Connexin43 repression following epithelium-to-mesenchyme transition in embryonal carcinoma cells requires Snail1 transcription factor

Received May 31, 2006; accepted in revised form August 4, 2006

Abstract Embryonic stem (ES) cells and embryonal carcinoma (EC) cells express high amounts of functional connexin43 (Cx43). During mesoderm formation and subsequent cardiac differentiation, Cx43 is initially down-regulated but is up-regulated again as the emerging cardiomyocytes mature. In this study, we investigated the regulation of Cx43 expression during early phases of differentiation in F9 and P19 EC cells. We found a striking inverse correlation between the expression of Cx43 and that of the transcriptional repressor Snail1. No clear relationship was found with Smad-interacting-protein1 (SIP1), another transcription factor inducing epithelium-to-mesenchyme transition (EMT). Promoter-reporter assays indicated Cx43 repression at the promoter level by ectopically expressed Snail1. To establish whether the Cx43 down-regulation depends on endogenous Snail1, MES-1 cells, differentiated derivatives of P19 EC, were stably transfected by an siRNA construct silencing Snail1 expression. This resulted in a mesenchyme-to-epithelium transition, which was accompanied by increased levels of Cx43 mRNA and protein and enhanced metabolic and electrical coupling. We conclude that Snail1-mediated EMT results in a Cx43 repression.

Key words connexin · snail · epithelium-mesenchyme transition · cadherin · gap junction

Introduction

Gap junctions allow the direct exchange of small metabolites and ions between adjacent cells. They are indispensable in many biological processes, from action potential propagation in the heart to secretion, development, and tissue repair (Sáez et al., 2003; Stout et al., 2004; Wei et al., 2004). Gap junctions are formed by two hemichannels on adjacent cells, each consisting of six connexin (Cx) proteins arranged around a central pore. The docking of the two hemichannels results in the creation of a functional cell-to-cell channel. To date, at least 20 Cx isoforms have been identified in the genomes of mouse and humans (Söhl and Willecke, 2003). Although expression is particularly prominent in the working myocardium of the heart, Cx43 is expressed widely in multiple tissues of all mammalians.

Cell–cell interactions are formed by different classes of proteins resulting in the formation of adherence junctions, desmosomes, and tight junctions. The functional integrity of cell–cell junctions is critically important for Cx43-mediated gap-junction formation (Jongen et al., 1991). However, the underlying molecular determinants such as the cadherin isoform, may be redundant or interchangeable (Ohsugi et al., 1997).
Furthermore, it was shown that Cx43 interacts with cell–cell junction-associated proteins such as cadherins, α- and β-catenins, p120ctn, and Zonula Occludens-1 (ZO-1), for a review, see Giepmans, 2004.

An unexpected finding in stem cell biology has been the observation that undifferentiated pluripotent cells are connected by functional gap junctions. Cx43 was found to be expressed in pluripotent human embryonic stem (ES) cells (Wong et al., 2004; Huetten et al., 2006), mouse ES cells (Oyamada et al., 1996, Van Kempen et al., 2003), and P19 embryonal carcinoma (EC) cells (Van der Heyden et al., 1998). Upon differentiation in aggregates known as embryo bodies (EBs) in which cardiomyocytes form spontaneously (Boheler et al., 2002), the amount of Cx43 expressed decreased, and many premature, embryonic-like cardiomyocytes display no detectable Cx43 (Van Kempen et al., 2003). Upon cardiomyocyte maturation, Cx43 levels increase again (Van Kempen et al., 2003). This recapitulates Cx43 expression during embryonic formation in vivo (Van Kempen et al., 1991, 1996; Delorme et al., 1997).

In mouse EB formation, an outer endoderm forms a skin around an ectoderm-like core. Subsequently, gastrulation-like events occur, resulting in mesoderm formation that eventually results in beating cardiomyocytes (Weitzer, 2006). In some ES cell lines, early mesoderm formation has been described as involving an epithelium-to-mesenchyme transition (EMT), mimicking that in the embryo (Behr et al., 2005). In general, an EMT is characterized by a dramatic change in cell–cell and cell–matrix interactions, which allow the cells to escape the epithelium and migrate through an extracellular matrix. In parallel, a transcriptional program is initiated to maintain the mesenchymal character of the cells. Many extracellular stimuli can evoke an EMT by activating at least four different transcription factor families. These include Smad-interacting protein1 (SIP1) and Snail1 (Thiery, 2003; Barral-Gimeno and Nieto, 2005; Radisky, 2005). Among the effects of Snail1 or SIP1 activity are direct transcriptional repression of the cell–cell adhesion genes E-cadherin, claudins, and occludin, which disrupt adherence and tight junctions, respectively (Cano et al., 2000; Ikenouchi et al., 2003; Vandewalle et al., 2005).

In a previous study, we used F9 EC cells to characterize Cx43 expression during a protein kinase A (PKA) signaling-mediated EMT (Van der Heyden et al., 2000). The F9 EC cells treated with retinoic acid (RA) and cyclic AMP (cAMP) recapitulate the transition made by extra-embryonic visceral endoderm into parietal endoderm in the embryo in vivo, one of the earliest developmental EMTs. We found that upon EMT, functional Cx43 expression that was detected decreased dramatically. This EMT was later associated with rapid Snail1 up-regulation (Veltmaat et al., 2000). This suggests a causal relation between Snail1 and Cx43.

Here, we hypothesize that Snail1-mediated EMT results in Cx43 down-regulation, and therefore could be responsible for low Cx43 levels in the resulting cell population. We have found that Snail1 expression negatively correlates with Cx43 in two different EC cell lines. We demonstrate that the Cx43 promoter is a target for Snail1 repression. By silencing endogenous Snail in MES-1 cells, we showed that Cx43 levels could be restored.

### Materials and methods

#### Cell culture and differentiation

P19 ES, END-2, MES-1, EPI-7 (Mummery et al., 1985, 1986), and F9 cells (Berstine et al., 1973) were maintained in DMEM/F12 (1:1; Gibco, Breda, the Netherlands) containing 10% fetal calf serum (Gibco), 2 mM l-glutamine, and 50 U/ml penicillin and 50 μg/ml streptomycin. P19s18 and F9 EC cells were cultured on 0.1% gelatin-coated surfaces.

F9 EC cells were induced to differentiate using 10−6 all-trans RA (Sigma, St. Louis, MO) for 5 days, with or without dibutyryl cAMP for the last 24 hr or less, as described previously (Van der Heyden et al., 2000).

#### Northern blotting

Total RNA was extracted with Trizol reagent (Gibco) according to the manufacturer’s instructions. Thirty microgram RNA was run on a 1.2% formaldehyde–agarose gel and transferred to a HybrBlot membrane (Qiagen, Venlo, the Netherlands). Blots were incubated with [32P]-labeled probes for murine Cx43 (protein-coding region) and GAPDH. Signal detection and analysis were performed using a PhosphoImager and ImageQuant software (Amersham Biosciences, Roosendaal, the Netherlands).

#### Semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR)

RNA was isolated using Trizol and 2 μg total RNA was reverse transcribed using oligo-dT and M-MLV-RT (Gibco) in a 20 μl reaction volume. Subsequently, 1 μl reaction volume (cDNA equivalent of 100 ng total RNA) was used in the PCR reaction. Primers, annealing temperature, product size, and optimized number of PCR cycles are depicted in Table 1. RT-PCR for determining Cx43 promoter usage and alternative splicing was performed according to Pfeifer et al. (2004). Products were analyzed in 1%–1.5% agarose, ethidium bromide-stained gels. β-tubulin was used as an internal standard.

#### Immunofluorescent staining

Indirect immunofluorescence was used to determine Cx43 and ZO-1 cellular localization. In brief, cells were washed with PBS, fixed for 25 min with 4% formaldehyde, permeabilized with 0.5% Triton X-100 for 5 min, and quenched with 50 mM glycine. Antibodies were diluted in NET-gel (50 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.2% gelatine) supplemented with 10% normal goat serum, followed by five washes with NET-gel. Primary and secondary antibodies were incubated overnight and for 2 hr, respectively. The antibodies used were Cx43 (Clone 2, Transduction labs, Lexington, KY; cat. nr. 610062), ZO-1 (Zymed, San Francisco, CA; catalog number 61-7300) as the primary antibody and Texas Red or fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse or anti-rabbit IgG.
rabbit (Jackson Immunoresearch, West Grove, PA) as the secondary antibody.

Western blotting

Cells were lysed in RIPA buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM Na2HPO4, 1% [v/v] Triton X-100, 1% [v/v] Na-deoxycholate, 0.1% [w/v] SDS, 1 mM EDTA, 50 mM NaF, 1 mM PMSF, 10 μg/ml aprotinin). Lysates were clarified by centrifugation at 14,000 × g for 5 min at 4 °C. Twenty microgram protein lysate was mixed with Laemmli sample buffer and proteins were separated by 7% or 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently electro-blotted onto a nitrocellulose membrane (BioRad, Veendael, the Netherlands). Equal loading was checked by reversible Ponceau staining. The antibodies were Cx43 (Zymed; catalog number 71-0700), ZO-1 (Zymed; catalog number 61-7300), Pan-cadherin (Sigma; catalog number C-3878), E-cadherin (Transduction Labs; catalog number 610181), β-catenin (BD Biosciences, Erembodegem, Belgium; catalog number 610153), β-Tubulin (Sigma; catalog number T-4026), and HA-tag (Sigma; catalog number H-9658). Proteins were visualized by using peroxidase-labeled secondary antibody (Jackson) and standard ECL procedures (Amersham Bioscience).

In attempts to visualize Cx26, Cx30.3, Cx45, and Snail1 at the protein level, we used Cx26 (Zymed #71-0500), Cx30.3 (Zymed #40-0000), Cx45 (a generous gift from Dr. T. H. Steinberg, Institute for Infectious Diseases, St. Louis, MO), and Snail1 (Santa Cruz, sc-10432; Heidelberg, Germany) antibodies. This, however, did not result in signals that unambiguously could be attributed to the presence of the Cx in question or Snail1, either caused by low levels of protein expression, antibody specificity or affinity, or a combination of factors (not shown).

Electrical and metabolic coupling

A symmetrical setup with two HEKA EPC-7 patch clamp amplifiers (HEKA Elektronik, Lambrecht, Germany) was used to measure electrical coupling between cells. Macroscopic gap junctional currents were recorded using a custom data acquisition program (kindly provided by Dr. J. G. Zegers, AMC, Amsterdam, the Netherlands) running on a G4 Apple Macintosh computer equipped with a 12-bit National Instruments PCI-MIO-16E-4 acquisition card. Current signals were low-pass filtered at 2.5 kHz and acquired at 10 kHz. Macroscopic gap junctional currents were elicited in cell pairs by applying small transjunctional voltage steps (10 mV) from holding potential (−50 mV). By using small pulses, junctional conductances were maximal and not inactivating. Offline analysis was performed using MacDaq 8.0 (kindly provided by Dr. A. C. G. van Ginneken, AMC, Amsterdam) and R 2.0.1 (Team RDC, 2005).

Gap junctional conductance (gJ) was defined as gJ = Ij/Vj, where Ij and Vj denote junctional current and transjunctional voltage, respectively. Electrophysiological experiments were conducted at 20 °C. Extracellular buffer was used as a modified Tyrode’s solution, containing (in mmol/l) NaCl 140, KCl 5.4, CaCl2, 1.8, MgCl2 1, HEPES 15, NaHCO3 35, glucose 6, pH 7.20/NaOH. Pipette buffer contained (in mmol/l) potassium gluconate 125, KCl 10, HEPES 5, EGTA 5, MgCl2 2, CaCl2 0.6, Na2ATP 4, pH 7.20/KOH. For dye injections, microelectrodes were filled with 4% (w/v) Lucifer Yellow in 150 mM LiCl3, 10 mM HEPES. Patch pipettes were pulled on a Narishige PC-10 puller and fire-polished. When filled with pipette buffer, pipette resistance ranged between 2 and 5 MΩ.

Luciferase assays

Cells were cultured on 25 cm2 dishes and subsequently transfected with 2 μg of luciferase reporter construct, either pGL3-CX43 (−1338/+281; Teunissen et al., 2003) or E-cadherin promoter (Batlle et al., 2000), and 1 μg of pSG-HA-Snail1 (C-terminal HA-tagged murine Snail1 [accession number X67253], a generous gift from Dr. A. García de Herreros, Barcelona, Spain) when indicated, using the Calcium-Phosphate method. As a transfection control, pRL-CMV (Promega, Leiden, the Netherlands) was co-transfected. After ~ 20 hr, cells were washed with phosphate-buffered saline (PBS) and medium was replaced by normal culture medium. Forty-eight hours post-transfection, cells were washed with PBS and harvested with passive lysis buffer (Promega) and assayed for luciferase activity using the Promega Dual Luciferase Reporter Assay system on a Lumat LB9507 luminometer (EG&G Berthold, Bad Wildbad, Germany). Transfections were carried out in duplicate and repeated at least three times independently. Promoter activity is given as fold activity ± SEM, relative to non-promoter containing pGL3-basic.

siRNA interference

For construction of siRNA vectors, we essentially used the system described by Van de Wetering et al. (2003). Oligonucleotides were based on murine Snail1 (accession number NM_011427), for SiSnail 5′-GATCCGCGCGCCACAGCTATAGCGAATTCGAAGCACGAGCTCGCTATAGG-3′ and 5′-GCTGTCTCTTCTTGTGCGGCGG-3′; for SiSnai2 5′-GATCCGCGCGCCACAGCTATAGCGAATTCGAAGCACGAGCTCGCTATAGG-3′ and 5′-GCTGTCTCTTCTTGTGCGGCGG-3′; and for SiSnai3 5′-GATCCGCGCGCCACAGCTATAGCGAATTCGAAGCACGAGCTCGCTATAGG-3′ and 5′-GCTGTCTCTTCTTGTGCGGCGG-3′.
ACTCGGATGTAAGATCTTGAATCTTCACATCCGAGTGGTGGG-3'. For oligonucleotide annealing, 2 μg sense and antisense oligo was mixed in a volume of 60 μl, and heated at 90°C for 3 min and slowly cooled to 37°C and incubated for 1 hr at 37°C. Once annealed, BamHI and HindIII sticky ends were formed at the 5' and 3' part of each double-stranded oligo, respectively. The H1 promoter and Zeocin resistance gene containing pTER+ vector was digested with BglII and HindIII, and double-strand oligos were ligated. The resulting pTER+–siRNA constructs were verified by sequencing.

MES-1 cells were transfected with empty vector pTER+, or pTER+ vectors containing siSnai1 or siSnai3 using Lipofectamine (Invitrogen, Paisly, UK) according to the manufacturer's protocol. Two days following transfection, selection using 500 μg/ml Zeocin (Invitrogen) was started. After 3 weeks, stable pools were subjected to limited dilution in 96-well plates (average of 0.5 cell/well). Growing clones were subsequently transferred into 24- and six-well plates. Stable clones were selected for further analysis on the basis of Snai1 expression as detected by RT-PCR.

**Results**

Cx43 down-regulation in F9 primitive to parietal endoderm EMT is preceded by Snai1 up-regulation

In a previous study, we observed a strong decrease in Cx43 expression at the mRNA, protein, and functional level in F9 EC cells undergoing EMT (Van der Heyden et al., 2000). In a similar study, it was found that this EMT was associated with a rapid up-regulation of the zinc finger transcriptional repressor Snai1 (Veltmaat et al., 2000). To investigate the kinetics of these two processes, we differentiated F9 EC cells into primitive endoderm-like cells by addition of RA (Figs. 1A,1B). This resulted in up-regulation of Cx43 mRNA and protein expression (Figs. 1C,1D). A small increase in Snai1 mRNA was also found, likely the result of spontaneous transdifferentiation into parietal endoderm (Fig. 1C). Thirty minutes after dibutyryl-cAMP addition, which initiates parietal endoderm differentiation (Figs. 1A,1B), a massive increase in Snai1 mRNA was observed (Fig. 1C). This was maintained for at least 24 hr (Fig. 1C). The increase in Cx43 mRNA expression was transient and expression had returned to low levels at 24 hr. Cx43 protein levels were similarly decreased in time (Fig. 1D). Although direct interaction of Cx43 with the tight junction protein ZO-1 has been established (reviewed in Giempans, 2004), we observed an inverse correlation when comparing F9EC and F9PrE with respect to Cx43 and ZO-1 expression.

From these data, we conclude that F9 EC cells undergoing primitive to parietal endoderm EMT are characterized by Cx43 down-regulation, which is preceded by Snai1 up-regulation.

Cx43 down-regulation correlates with increasing Snai1 levels in P19 EC cells and their differentiated derivatives

Pluripotent P19 EC cells can be induced to differentiate in derivatives of all three germ layers (reviewed in Van der Heyden and Defize, 2003) recapitulating early differentiation events in embryonic development. We compared undifferentiated P19s18 EC cells with three derivative cell lines of endodermal (END-2), mesodermal (MES-1), and ectodermal (EPI-7) characteristics (Mummery et al., 1985, 1986). P19s18 EC, END-2, and EPI-7 display an epithelial-like morphology when grown as monolayer cultures, in contrast to MES-1 cells, which have a mesenchymal-like morphology. mRNA was isolated from all four-cell lines and Cx43 mRNA was detected by Northern blotting and quantified with respect to GAPDH. The highest Cx43 mRNA levels were detected in P19s18 EC cells (100 arbitrary units [a.u.]), while lower levels were found in EPI-7 (32 a.u.), END-2 (15 a.u.), and MES-1 (4 a.u.) cells, respectively (Fig. 2A). Similar results were found by semi-quantitative RT-PCR (Fig. 2B). Furthermore, RT-PCR revealed an almost perfect negative correlation between Cx43 and Snai1 expression. Some positive relation was found between this Cx and SIP1, another transcriptional inducer of EMT, when comparing the three differentiated derivatives. However, by including P19EC in this comparison, this correlation becomes less clear. Finally, Cx26, Cx30.3, and Cx45 were detected. A perfect positive correlation between Cx30.3 and Snai1 mRNA was observed, but neither Cx26 nor Cx45 displayed a strong correlation with either Snai1 or SIP1 (Fig. 2B).

Cx43 mRNA expression was partially reflected in protein expression, which indicates additional post-transcriptional regulation of expression levels. The lowest levels of Cx43 were found in MES-1 cells (Fig. 2C). Here, a positive correlation was found with ZO-1 and to some extent with Pan-cadherins, in contrast to F9 cells. Remarkably, E-cadherin was only detected in undifferentiated P19s18 EC cells, and not in its differentiated derivatives. However, E-cadherin is a well-known target of Snai1 repression, and its absence in the differentiated derivatives is in accordance with Snai1 expression in these cell lines. No differences in β-catenin were observed.

We next assessed the cellular localization of Cx43 in the four different cell lines (Fig. 3), and to reveal the cell borders, ZO-1 co-staining was performed. In P19s18 EC cells, the majority of Cx43 was located intracellularly; however, Cx43 was also located at the borders between cells in gap-junctional-like structures. In END-2 and EPI-7, Cx43 was mainly localized at the cell borders, apparently in gap junctions. In MES-1 cells, however, Cx43 was mainly intracellular, although some gap-junctional-like staining could be seen.

Finally, functional gap-junctional coupling was established by both Lucifer Yellow dye coupling and electrotonic coupling. Dye spreading assays indicated the strongest gap-junctional coupling in P19s18 EC cells (Figs. 4A,4B). Two minutes after dye injection, Lucifer Yellow was visible on average in 15.8 ± 2.4 (SEM;
added at day 5 for the indicated time (hours). In response to RA, resulting F9PrE cells express Cx43. Subsequent differentiation into PE, characterized by an EMT results in a rapid increase (within 0.5 hr) of Snail1. Maximal down-regulation of Cx43 is observed during the 24-hr period and is most obvious at 6 hr of dbcAMP stimulation. No PCR product was obtained without prior reverse transcriptase reaction (-RT). β-Tubulin serves as input control. (D) Western blot analysis of Cx43, ZO-1, and E-cadherin in differentiating F9 EC cells. Cell stimulation protocol as in (C). Cx43, displaying multiple bands of differently phosphorylated states, is up-regulated in response to RA-mediated PrE and down-regulated in response to dbcAMP mediated PE differentiation. ZO-1 expression is strong in F9 EC cells and decreased in PrE and PE cells. E-cadherin is down-regulated following 24-hr stimulation with dbcAMP. Total protein serves as control.

N = 20) neighboring cells for P19s18 EC cells. In END-2 and EPI-7 cells, on average dye spread into 9.1 ± 1.3 (N = 20) and 7.4 ± 1.0 (N = 20) adjacent cells, respectively, while MES-1 cells were poorly coupled with only 3.0 ± 0.5 (N = 20) positive cells. As dye-coupling depends to some extent on the Cx isoform involved and on the size of the cells, we next compared electrotonic coupling of P19s18 EC and MES-1 cells. P19s18 EC cell pairs exhibited a significantly higher level of electrical coupling (37.3 ± 7.0 nS [N = 17]) as compared with MES-1 cells (17.3 ± 3.0 nS [N = 17], p < 0.015).

These results illustrate that upon differentiation of pluripotent P19s18 EC cells, Cx43 expression was decreased at mRNA and protein levels and probably also at the functional level. The strongest decrease was seen in MES-1 cells. Furthermore, Cx30.3 mRNA is reciprocally up-regulated in these cell lines. Finally, a strong correlation was found between the alternate Cx43/Cx30.3 expression and that of Snail1.

The Cx43 promoter activity matches Cx43 mRNA levels and is repressed by ectopic Snail1.

The Cx43 gene was originally considered as a two-exon gene driven by one promoter. Only recently it has been
recognized that Cx43 transcription can be mediated by three promoters (P1–P3) and that Cx43 pre-mRNA is subjected to alternative splicing (Pfeifer et al., 2004). Promoter P1, which was the first identified Cx43 promoter, however, appears superior in activity compared with P2 and P3 (Pfeifer et al., 2004). Nevertheless, we questioned whether P19s18 EC and its derivatives may use alternative promoters, and/or generate different mRNAs by alternative splicing. Therefore, mRNA was subjected to RT-PCR using different primers to distinguish between promoter usage and alternative splicing. This analysis provided no evidence for alternative promoter use or alternative splicing between the four cell lines (Fig. 5A).

To further investigate the level at which the negative regulation of Cx43 in P19s18 EC cell differentiation occurred, we determined Cx43 P1 promoter activity in undifferentiated P19s18 EC cells and the differentiated derivatives. The rat Cx43 P1 promoter reporter was transfected into all four cell lines, and the relative reporter activity was determined by luciferase activity (Fig. 5B). The strongest promoter activity was found in P19s18 EC cells (26.1 ± 3.2 [SEM] fold over non-promoter containing luciferase reporter), followed by END-2 (12.9 ± 0.8 fold) and EPI-7 (11.3 ± 1.0 fold) cells. The lowest promoter activity was found in MES-1 cells (7.3 ± 0.5 fold). Therefore, differences in promoter activity may account for the differences in Cx43 mRNA expression.

As a strong inverse correlation was found between Cx43 and the transcriptional repressor Snail1, we next investigated whether Snail1 co-expression would lead to Cx43 P1 promoter down-regulation. Co-transfection of 1 μg of Snail1 expression construct resulted in inhibition of the rat Cx43 P1 promoter by 58.4% ± 4.3% in P19 EC cells. As a control, we used the E-cadherin promoter as an established Snail1 target gene (Batlle et al., 2000), which was inhibited by 77.6% ± 3.1%. As Snail1 is an E-box (CANNTG)-binding transcription factor, we established whether Snail1-specific E-boxes (CA(C/G)(C/G)TG; Batlle et al., 2000; Ikenouchi et al., 2003) are present in the Cx43 P1 promoter. The murine and rat Cx43 promoter contained nine and four E-box consensus sequences, respectively, of which two and one conform to the Snail1-specific E-boxes, respectively (Fig. 5D). Therefore, we conclude that the Cx43 promoter is a target gene for Snail1, whose regulation might be by direct Snail binding to its E-boxes.

**Fig. 2** P19s18 and its derivatives display a negative correlation between Snail1 and connexin43 (Cx43) expression while Cx30.3 is positively correlated with Snail1. (A) Detection of Cx43 expression in P19s18 embryonal carcinoma (EC) cells and its derivative cell lines by Northern blotting. Signal of 18S and 28S rRNA is visible above and beneath the Cx43 signal. GAPDH serves as input control. Phospholmager mediated quantification indicates strongest expression in P19s18 EC (100 arbitrary units [a.u.]) and lower expression in END-2 (15 a.u.), MES-1 (4 a.u.), and EPI-7 (32 a.u.) cells respectively. (B) Semi-quantitative reverse transcriptase polymerase chain reaction analysis of Snail1, SIP1, Cx43, Cx30.3, Cx26, and Cx45 on P19s18 EC and its derivative cell lines. β-tubulin serves as input control. (C) Western blot analysis of Cx43, ZO-1, pan-cadherin, E-cadherin, and β-catenin expression in P19s18 EC cells and its derivatives.
Silencing of endogenous Snail1 in MES-1 cells results in mesenchyme-to-epithelium transition, Cx43 up-regulation, and enhanced gap-junctional communication.

To confirm a causal relationship between Snail1 and Cx43 expression, we investigated whether inhibition of endogenous Snail1 would increase Cx43 expression.

We constructed three different Snail1 siRNA-silencing expression constructs. Ectopic expression of HA-tagged Snail1 resulted in detectable Snail1 expression on Western blot (Fig. 6A). Co-transfection of silencing construct siSna1 or siSna2 resulted only in a very minor inhibition of Snail1 in this system. In contrast, co-transfection of siSna3 resulted in very efficient silencing of ectopic Snail1 (Fig. 6A). Next, MES-1 cells were stably transfected with either empty siRNA expression vector (pTER+), or silencing constructs containing either siSna1 or siSna3. pTER+ overexpression resulted in no difference in cell morphology (Fig. 6B). siSna1 overexpression resulted in a moderate change in cell morphology as the cells appeared larger and more flattened. siSna3a overexpression resulted in a total mesenchyme-to-epithelium transition (Fig. 6B).

Subsequently, RNA was isolated from stably transfected cells (total pools) or isolated clones from these pools, and subjected to semi-quantitative RT-PCR (Fig. 6C). Upon expression of siSna1, Snail1 was only down-regulated to a very limited extent. Expression of siSna3 resulted in a strong reduction of Snail1 expression. Upon silencing of Snail1, Cx43 was up-regulated, which was most obvious in siSna3-expressing cells. Western blot analysis of the same pools and clones revealed a minor up-regulation of Cx43 in siSna1 and stronger Cx43 up-regulation in siSna3 (Fig. 6D). Pan-cadherins and ZO-1 were up-regulated in both siSna1 and siSna3 pools and clones, but the strongest effects were seen in siSna3 pools and clones. Restoration of E-cadherin expression was found only in siSna3 pools and clones (Fig. 6D). Finally, we found that siSna3 clones exhibited increased levels of gap-junctional communication as measured by dye coupling (9.3 ± 0.9 [n = 10], 9.4 ± 1.2 [n = 9], and 14.7 ± 1.6 [n = 11]) coupled neighboring cells for pTER+cl5, siSna1 cl1, and siSna3 cl2, respectively; Fig. 6E) and electrotonic coupling (8.6 ± 1.7 [n = 8] and 14.8 ± 2.0 [n = 9]) nS for pTER+cl5 and siSna3 cl2, respectively; Fig. 6F) when compared with MES-1 cells overexpressing the empty pTER vector or siSna1.

From these results, we conclude that there is a causal, reciprocal relationship between endogenous Snail1 and Cx43 expression.

**Discussion**

The results presented here indicated the existence of a Cx43 down-regulation during an EMT that is associated with a rapid up-regulation of Snail1. In a recent study, it was shown that ectopic SIP1 induces EMT in human epithelial cells, which is accompanied by direct down-regulation of P-cadherin, Claudin 4, and Cx26 promoter activity (Vandewalle et al., 2005). In our
P19s18 EC and derivative cell lines, SIP1 is endogenously expressed, but there was little correlation between expression levels and Cxs (Fig. 2B) in contrast to the effect induced by Snail1. Moreover, Cx26 is most highly expressed in MES-1 cells, which displayed the highest level of SIP1, although we have not determined the functional activity of SIP1 in these cells. Nevertheless, our study and that of Vandewalle et al. (2005) indicate that EMT-inducing transcription factors, either ectopic SIP1 or endogenous Snail1, repress a set of genes whose products function in cell–cell adhesion, which now also include cell–cell channel proteins like Cx26 and Cx43.

Dye-coupling studies in the developing mouse embryo gave rise to the concept of communication compartments, in which the early mouse embryo and its extra-embryonal tissues are divided into many discrete regions that display intraregional, but no interregional dye coupling (Kalimi and Lo, 1988, 1989). This would enable the exchange of differentiation factors, and eventually determining cell fate, between the cells of one tissue type and excluding the neighboring type. It was established that the visceral extra-embryonic ectoderm expresses high levels of Cx43 (Dahl et al., 1996; Van der Heyden et al., 2000), while no Cx43 is found in the parietal endoderm (Van der Heyden et al., 2000). Differentiation of F9 EC cells represents the first embryonic EMT, i.e., the transition of visceral (or primitive) extra-embryonic to parietal endoderm, in development. The observed Cx43 down-regulation (Fig. 1) may separate the communication capacity between these two tissue types.

With respect to ZO-1 expression in relation to Snail1, our data confirmed the study of Ikenouchi et al. (2003) in which a redistribution, but no down-regulation of ZO-1, was observed in response to Snail1 expression. A
Fig. 6 Snail1 silencing in MES-1 cells induces a mesenchyme–epithelium transition, connexin43 (Cx43) up-regulation and enhanced functional gap-junctional coupling. (A) Western blot analysis of COS-7 cells co-transfected with HA-tagged Snail1 with empty pTER+ and three different Snail1 silencing siRNA expressing pTER+ constructs. Snail1 expression is detected by anti-HA-tag antibody. Control is non-transfected COS-7 cells. Strongest silencing is observed with siSna3. (B) Phase-contrast images of native MES-1 cells and MES-1 clones expressing either empty pTER+ (clone 5), siSna1 expressing pTER+ (clone 1) or siSna3 expressing pTER+ (clone 2). siSna3 expressing cells display an epithelium-like morphology. Scale bar = 20 μm. (C) Semi-quantitative reverse transcriptase polymerase chain reaction for Snail1 and Cx43 on MES-1 pools and selected clones expressing pTER+, siSna1, or siSna3. Snail1 expression is strongly decreased in siSna3 expressing pools and clones, while Cx43 is up-regulated. (D) Western blot analysis of Cx43, pan-cadherin, E-cadherin, and Zonula Occludens-1 (ZO-1) expression on same-cell pools/lines as in (C). (E) Localization of Cx43 and ZO-1 in clones expressing empty pTER+ (clone 5), siSna1 (clone 1), and siSna3 (clone 2). In siSna3 expressing cells, Cx43 is strongly expressed at the cell borders and co-localizes with ZO-1. pTER+ and siSna1 expressing cells display mainly intracellular Cx43 and ZO-1 expression. Scale bar = 20 μm. (F) Lucifer Yellow dye-coupling quantification in MES-1 cell clones expressing pTER+, siSna1 or siSna3. Increased coupling is found in siSna3 clone 2. *p < 0.05 with respect to pTER+ cl 5 and siSna1 cl 1 (ANOVA Holms–Sidak test). (G) Electrical coupling of MES-1 cell clones expressing either empty pTER+ (clone 5) or siSna3 (clone 2). *p < 0.05 (Student’s t-test).
direct interaction of Cx43 and ZO-1 has been observed by several researchers (for a review, see Giepmans, 2004). Our data suggest that the existence of such an interaction strongly depends upon the cell–cell junctional localization of ZO-1. While Snail1-mediated redistribution of ZO-1 (Ikenouchi et al., 2003) seems to depend primarily on the decrease of tight junction proteins claudins and occludins, down-regulation of Cx43 may be a primary effect of Snail1-dependent transcriptional repression.

The role of cadherins with respect to Cx43 expression and localization is still enigmatic. Cx43-mediated gap junctional communication in mouse epidermal cell lines correlates with E-cadherin, but not with N-cadherin expression, and gap-junctional communication (and epithelial morphology) could be restored in E-cadherin negative cell lines by ectopic E-cadherin expression (Jongen et al., 1991). On the other hand, E-cadherin-negative mouse blastocysts display normal Cx43 distribution in the trophectoderm (Ohsugi et al., 1997). Furthermore, gap-junctional coupling in L cells was inhibited by E-, P-, or N-cadherin overexpression, while in the same study coupling was increased by N-cadherin expression in hepatoma cells (Wang and Rose, 1997). It became clear that in regenerating hepatocytes, cadherin-based cell–cell adherence junctions act as foci for gap junctional assembly, although similar foci were observed at sites of tight junction formation (Fujimoto et al., 1997). Apparently, the relationship between cadherin mediated cell–cell adhesion and gap-junctional communication may be very differentially dependent on the cell type involved. In our study, we see no relationship between E-cadherin and Cx43 expression in F9 EC, PrE, and PE cells (Fig. 1C) or P19s18 and its derivatives (Fig. 2C), while in the latter cell types, some correlation in expression levels was observed when using a Pan-cadherin antibody (Fig. 2C). Also, in MES-1 cells expressing sSiSnal and sSiSnal3, a positive correlation is found between Pan-cadherin and Cx43 staining, while a correlation with E-cadherin was less clear. Our observation that Cx43 is repressed by Snail1 and the fact that E-cadherin, and perhaps other cadherins too, form a direct target for Snail1 may explain these discrepancies. Depending on the relative role of Snail1 and other transcriptional regulators of both Cx43 and E-cadherin in a given cell type, different or even opposite effects may be expected.

Although speculative, the Snail1-associated Cx43 down-regulation as observed in F9 and P19 EC cells might occur as a general phenomenon in many EMTs during differentiation, organogenesis, and tissue repair mechanisms.

Acknowledgments Christine Mummery is thanked for the P19s18 EC, END-2, MES-1, and EPI-7 cells and for critically reading the manuscript. We thank Bas Defize for sharing Snail1 expression and E-cadherin reporter constructs and for his valuable advice.

This study is supported by the Technology Foundation (STW program DPTE, Grant #MKG5942, MvdH and Grant UGT.6746, TvV), the Netherlands Heart Foundation (Grant 2003B07304, TdB, BK), the FP6 (Framework Program LSBB-CT-2004-502988) of the European Committee (BK), and the Netherlands Organization for Scientific Research (NWO, Grant 916.36.012, TvV).

References


