been suggested that Cx43 may act as an adhesion molecule itself[18-22]. In a mouse model for wound healing, Cx43 knockdown reduced inflammation, seen both macroscopically, as a reduction in swelling, redness and wound gape, and microscopically, as a significant decrease in neutrophil numbers in the tissue around the wound. In addition, Cx43 knockdown resulted in an increased rate of migration of cells into the wound area, leading to increased wound healing capacity compared to wild type mice[23]. However, given the diverse effects of Cx43 knockdown on wound pathology, this may not be a direct effect of Cx43 knockdown on the migration machinery.
In this study, we set out to determine the importance of Cx43 for diverse aspects of cell behaviour. To this end, we stably knocked down Cx43 in Rat-1 fibroblasts. These cells express Cx43 as the only connexin and form functional and regulatable gap junctions, which makes them a perfect model system. Under control conditions, these cells are contact-inhibited. We found that Cx43 knockdown reduced the rate of cell migration in an *in vitro* wound healing assay. This effect was independent of Cx43 expression or function, but appeared to be an indirect effect of Cx43 knockdown on N-cadherin expression.

## Results

*Knockdown of Cx43*

To investigate the importance of Cx43 expression for cell behaviour, we knocked down Cx43 in Rat-1 fibroblasts. We previously reported that stable knockdown of Cx43 results in Cx43 depletion, and cell-cell communication is completely downregulated. Actin staining revealed no changes in gross cytoskeletal arrangement or cell morphology following Cx43 knockdown (chapters 2 and 3 of this thesis and [20]).

*Cx43 knockdown does not affect cell proliferation or contact inhibition*

To study whether Cx43 knockdown affects cell growth or contact inhibition, we measured the rate of cell proliferation (Fig. 1A). There was no significant difference in the growth rate of control and Cxmin cells; the number of cells for both cell lines reached a plateau, indicative of contact inhibition.
It was previously reported that addition of endothelin to Rat-1 cells stimulates anchorage-independent growth in the presence of epidermal growth factor (EGF)[24].

We tested whether Cx43 knockdown affects the ability of Rat-1 cells to form colonies in soft agar. Control cells and Cxmin cells were plated in soft agar in 8% FCS alone or in the presence of endothelin (ET), or EGF, or both stimuli. After two weeks, colony outgrowth was monitored (for images, see Fig. 1B). Consistent with previous findings, both endothelin and EGF induced colony formation, while the combination of both stimuli enhanced colony outgrowth even more. In Cxmin cells, we observed enhanced colony outgrowth similar to that in control cells (Fig. 1B). We conclude that Cx43 knockdown does not affect ET/EGF induced anchorage-independent growth of Rat-1 cells.

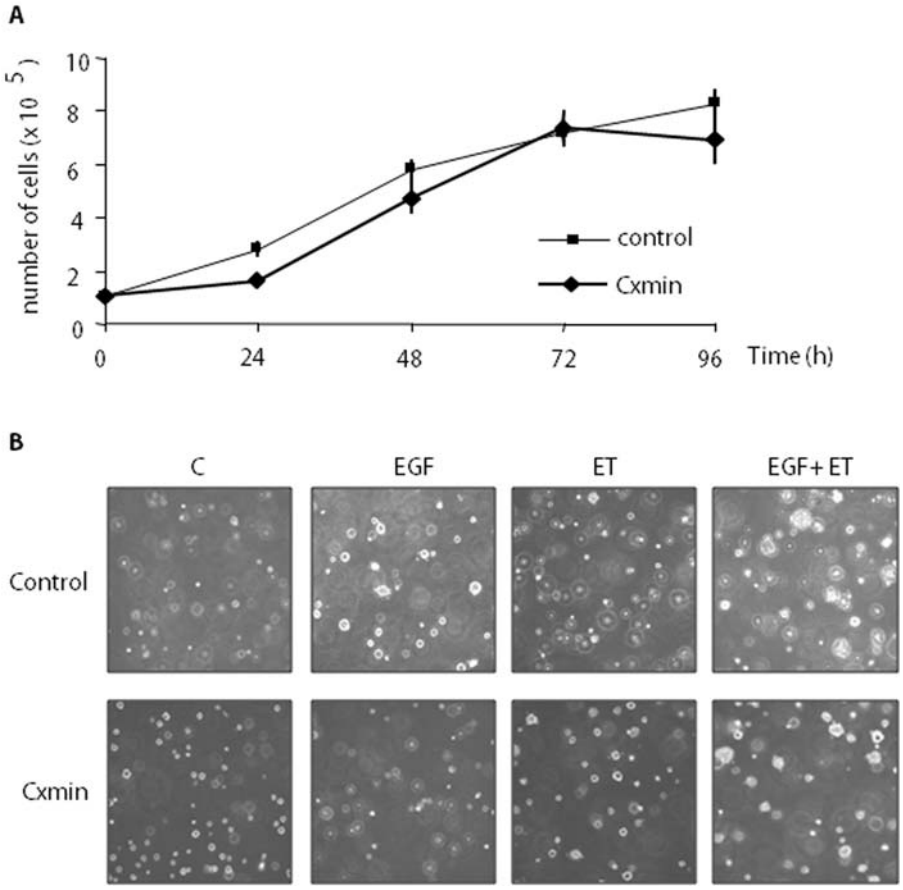


**Figure 1. Cx43 knockdown does not affect cell proliferation or contact inhibition.**
A: Graph showing the proliferation of both control and Cxmin cells. y-axis: number of cells ($x10^5$), x-axis: time (h)
B: Wide field images of Rat-1 cells growing in soft agar in the presence of 8% FCS. Pictures were taken two weeks plating Left: control cells, right: Cxmin cells. C: control, EGF: incubation with EGF (5ng/ml), ET: incubation with endothelin (50 nM), EGF + ET: incubation with EGF (5 ng/ml) and endothelin (50 nM).

*Cx43 knockdown reduces the rate of cell migration*

We performed an *in vitro* wound healing assay to investigate the effect of Cx43 knockdown on cell migration. For this assay, cells were grown to confluency and then serum starved for 8 hrs. Subsequently, the monolayer was scratched with a pipette tip. The cells were allowed to close the wound through migration during 16 hours in the presence of 4% FCS. Cells at the edge of the wound and cells within the monolayer communicated normally with their neighbours (as measured by Lucifer yellow diffusion following microinjection[25,26], data not shown). Cx43 was localized to cell-cell contacts and to the perinuclaer region, similar to the distribution of Cx43 in confluent cells (Fig. 3A). At later time points, when the intracellular structure at the rim of the wound became looser, Cx43 was still detected at sites of cell-cell contact (Fig. 3A). So, during migration, cells are contacted and communicate continuously, which is consistent with a previous study[27].

The surface of the wound before and after migration was measured, and the difference was used as a measure for migration. Figure 2A shows pictures of the scratches before (left) and after (right) migration. Quantification of the relative migration of both control and Cxmin cells shows that knockdown of Cx43 leads to a reduction of migration speed to 52% of that in control cells (Fig. 2B). We conclude that knockdown of Cx43 reduces migration of contacted Rat-1 fibroblasts.

*Cx43 knockdown affects the formation of stress fibres and focal adhesions.*

Directional cell migration requires polarization of the cells toward the desired direction of migration[28]. Therefore, decreased migration ability may be explained by

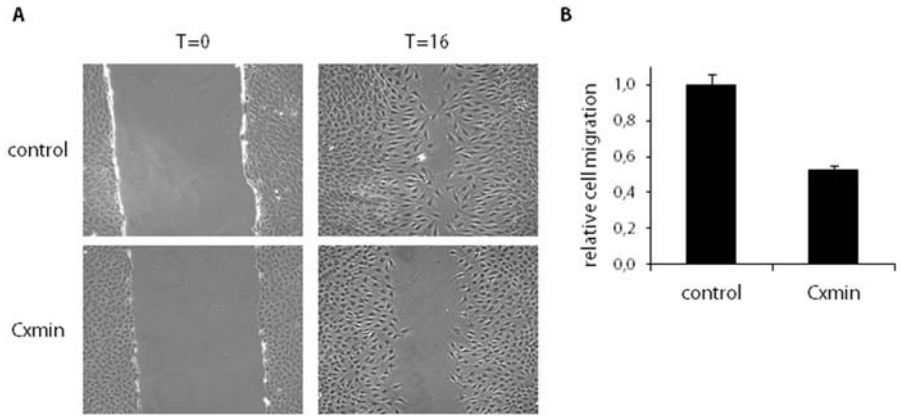


**Figure 2. Knockdown of Cx43 inhibits cell migration.**
Cells were grown to confluency, serum starved for 8 hours and then scratched with a yellow pipette tip. Cells were left to fill the scratch for 16 hours, images were taken at time point 0 and after 16 h. The reduction of the scratch surface is a measure for migration.
A: Wide-field images of control (top) and Cxmin (bottom) cells at T=0 (left) and T=16 (right)
B: Bar diagram showing relative rate of migration. Migration of the control cells was set to 1.

a defect in cell polarisation. We investigated whether knockdown of Cx43 affects the ability of Rat-1 cells to polarise. Control and Cxmin cells were fixed at various time points after the start of the wound healing assay. As a marker for polarisation, we visualized the orientation of the microtubule organizing centre (MTOC) by staining for α-tubulin. All cells which have their MTOC oriented within 45° from the direction of migration were marked (green dots, Fig. 3B). In both control and Cxmin cells at all time points, all cells at the wound edge and most cells in the second row were polarised, while the MTOC orientation in the cells further from the scratch appeared to be random.

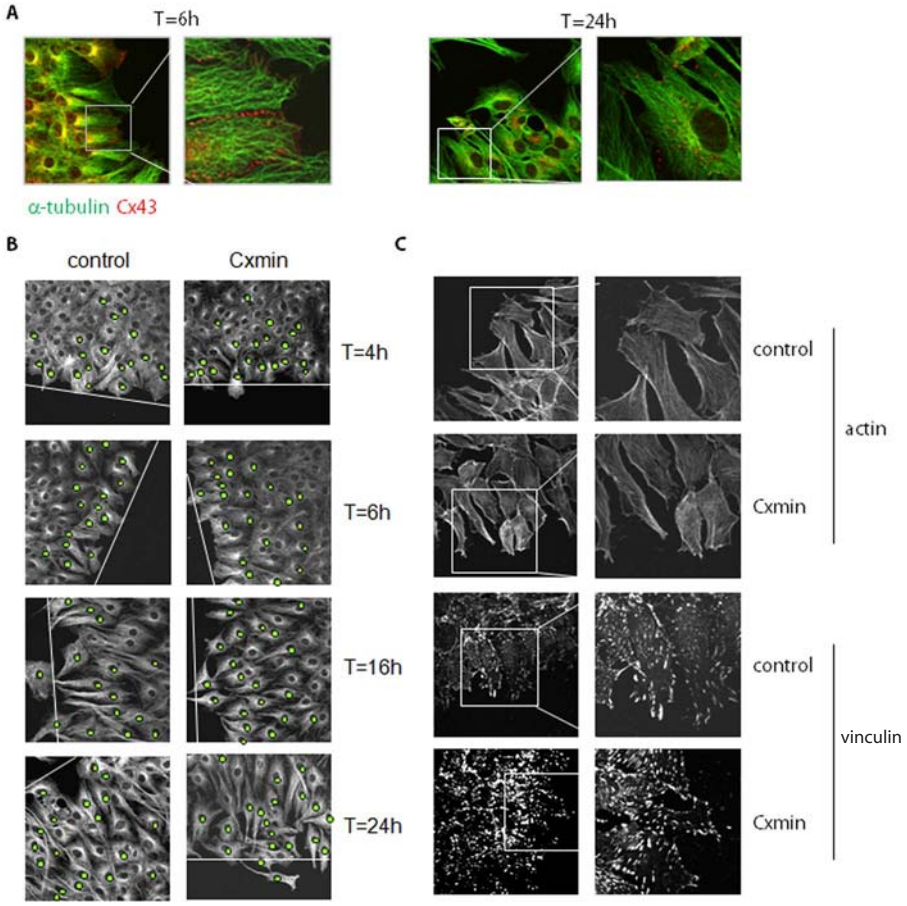


**Figure 3. Cx43 knockdown affects polarised cell movement.**
A: Confocal images of Rat-1 cells 6 and 24 hours after the start of a scratch assay. Cells on coverslips were fixed and stained for Cx43 (red) and α–tubulin (green)
B: Confocal images of control (left) or Cxmin (right) cells, stained for α-tubulin, fixed at several time points after start of the scratch assay. Cells with their MTOC oriented in the direction of migration are marked with a green dot.
C: Confocal images of cells fixed 8 hours after start of the scratch assay, stained for either actin (top) or vinculin (bottom).

Next, we compared actin stress fibre formation and organisation during the migration of control and Cxmin cells (Fig. 3C, top). We found that control cells were more elongated than Cxmin cells, especially after 16h of migration, and that more rows of cells were elongated. Furthermore, stress fibres in control cells were long and oriented in the direction of migration, whereas in Cxmin cells the stress fibres were shorter and more randomly oriented.
The ability to form focal adhesions was studied by staining for vinculin. Cxmin cells appeared to form more focal adhesions than control cells (Fig. 3C, bottom), suggesting that Cxmin cells are better attached, and thereby may hamper migration. Thus, Cxmin cells are still able to polarize, but not to same extent as control cells.

*Knockdown of Cx43 reduces N-cadherin expression*

N-cadherin is the only cadherin that is expressed by Rat-1 fibroblasts. Western blots of total cell lysates show that N-cadherin was strongly downregulated in Cxmin cells (Fig. 4A). This was confirmed by RT-PCR (Fig. 4B), indicating that regulation of N-cadherin expression by Cx43 knockdown takes place at the transcriptional level. N-cadherin is known to be essential for migration of neuronal cells and fibroblasts, and is associated with tumour aggressiveness and metastatic potential[29].
Expression levels of the Cx43-interaction partner ZO-1, focal adhesion marker vinculin, and cell-cell contact protein β-catenin were not affected by Cx43 knockdown (Fig. 4A).

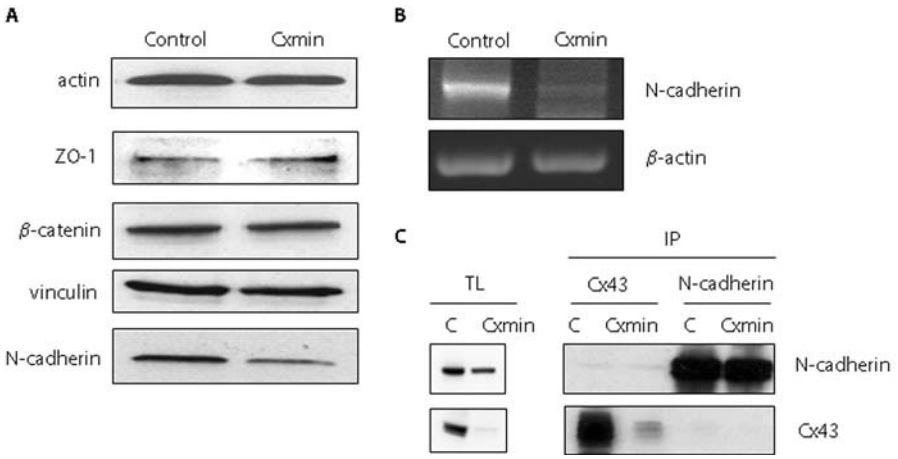


**Figure 4. Knockdown of Cx43 inhibits N-cadherin expression.**
A: Western blots of total lysates from control (left) and Cxmin (right) cells, showing downregulation of N-cadherin protein, but not of ZO-1, β-catenin and vinculin. Actin was used as loading control.
B: RT-PCR showing downregulation of N-cadherin on mRNA level (top) left: control cells, right: Cxmin cells, RT-PCR of β-actin is used as input control (bottom)
C: No direct interaction between Cx43 and N-cadherin. TL: total lysates blotted for N-cadherin (top) or Cx43 (bottom). IP: Either Cx43 (left 2 lanes) or N-cadherin (right 2 lanes) was immunoprecipitated from control and Cxmin cells. Immunoprecipitates were blotted for Cx43 (bottom) and N-cadherin (top).

To ensure that the effect of Cx43 knockdown on N-cadherin expression is not an off-target effect, we designed two additional shRNA constructs (B and C) against Cx43. We stably knocked down Cx43 in Rat-1 cells, using these constructs and blotted total lysates for Cx43 and N-cadherin (Fig. 5A). In these cell lines, Cx43 expression was knocked down below detection level and resulted in a complete lack of GJC. We found that N-cadherin was downregulated to the same extent as in the Cxmin cells, ruling out an off-target effect of the Cx43 shRNA construct. In the wound healing assay, we found that the Cxmin B and Cxmin C cell lines were impaired in their ability to migrate to the same extent as the original Cxmin cells (Fig. 5B). Thus, the effect of Cx43 knockdown on migration coincides with downregulation of N-cadherin.

Cx43 has been reported to colocalise with N- and E-cadherin and suggested to interact with cadherins, but the evidence is primarily based on colocalisation[30-33]. In Rat-1 cells, Cx43 co-localised with N-cadherin (Fig. 6C). We investigated if Cx43 and N-cadherin are part of the same protein complex by performing co-immunoprecipitations. We precipitated Cx43 or N-cadherin from both control and Cxmin cells and immunoblotted for Cx43 and N-cadherin (Fig. 4C). N-cadherin did not co-IP with Cx43 (Fig. 4C, left two lanes), nor did Cx43 with N-cadherin (right two lanes). So, we found no evidence for a direct interaction between Cx43 and N-cadherin.

*Reconstitution of Cx43 expression does not rescue N-cadherin expression or migration.*

We asked whether the effect of Cx43 knockdown on migration and N-cadherin expression is communication-dependent. We reconstituted Cx43 expression in Cxmin cells with RNAi- resistant Cx43. We reported previously that GJC in these cells was fully restored and Cx43 was distributed in the same pattern as in the parental Rat-1 cells[26]. Cxmin cells re-expressing Cx43 migrated at the same rate as Cxmin cells (Fig. 5B). Western blot analysis of total lysates showed that N-cadherin expression was not restored in these cells (Fig. 5A). This suggests that Cx43 expression and cell-cell communication are not the key factors in inhibition of migration by knockdown of Cx43 and suggests a correlation between N-cadherin expression and migration speed.

*N-cadherin knockdown mimics the Cx43 knockdown cell migration phenotype*

We studied the effect of N-cadherin knockdown on Cx43 expression, Cx43-based GJC and cell migration. We stably knocked down N-cadherin in Rat-1 cells using two different shRNA constructs. N-cadherin expression in these cells was reduced to ~80% of that in control cells, while Cx43 was still expressed at normal levels (Fig. 6A). To investigate whether N-cadherin knockdown impaired Cx43 function, we measured gap junctional communication by monitoring the diffusion of Lucifer yellow after microinjection. We found that N-cadherin knockdown cells

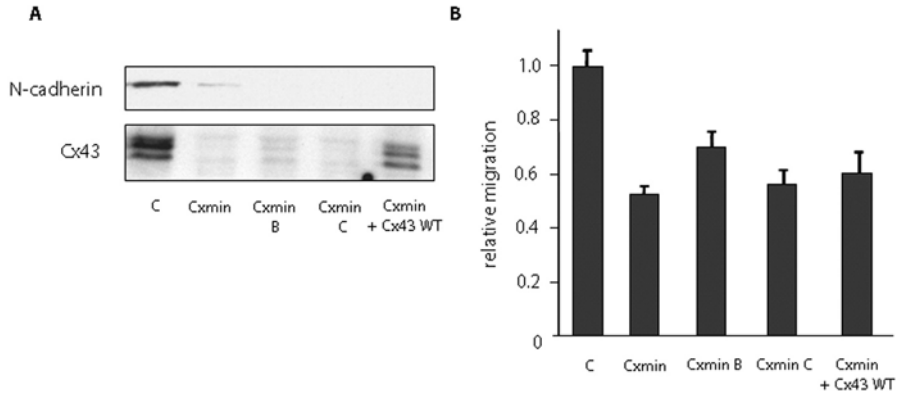


**Figure 5. Rescue of Cx43 expression affects neither N-cadherin expression, nor cell migration.**
A: Western blot showing downregulation of Cx43 by two additional shRNA constructs (3rd and 4th lanes) and reconstitution of Cx43 expression in Cxmin cells (5th lane) (bottom) and N-cadherin expression in these cells (top), compared to control (1st lane) and Cxmin (2nd lane) cells.
B: Bar diagram showing the relative migration of the diverse cell lines from Fig.A. Migration of the control cells was set to 1.

communicated to the same extent as control cells and that GJC could still be inhibited by endothelin and TRP (Fig. 6B and [20]). We immunostained the cells for Cx43 and N-cadherin and found that, while N-cadherin knockdown cells are flatter and form looser contacts, Cx43 accumulated in the perinuclear region and at cell-cell contacts in a punctate fashion similar to control cells (Fig. 6C). Finally, we studied the effect of N-cadherin knockdown on migration in the in vitro wound healing assay (Fig. 6D). In both N-cadherin knockdown cell lines, migration was reduced to ~50% of that in control cells, similar to the effect of Cx43 knockdown (Fig. 6E). We conclude that reduced N-cadherin expression is sufficient to decrease the migration rate to that of Cx43 knockdown cells.

## Discussion

Gap junctional communication (GJC) is essential for tissue homeostasis, growth control and coordinated cell behaviour[1,12,34,35]. Connexins, the building blocks of gap junctions, have been suggested to be tumour suppressors [9,10,34,36]. Loss of communication is associated with loss of growth control, contact inhibition and cell transformation. In this study, we investigated the importance of Cx43 expression on diverse aspects of cell behaviour. We found that knockdown of Cx43 and GJC did not affect cell growth, contact inhibition, cell morphology or cell transformation.
However, in three independent Cxmin cell lines, we observed a ~50% decrease in migration speed of contacted cells. The decrease in migration rate of Cxmin cells

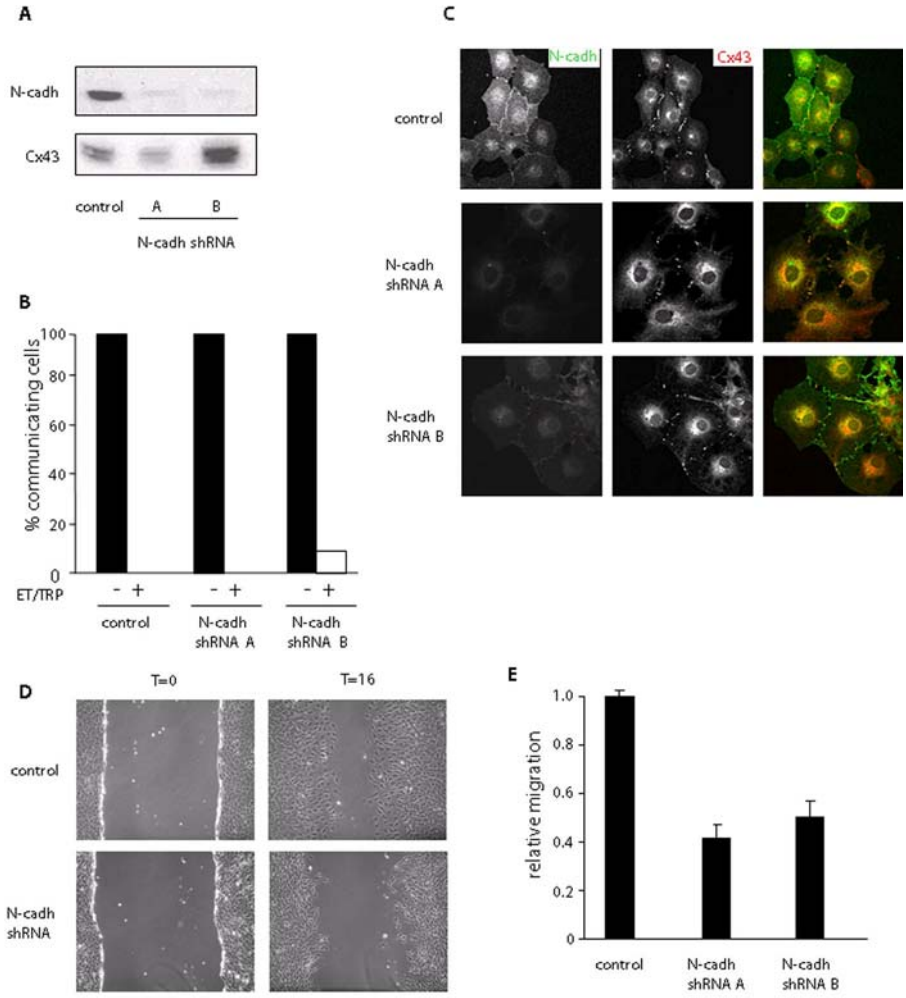


**Figure 6. Knockdown of N-cadherin mimics the effect of Cx43 knockdown on cell migration.**

A: Western blot showing knockdown of N-cadherin by two different shRNA constructs (top) and the expression of Cx43 in these cells.

B: Bar diagram showing the gap junctional communication of N-cadherin knockdown and control cells, before and after stimulation with endothelin (50 nM).

C: Confocal images of control and N-cadherin knockdown cells. Left (green): N-cadherin, Middle (red): Cx43.

For the migration assay, cells were grown to confluency, serum starved for 8 hours and then scratched with a yellow pipette tip. Cells were left to fill the scratch for 16 hours, images were taken at time point 0 and after 16 h. The reduction of the scratch surface is a measure for migration.

D: Wide-field images of control (top) and N-cadherin knockdown (bottom) cells at T=0 (left) and T=16 (right).

E: Bar diagram showing relative migration of control and N-cadherin knockdown cells. Migration of the control cells was set to 1.

was accompanied by reduced cell elongation and increased focal adhesion formation during migration. In all three Cxmin cell lines, we observed a strong downregulation of N-cadherin expression at the transcriptional level. Rat-1 fibroblasts express N-cadherin as only cadherin. N-cadherin is associated with numerous processes, such as cell-cell adhesion, differentiation, embryonic development, invasion and migration[37]. Although the exact mechanism is unknown, it is generally accepted that N-cadherin is essential for migration of neuronal cell and fibroblasts[37]. Given the less elongated shape of Cxmin cells in the wound healing assay and the disturbance in stress fibre alignment, it seems likely that there is a defect in Rho activation. However, it is unknown how N-cadherin expression is linked to stress fibre organisation, focal adhesion formation or activation of Rho family GTPases.

Reconstitution of Cx43-based GJC did not rescue the migration phenotype, suggesting that that the decrease in migration is independent of GJC and Cx43 expression. However, N-cadherin expression was not rescued by expression of Cx43. We hypothesize that the Cxmin cells had reached a new steady state, in which N-cadherin expression was lower than in control cells. Re-introduction of Cx43 expression did not increase the need for the cells to increase N-cadherin levels. Our results fit the growing notion that Cx43 not only functions as a channel protein, but also is an essential player in a multi-protein complex. This is not only important for regulation of GJC, but may also have a broader cellular effect, through signals emanating from (proteins in) this complex[17,19,38,39]

Furthermore, in N-cadherin knockdown cell lines, Cx43 levels and GJC were not affected. In the wound healing assay, N-cadherin knockdown cells showed a decrease in migration rate, comparable to that in Cxmin cells. Thus, downregulation of N-cadherin expression can explain the decrease in migration speed in Cxmin cells.

In summary, Cx43 influences cell migration through a mechanism that is independent of gap junctional communication, but rather acts by influencing the expression of N-cadherin at the transcriptional level through an as-yet-unknown mechanism.

## Materials and methods

*Reagents*

Materials were obtained from the following sources: endothelin, thrombin receptor-activating peptide (TRP; sequence SFLLRN), actin monoclonal, Cx43 polyclonal, vinculin polyclonal and α-tubulin monoclonal antibodies from Sigma (St. Louis, MO); HRP-conjugated secondary antibodies from Cell Signalling and secondary antibodies for immunofluorescence (goat-anti-mouse, Alexa488 and goat-anti-rabbit, Alexa594) from Molecular Probes. N-cadherin and β-catenin monoclonal antibodies from BD Transduction Laboratories. ZO-1 monoclonal antibody from Zymed.

*Cell culture, cell proliferation and cell-cell communication assays*

Cells were cultured in DMEM containing 8% fetal calf serum, L-glutamine and antibiotics. Proliferation rate of the cells is determined by plating $1x10^5$ cells in a 35mm dish in triplo for each time point. Cells are counted on 4 consecutive days with an automatic cell counter. From these data, the growth curve is calculated using Excell. For cell-cell communication assays, cells were grown in 35mm dishes and serum starved for at least 4 hrs prior to experimentation. Monitoring the diffusion of Lucifer Yellow (LY) from single microinjected cells was done as described before[25,26] .

*RNA Interference*

To generate Cx43-deficient Rat-1 cells, Cx43 was knocked down by stable expression of retroviral pSuper[40] ( pRS) containing the shRNA target sequence GAAGCAGATTGAAATCAAGAA (Cxmin B) or TCTCGCTTTGAACATCATTGA (Cxmin C). pRS-Cx43 was transfected into Phoenix-Eco package cells and the supernatant containing viral particles was harvested after 72 hrs. For infection, cells were incubated with 1 ml of viral supernatant supplemented with 10 µl Dotap (Roche; 1 mg/ml). 48 hrs after infection, cells were selected on puromycin (2 µg/ml, Sigma) for 1 week. Single cell-derived colonies were tested for Cx43 expression and communication. A clone expressing <5% of residual Cx43 and completely lacking cell-cell coupling was used for further experiments. N-cadherin was stably knocked down by retroviral expression of N-cadherin shRNA. Two different target sequences were selected (CCGATCAACTTGCCAGAAA and TGACTGAGGAGCCGATGAA). Stable knockdown clones were produced as described above. In all cases, a non-functional shRNA was used as control.

*Construction and expression of shRNA resistant Cx43 cDNA*

Cx43 cDNA was cloned into pEntr 1A (Invitrogen) by BamHI/Xho restriction and subsequently cloned into pAd/Dest/CMV adenoviral expression vector (gateway system, invitrogen) by homologues recombination. To prevent targetting of this construct by Cx43 directed shRNA, we made two silent mutations in the RNAi target site, using PCR-SDM (primers: F: CCGCTGGAGGGAAGGTGTGGTTGTCCGTGCTCTTCATATTC, R: GAATATGAAGAGCACGGACAACCACACCTTCCCTCCAGCGG). Virus was produced in 293A packaging cells according

to standard procedures. Supernatant containing virus particles was titrated on Rat-1 cells to determine the amount needed for Cx43 expression at levels comparable to endogenous Cx43 expression in Rat-1 cells.

*Soft agar assay*

$1x10^5$ cells were plated in 2 ml of growth medium, containing 0.4% low melting point agar in a 35mm dish. Two weeks after plating, digital images were taken on a Zeiss Axiovert 200M microscope using a 10x NA 0.25 objective. Axiovision software was used to capture the images.

*In vitro wound healing assay*

To ability of contacted Rat-1 fibroblasts to migrate is investigated with an *in vitro* wound healing assay: cells are grown to confluency in 35mm dishes in triplo. Before starting the assay, cells are serum starved for 8 hours. The monolayer is then scratched with a yellow pipette tip and cells are left to migrate into the created gap for 16 hours in the presence of 4% FCS. At T=0 and T=16h, digital pictures were taken on a Zeiss Axiovert 200M microscope using a 10x NA 0.25 objective. Axiovision software was used to capture the images. The surface of the scratch area on each picture is measured using Image J. The difference in scratch surface between T=16 and T=0 (as calculated using Excell) is a measure for migration.

*SDS-PAGE, immunoblotting and immunoprecipitation*

Cells were harvested in Laemmli sample buffer (LSB), boiled for 10 min. and subjected to immunoblot analysis according to standard procedures. Filters were blocked in TBST/5% milk, incubated with primary and secondary antibodies, and visualized by enhanced chemoluminescence (Amersham Pharmacia). For immunoprecipitation, cells were harvested in lysis buffer containing1% NP-40, 0.25% sodium desoxycholate, supplemented with protease inhibitor cocktail (Roche). Lysates were spun down and the supernatants were subjected to immunoprecipitation using protein A-conjugated antibodies for 4 hrs at 4°C. Proteins were eluted by boiling for 10 min. in LSB and analyzed by SDS page and immunoblotting according to standard protocols.

*Immunostaining and fluorescence microscopy*

Cells grown on coverslips were fixed in methanol for 15 min. Samples were blocked and in PBS containing 1.5% BSA for 20 min. Subsequently, samples were incubated with primary and secondary antibodies for 30 min. each in PBS/1.5% BSA, washed five times with PBS and mounted in Immumount (Thermo Scientific). Confocal fluorescence images were obtained on a Leica TCS NT (Leica Microsystems, Heidelberg, Germany) confocal system, equipped with an Ar/Kr laser. Images were taken using a 63x NA 1.32 oil objective. Standard filter combinations and Kalman averaging were used. Processing of images for presentation was done on a PC using the software package Photoshop (Adobe Systems Incorporated Mountain View, California, USA).

*RT-PCR*

Total RNA is isolated from subconfluent control and Cxmin cells using the RNeasy kit (Qiagen). mRNA is converted to cDNA according to standard procedures. The primers that were used for PCR of N-cadherin are: Forward: GATCGAATTCCTCTAGAATGTGCCGGATAGCGGGAGCGC and Reverse: CACAAGTCTCGGCCTCTTG

**Reference list**

1. Goodenough, D. A., Goliger, J. A. & Paul, D. L. Connexins, Connexons, and Intercellular Communication. Annual Review of Biochemistry 65, 475-502 (1996).
2. Saez, J. C., Berthoud, V. M., Branes, M. C., Martinez, A. D. & Beyer, E. C. Plasma membrane channels formed by connexins: their regulation and functions. Physiol Rev 83, 1359-1400 (2003).
3. Harris, A. L. Emerging issues of connexin channels: biophysics fills the gap. Q Rev Biophys 34, 325-472 (2001).
4. Wei, C., Xu, X. & Lo, C. Connexins and cell signaling in development and disease. Annual Review of Cell and Developmental Biology 20, 811-838 (2004).
5. Laird, D. W. Life cycle of connexins in health and disease. Biochem. J. 394, 527-543 (2006).
6. Lai-Cheong, J. E., Arita, K. & McGrath, J. A. Genetic diseases of junctions. J Invest Dermatol 127, 2713-2725 (2007).
7. Naus, C. C. Gap junctions and tumour progression. Can J Physiol Pharmacol 80, 136-141 (2002).
8. King, T. J. & Lampe, P. D. The gap junction protein connexin32 is a mouse lung tumor suppressor. Cancer Res 64, 7191-7196 (2004).
9. Rose, B., Mehta, P. P. & Loewenstein, W. R. Gap-junction protein gene suppresses tumorigenicity. Carcinogenesis 14, 1073-1075 (1993).
10. Ruch, R. J., Cesen-Cummings, K. & Malkinson, A. M. Role of gap junctions in lung neoplasia. Exp Lung Res 24, 523-539 (1998).
11. Snyder, A. R. Review of radiation-induced bystander effects. Hum Exp Toxicol 23, 87-89 (2004).
12. Reaume, A. G. et al. Cardiac malformation in neonatal mice lacking connexin43. Science 267, 1831-1834 (1995).
13. Beardslee, M. A., Laing, J. G., Beyer, E. C. & Saffitz, J. E. Rapid turnover of connexin43 in the adult rat heart. Circ Res 83, 629-635 (1998).
14. Rohr, S. Role of gap junctions in the propagation of the cardiac action potential. Cardiovascular Research 62, 309-322 (2004).
15. Lo, C. W., Waldo, K. L. & Kirby, M. L. Gap junction communication and the modulation of cardiac neural crest cells. Trends Cardiovasc Med 9, 63-69 (1999).
16. Huang, G. Y. et al. Gap junction-mediated cell-cell communication modulates mouse neural crest migration. J Cell Biol 143, 1725-1734 (1998).
17. Elias, L. A., Wang, D. D. & Kriegstein, A. R. Gap junction adhesion is necessary for radial migration in the neocortex. Nature 448, 901-907 (2007).
18. McDonough, W. S., Johansson, A., Joffee, H., Giese, A. & Berens, M. E. Gap junction intercellular communication in gliomas is inversely related to cell motility. Int J Dev Neurosci 17, 601-611 (1999).
19. Lin, J. H. et al. Connexin 43 enhances the adhesivity and mediates the invasion of malignant glioma cells. J Neurosci 22, 4302-4311 (2002).
20. Bates, D. C., Sin, W. C., Aftab, Q. & Naus, C. C. Connexin43 enhances glioma invasion by a mechanism involving the carboxy terminus. Glia 55, 1554-1564 (2007).
21. Cai, J., Jiang, W. G. & Mansel, R. E. Gap junctional communication and the tyrosine phosphorylation of connexin 43 in interaction between breast cancer and endothelial cells. Int J Mol Med 1, 273-278 (1998).
22. Elzarrad, M. K. et al. Connexin-43 upregulation in micrometastases and tumor vasculature and its role in tumor cell attachment to pulmonary endothelium. BMC Med 6, 20 (2008).

23. Qiu, C. et al. Targeting connexin43 expression accelerates the rate of wound repair. Curr Biol 13, 1697-1703 (2003).
24. Muldoon, L. L., Pribnow, D., Rodland, K. D. & Magun, B. E. Endothelin-1 stimulates DNA synthesis and anchorage-independent growth of Rat-1 fibroblasts through a protein kinase C-dependent mechanism. Cell Regul 1, 379-390 (1990).
25. Postma, F. et al. Acute loss of Cell-Cell Communication Caused by G Protein-coupled Receptors: A Critical Role for c-Src. J. Cell Biol. 140, 1199-1209 (1998).
26. van Zeijl, L. et al. Regulation of connexin43 gap junctional communication by phosphatidylinositol 4,5-bisphosphate. J Cell Biol 177, 881-891 (2007).
27. Defranco, B. H. et al. Migrating cells retain gap junction plaque structure and function. Cell Commun Adhes 15, 273-288 (2008).
28. Grande-Garcia, A. & del Pozo, M. A. Caveolin-1 in cell polarization and directional migration. Eur J Cell Biol 87, 641-647 (2008).
29. Nagi, C. et al. N-cadherin expression in breast cancer: correlation with an aggressive histologic variant--invasive micropapillary carcinoma. Breast Cancer Res Treat 94, 225-235 (2005).
30. Petridou, S. & Masur, S. K. Immunodetection of connexins and cadherins in corneal fibroblasts and myofibroblasts. Invest Ophthalmol Vis Sci 37, 1740-1748 (1996).
31. Fujimoto, K. et al. Dynamics of connexins, E-cadherin and alpha-catenin on cell membranes during gap junction formation. J Cell Sci 110 ( Pt 3), 311-322 (1997).
32. Nambara, C., Kawasaki, Y. & Yamasaki, H. Role of the cytoplasmic loop domain of Cx43 in its intracellular localization and function: possible interaction with cadherin. J Membr Biol 217, 63-69 (2007).
33. Hartsock, A. & Nelson, W. J. Adherens and tight junctions: structure, function and connections to the actin cytoskeleton. Biochim Biophys Acta 1778, 660-669 (2008).
34. Beyer EC., Paul DL. & Goodenough DA. Connexin family of gap junction proteins. J Membr Biol 116, 187-194 (1990).
35. Bergoffen, J. et al. Connexin mutations in X-linked Charcot-Marie-Tooth disease. Science 262, 2039-2042 (1993).
36. Hotz-Wagenblatt, A. & Shalloway, D. Gap junctional communication and neoplastic transformation. Crit Rev Oncog 4, 541-558 (1993).
37. Derycke, L. D. & Bracke, M. E. N-cadherin in the spotlight of cell-cell adhesion, differentiation, embryogenesis, invasion and signalling. Int J Dev Biol 48, 463-476 (2004).
38. Giepmans, B. Gap junctions and connexin-interacting proteins. Cardiovascular Research 62, 233-245 (2004).
39. Theis, M., Sohl, G., Eiberger, J. & Willecke, K. Emerging complexities in identity and function of glial connexins. Trends Neurosci 28, 188-195 (2005).
40. Brummelkamp TR, B. R. A. R. A System for Stable Expression of Short Interfering RNAs in Mammalian Cells. Science 296, 550-553 (2002).