The Tumor-Suppressive Function of Connexin43 in Keratinocytes Is Mediated in Part via Interaction with Caveolin-1

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Abstract

Connexin43 (Cx43) is known to have tumor-suppressive effects, but the underlying mechanisms are still poorly understood. In keratinocytes, we previously showed that the COOH-terminal domain of Cx43 directly interacts with the tumor suppressor Cav-1. We now show that rat epidermal keratinocytes (REK) that are deficient in Cx43 present features of epithelial-to-mesenchymal transition and are more invasive than their control counterparts, whereas overexpression of Cx43 inhibited the 12-O-tetradecanoyl-phorbol-13-acetate (TPA)– and epidermal growth factor (EGF)–induced invasive properties. Carbenoxolone did not alter the inhibitory effect of Cx43 against TPA- and EGF-induced cell invasion, indicating the involvement of a gap junctional intercellular communication–independent mechanism. Interestingly, the association of Cx43 with Cav-1 was found to be reduced after TPA and EGF treatment. Accordingly, the colocalization of Cx43 with Cav-1 was diminished in cells from a human epidermal squamous cell carcinoma, as well as in sections from human keratinocyte tumors, suggesting that Cx43/Cav-1 interaction plays a protective role against keratinocyte transformation. As opposed to cells that overexpress Cx43-GFP, invasion could be induced in rat epidermal keratinocytes that overexpress a GFP-tagged truncated mutant of Cx43 (Δ244-GFP) that we previously showed not to interact with Cav-1, as well as in cells that overexpressed Cx43-GFP but were reduced in Cav-1. Our data show that Cx43 possesses tumor-suppressive properties in keratinocytes and provide the first evidence that the Cx43/Cav-1 interaction is altered in keratinocyte transformation processes, as well as in human keratinocyte tumors, and that this association might play a role in Cx43-mediated tumor suppression.

Introduction

Gap junctions are specialized intercellular membrane channels that allow direct transfer of small molecules to selectively pass from one cell to another (1). Consequently, gap junctional intercellular communication (GJIC) plays a critical role in the coordination of development, tissue function, and cell homeostasis (2). Gap junction channels are composed of trans-membrane proteins, called connexins (Cx; ref. 2). Among the 21 Cx members found in human, Cx43 is the most abundant gap junction protein in a wide spectrum of tissues, including the epidermis (3).

In cancer, loss of GJIC has been shown to facilitate tumorigenesis and enable the autonomous cell behavior associated with transformed cells (4). Many tumor lines show deficient or aberrant GJIC and/or loss of Cx expression, whereas restoration of Cx in cancer cells could reduce their neoplastic potential (4). Decreased Cx expression has been observed in human neoplasia of various organs (4, 5). Additionally, genetically engineered mice lacking Cx32 exhibit increased susceptibility to radiation and chemically induced liver and/or lung tumorigenesis (6–8). These studies strongly suggest that gap junctions serve a tumor suppressor role and whereas Cx43 has long been viewed as a tumor suppressor, at least in some tissues and microenvironments, the underlying mechanisms are still largely unknown.

We and others have reported that the tumor-suppressive effects of Cx could be GJIC-independent (9–14). One proposed mechanism by which Cx may act as a tumor suppressor in a GJIC-independent manner is through binding to proteins that regulate carcinogenesis. We have recently shown by coimmunoprecipitation and colocalization experiments that caveolin-1 (Cav-1) interacts with Cx43 in rat epidermal keratinocytes (REK) and that these molecules colocalize in human epidermis (15). Our Far Western analysis and utilization of Cx43 mutants revealed that this association is direct and occurs through the COOH-terminal domain of Cx43 (15). Interestingly, Cav-1 has been identified as a skin tumor suppressor in vivo (16). In this study, we show that
Cx43 possesses tumor-suppressive functions in keratinocytes through a process that is GJIC-independent and involves its COOH-terminal domain. Our data provide the first evidence that the Cx43/Cav-1 interaction is dysregulated in keratinocyte transformation processes in vitro, as well as their co-segregation profile in human skin cancer cell lines and tumor biopsies. Together, these results suggest that the tumor-suppressive properties of Cx43 involve its association with Cav-1.

**Materials and Methods**

**Cell culture.** REKs (17), as well as the cells derived from normal human skin (Hs 456.Sk) and human epidermal squamous cell carcinoma (SCC-13) were cultured in DMEM containing 10% fetal bovine serum (FBS; Invitrogen Corporation). Hs 456.Sk cells were obtained from American Type Culture Collection, whereas SCC-13 cells were a kind gift from Dr. M. Denning (Loyola University, Chicago, IL).

**shRNA constructs and retroviral infections.** A Cx43-targeted shRNA construct (GGTGTGGCTGTCAGTACTT), which was typically the most efficient at reducing Cx43 level in REKs, together with a control vector containing a nonsense sequence, was used to make viral supernatants and infect REKs (18). Generation of stable REK cell lines overexpressing the human Cx43-GFP or the Δ244-GFP Cx43 mutant was previously described (15, 19). Stable REK cell lines expressing Cx43-GFP and reduced in Cav-1, together with cells that overexpress Cx43-GFP and a control vector containing a nonsense sequence, were generated by retroviral infection (15).

**Human tissue samples.** Tissue collection was performed at the Vancouver General Hospital Skin Care Center, University of British Columbia, in accordance with the ethical principles set forth in the Declaration of Helsinki and as instituted at the University of British Columbia, Vancouver, British Columbia, Canada. Tumor samples were collected during the resection of a cutaneous malignancy and, during the reconsolidation of epithelial-to-mesenchymal transition. Cx43-reduced REKs were seeded at 2 × 10^5 cells in six-well plates with or without coverslips. After 24 hours, cells were treated with either a combination of transforming growth factor β1 (TGF-β1; 3 ng/mL; R&D Systems) and 12-O-tetradecanoylphorbol-13-acetate (TPA; 100 ng/mL; Sigma; ref. 20).

**TPA, epidermal growth factor, and carbenoxolone treatments.** Cells were starved overnight in DMEM containing 0.5% FBS and then treated for 24 hours with 20 ng/mL of TPA in DMEM supplemented with 10% FBS, or with 10 ng/mL of human recombinant epidermal growth factor (EGF; Clonetics) in DMEM supplemented with 0.5% FBS. For some experiments, cells were treated with 30 μmol/L of carbenoxolone for 24 hours.

**Western blotting.** Cell lysates were obtained as previously described (21). After separation by SDS-PAGE, proteins were transferred to nitrocellulose membranes and immunoblotted with primary antibodies [1:1,000; vimentin (mAB3400, Millipore), E-cadherin (C20820, BD Transduction Laboratories), Cav-1 (clone pAb, BD Transduction Laboratories), Cx43 (C6219, Sigma), or mouse anti-Cx43NT; ref. 22]. Infrared fluorescent-labeled secondary antibodies [1:5,000, IRDye 800 (Rockland Immunochemicals) or Alexa 680 (Molecular Probes) anti-rabbit or anti-mouse] were used, and immunoblots were quantified using the Odyssey Infrared-Imaging System (Lincor). The membranes were reprobed for β-actin (1:5,000, AC-15; Sigma) for normalizing protein loading. The relative intensities of the bands represent the average ± SD of at least three independent Western blots.

**Immunofluorescence.** Cells were fixed with 3.7% formaldehyde, permeabilized with 0.1% saponin (23), and labeled for Cav-1 (1:100). For immunolocalization of vimentin and E-cadherin, cells were labeled with antivimentin (1:100, MAB3400; Millipore) or anti-E-cadherin (1:100, C20820; BD Transduction Laboratories) Alexa Fluor488–conjugated (1:500; Invitrogen) and Texas red–conjugated anti-mouse IgG (1:200; Jackson Immunoresearch) were used as secondary antibodies.

**Human skin and tumor sections (5 μm thick)** were deparaffinized in xylene and rehydrated in graded alcohols. Antigen retrieval and colabeling of Cx43 and Cav-1 were performed (15). In all cases, Hoechst 33342 (Molecular Probes) was used as a nuclear stain, and labeling was visualized with a Zeiss LSM 510 META inverted confocal microscope. Where expression level comparisons were made by densitometry between samples, image acquisition was performed in sequence with the microscope settings kept constant. Quantification was performed using the Zen LSM Image Analysis software (Zeiss). Positive labeling in normal epidermis was used to optimize the identification of cutoffs for positive labeling in the absence of saturation as well as background. The analyzed area in normal samples represents the epidermis. In SCC samples, it represents the core of the tumor, and in basal cell carcinomas (BCC), it represents either the tumor core ("BCC core") or the peripheral epidermis adjacent to the tumor ("BCC periphery"). Results are presented as the average of relative densitometric units ± SD of between 8 and 15 different human samples.

**Cell invasion.** Cells (2 × 10^5) were plated in serum-free DMEM on top of 8.0 μm pore size Transwell (BD Biosciences) filters previously coated with Matrigel (BD Transduction Laboratories) diluted 1:6 in serum-free DMEM. DMEM containing 10% FBS with or without 20 ng/mL of TPA or 0.5% FBS with or without 10 ng/mL of EGF was added in the lower chambers. After 24 hours, cells on the bottom of the filters were fixed, stained with Hoechst 33342, and counted in 10 microscope fields using OpenLab software (Improvement). Results are presented as the average number of cells that invaded and represent the average ± SD of at least four independent assays.

**Cell proliferation.** Incubation with bromodeoxyuridine (BrdUrd), labeling of BrdUrd-positive nuclei, imaging, and calculation of the proliferation index were done as previously described (24).
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Results

Cx43-reduced REKs present epithelial-to-mesenchymal transition features. The process of epithelial-to-mesenchymal transition (EMT), in which epithelial cells lose their characteristic features and acquire a mesenchymal phenotype, is a fundamental process of cancer and involves a delocalization of the cell membrane protein E-cadherin and the induction of vimentin (20). Although TGF-β1 treatment with either TPA or EGF has been reported to trigger EMT in the human keratinocyte cell line HaCaT (20), the combination of TGF-β1 with TPA (Fig. 1A, left) or EGF (data not shown) did not alter vimentin or E-cadherin levels in REKs. However, we observed that Cx43-reduced REKs, which display ∼50% decrease in Cx43 (Fig. 1A) with little evidence for gap junction plaques (21), exhibited EMT features as defined by significantly more cells expressing vimentin compared with controls (Fig. 1A and B, red). Vimentin expression was not further enhanced by treating Cx43-reduced cells with TGF-β1 and TPA (Fig. 1A, left). Although E-cadherin levels were not affected by the reduction of Cx43 expression (Fig. 1A), its localization to the plasma membrane (Fig. 1C, green, arrows) was less evident with E-cadherin found in intracellular compartments (Fig. 1C, arrowheads). These data indicate that the reduction of Cx43 induces EMT characteristics in REKs.

Reduction of Cx43 levels augments, whereas overexpression of Cx43-GFP inhibits, TPA- and EGF-induced cell invasion. To assess whether Cx43 has tumor-suppressive functions in keratinocytes, we used Cx43-reduced REKs and REKs that overexpress Cx43-GFP and submitted them to TPA- or EGF-induced invasion assays. TPA and EGF stimulated the invasion of wild-type and control REKs (Fig. 2, left), which was further enhanced in Cx43-reduced cells (Fig. 2A and B, left). Fittingly, TPA- and EGF-induced invasion were inhibited in REKs that overexpressed Cx43-GFP (Fig. 2C and D, left) indicating that an increased level of Cx43 was protective against processes associated with cell transformation.

To verify that the effects observed upon invasion were not due to a modulation of REKs proliferation, BrdUrd incorporation was measured under the same conditions. EGF had no effect on proliferation (Fig. 2B and D, right), although it was slightly reduced by TPA (Fig. 2A and C, right). Cx43-reduced REKs proliferated less than their control counterparts in the presence of TPA (Fig. 2A, right), whereas there was no diminution of cell proliferation in Cx43-GFP overexpressing cells (Fig. 2C, right). Reduction or overexpression of Cx43 did not affect cell proliferation in the presence of EGF (Fig. 2B and D, right). These data indicate that the effects of modulating Cx43 expression on REKs invasion are not due to proliferation. Together with our results showing that Cx43-reduced REKs present EMT features, our invasion data indicate that Cx43 possesses functions associated with tumor suppression in keratinocytes.

Cav-1 was diminished in TPA- or EGF-treated REKs (Fig. 4C) did not alter the protective effect of Cx43 overexpression against TPA- and EGF-induced invasion (Fig. 3D). Altogether, these data reveal that the inhibitory effect of Cx43 against REK invasion is GJIC-independent.

TPA and EGF treatment reduce the Cx43/Cav-1 association in REKs. One proposed mechanism by which Cx may act as a tumor suppressor in a GJIC-independent manner is through binding to proteins that regulate tumorigenesis. Because we previously showed that the skin tumor suppressor Cav-1 (16) interacts with Cx43 in keratinocytes (15), we now wanted to determine whether this association was altered by TPA and EGF.

As shown in Fig. 4, whereas Cx43-GFP (green) is present at the plasma membrane (arrows) and the intracellular compartment (arrowheads) of REKs, its colocalization with Cav-1 (red) was primarily observed in the perinuclear region (Fig. 4A and B, left, yellow, arrowheads; ref. 15). In accordance with published data (26–29), TPA induced Cx43-GFP internalization, as the majority of Cx43-GFP was found in intracellular compartments (Fig. 4A, arrowheads) previously identified as early and late endosomes, as well as lysosomes (30). On the other hand, Cav-1 was largely redistributed to the plasma membrane (Fig. 4A, arrows). In EGF-treated REKs, which presented an elongated morphology (Fig. 4B), Cx43-GFP was mostly found in intracellular punctate structures (Fig. 4B, double arrows), such as early and late endosomes, and lysosomes (30). Cav-1 was also mainly detected in intracellular punctuate structures of EGF-treated REKs (Fig. 4B). Overlay images indicate that the majority of Cx43/Cav-1 colocalization was lost in TPA- or EGF-treated cells, but could still be observed in the intracellular compartment of a few cells (Fig. 4A and B, yellow, arrowheads).

In parallel studies, the coimmunoprecipitation of Cx43 with Cav-1 was diminished in TPA- and EGF-treated REKs (Fig. 4C). Although the total expression of Cx43 and Cav-1 remained unaltered (Fig. 4D), TPA and EGF transiently stimulated Cx43

(mAb clone 2297, BD Transduction Laboratories; ref. 15). Results are presented as a ratio of the intensity of Cx43 that was communoprecipitated to the intensity of Cav-1 immunoprecipitated, and represent the ratio ± SD of at least three independent experiments.

Dye transfer. In the case of wild-type REKs, REKs expressing the empty vector (V) or REKs overexpressing Cx43-GFP, one cell within a confluent cluster of cells was pressure-microinjected with 1% Lucifer yellow (Molecular Probes; ref. 15). At least 29 microinjections were performed for each experimental condition.

Statistics. All statistical data were analyzed using two-tailed Student’s t test, except for the data comparing Cx43 and Cav-1 levels and their colocalization in human skin samples, which were analyzed using one-way ANOVA followed by Tukey’s test for significance.
phosphorylation (31, 32). Together, these data revealed that Cx43/Cav-1 association was reduced after treatment with two inducers of transformation, TPA and EGF.

**Cx43 and Cav-1 expression, as well as their colocalization, are reduced in human skin keratinocyte tumors.** Our next goal was to confirm that the diminution of Cx43/Cav-1 interaction seen in transformation-associated processes in REKs is also observed in human skin cancer. Similar to REKs, Cx43 (green) and Cav-1 (red) were found colocalized in a perinuclear pattern in cells derived from normal human skin (Hs 456.Sk; Fig. 5A). On the other hand, Cx43 and Cav-1 were dispersed within the cytoplasm of cells from human epidermal SCC-13 (Fig. 5A). Cav-1 levels were particularly low compared with cells from normal skin and its colocalization with Cx43 was not detected (Fig. 5A). To further investigate the relationship between these two proteins, we assessed their localization and expression in sections from human keratinocyte tumors (BCC and SCC). As we previously reported (15), Cx43 (green) and Cav-1 (red) were detected, and partially colocalized, in the vital layers of human epidermis (Fig. 5B; yellow, arrowheads). As compared with keratinocytes from normal skin, Cx43 and Cav-1 levels were diminished in both BCCs and SCCs (Fig. 5B and C). Interestingly, we observed that the epidermis in the periphery (“P”) of the BCC cores (“C”) exhibited a higher level of Cx43 than normal skin (Fig. 5B and C), whereas Cav-1 expression was reduced in both areas (Fig. 5B and C). Importantly, the colocalization of Cx43 with Cav-1 was found to be significantly reduced.
in BCCs and SCCs (Fig. 5B and D). These data revealed that Cx43 and Cav-1 levels are diminished in human keratinocyte tumors, as well as their colocalization. Altogether, these data suggest that the Cx43/Cav-1 association is altered in human skin cancer.

**Overexpression of a truncated GFP-tagged Cx43 mutant (Δ244-GFP), or the reduction of Cav-1 in Cx43-GFP overexpressing cells, does not protect against induction of cell invasion.** Because the interaction of Cx43 with Cav-1 is altered in transformation-associated processes in vitro, a scenario likely to also occur in vivo, the next step was to determine whether Cav-1 was required for the tumor-suppressive functions of Cx43. We have previously shown that a GFP-tagged mutant of Cx43 (Δ244-GFP), truncated of its COOH-terminal tail, does not interact with Cav-1 (15). As shown in Fig. 6A (left), when expressed, this mutant was found in intracellular compartments (arrowheads) and at the plasma membrane (arrows), similar to Cx43-GFP and endogenous Cx43. Because the overexpressed Δ244-GFP Cx43 mutant traffics to the plasma membrane, allows dye transfer (data not shown), and does not affect endogenous Cx43 levels and/or phosphorylation (Fig. 6A), we tested whether the truncation of the COOH-terminal domain of Cx43 would alter its ability to regulate invasion. We found that the Δ244-GFP-overexpressing REKs are more invasive in the presence of 10% FBS, TPA, or EGF, as opposed to REKs that express wild-type Cx43-GFP (Fig. 6B). These results suggest that the interaction of the cytoplasmic COOH-terminal domain of Cx43 with binding partners might be involved in regulating the invasive properties of REKs.

**Figure 2.** Reduction of Cx43 level augments, whereas overexpression of Cx43-GFP inhibits, TPA- and EGF-induced cell invasion. TPA (A, left) and EGF (B, left) significantly increased the invasive properties of control REKs (Ctl shRNA), which is further enhanced in Cx43-reduced cells (n = 4; *; P < 0.05). Fittingly, overexpression of Cx43-GFP inhibits TPA- and EGF-induced cell invasion (C and D, left), as opposed to wild-type REKs (WT) or REKs that express the empty vector (V; n = 4; *; P < 0.05). TPA slightly reduced proliferation of control cells, which was further diminished in Cx43-reduced REKs (A, right, n = 4; *; P < 0.05) but not in cells overexpressing Cx43-GFP (C, right, n = 4; *; P < 0.05). EGF did not affect BrdUrd incorporation in these conditions (B and D, right, n = 3).
To specifically examine the role of Cav-1 in this process, we used REKs that overexpress Cx43-GFP, but that were reduced in Cav-1 by \( \sim 55\% \). As shown in Fig. 6C, Cx43-GFP localization as well as endogenous Cx43 level and/or phosphorylation were not affected by the diminution of Cav-1. There was no significant TPA-linked induction of cell invasion by REKs overexpressing Cx43-GFP, but cells were reduced in Cav-1 when compared with control cells (Fig. 6D, left). However, these cells were approximately twice more invasive then their control counterparts in the presence of EGF (Fig. 6D, right). Although the reduction of Cav-1 level alone might play a role in the induction of cell invasion, these results suggest cross-talk between Cav-1 and Cx43 in tumor suppression.

**Discussion**

Although Cx43 has been reported to act as a tumor suppressor in many cell lines, to the best of our knowledge, this is the first study demonstrating that Cx43 possesses tumor-suppressive function in keratinocytes. We and others have shown that the tumor-suppressive properties of Cx43 are GJIC-independent in some cells and systems, and suggested a possible involvement of binding to proteins that regulate carcinogenesis (9–14). However, no mechanism involving Cx43 binding partner has been identified to explain its tumor-suppressive function, which probably reflects the difficulty of identifying biological functions associated with protein-protein interactions. Thus, this work is the first to assign a biological function to a Cx43 interaction with such a binding partner. Indeed, we now provide evidence that the interaction of Cx43 with Cav-1 is disrupted in processes associated with keratinocyte transformation, and that this interaction might play a role in some of the tumor-suppressive properties of Cx43.

In this study, we used a Cx43- and Cav-1-positive, and non-invasive rat epidermal keratinocyte cell line that is phenotypically similar to basal keratinocytes in that they retain the ability to differentiate into organotypic epidermis (33). We subsequently used TPA and EGF to stimulate keratinocyte invasion to assess the effect of Cx43 overexpression or underexpression in a context where transformation is induced. Our data using these cell lines suggest that the level of Cx43 is important in the regulation of cell transformation. Indeed, REKs that are reduced in endogenous Cx43 present EMT features and are more invasive than their control counterparts, whereas overexpression of Cx43-GFP protects against the stimulation of cell invasion. Accordingly, we and others suggest that forced Cx43 expression has the potential to act as an inhibitor to tumor onset (11, 13, 34).

Although the transformation-protective effect of Cx43 could be assigned to enhanced GJIC, our results indicate that the mechanism at play in our model was GJIC-independent but dependent on the COOH-terminal tail of Cx43. Although this domain has been reported to be involved in the regulation of glioma invasion (35), the underlying mechanism is still unknown. The COOH-terminal tail of Cx43 is thought to be crucial in the control of gap junction function via phosphorylation-dependent control of gap junction assembly and gating, and
Figure 4. TPA and EGF reduce Cx43/Cav-1 association in REKs. REKs overexpressing Cx43-GFP (green) were treated with TPA (A) or EGF (B) and labeled with Cav-1 (red). Cx43-GFP was located at the cell surface (arrows) and in the perinuclear region indicative of the Golgi apparatus (arrowheads) of untreated cells, whereas Cav-1 was mainly perinuclear (arrowheads). Overlay images suggest that Cx43-GFP is colocalized (yellow, arrowheads) with Cav-1. TPA treatment resulted in a decrease of Cx43-GFP at the plasma membrane (arrows) and in an increase of intracellular Cx43-GFP (arrowheads). Intracellular Cav-1 could be detected in some cells (arrowhead), but many TPA-treated REKs showed Cav-1 at the plasma membrane (arrows). Following treatment with EGF, Cx43-GFP and Cav-1 were mainly detected as intracellular punctuate structures (double arrows). Cx43-GFP/Cav-1 colocalization (yellow, arrowhead) was not detected in the majority of TPA- and EGF-treated REKs (blue, nuclei; bar, 20 μm). In parallel experiments, the amount of Cx43 coimmunoprecipitated with Cav-1 was significantly reduced by TPA and EGF (C). Data are expressed as a ratio of the intensity of Cx43 that was coimmunoprecipitated (top gels) to the intensity of Cav-1 immunoprecipitated (bottom gels; n = 3–5, * P < 0.05). As shown in D, a transient phosphorylation of Cx43, as evidenced by the increase in the slower migrating P species and a decrease in the P0 species, was observed following the addition of TPA and EGF, whereas the overall expression levels of Cx43 and Cav-1 remained unchanged.
Figure 5. Cx43 and Cav-1 expression, as well as their colocalization, are reduced in human skin keratinocyte tumors. A, cells derived from human normal skin (Hs 456. Sk) or epidermal squamous cell carcinoma (SCC-13) were colabeled with Cx43 (green) and Cav-1 (red). In Hs 456. Sk, but not SCC-13 cells, Cx43 was found colocalized with Cav-1 in a perinuclear pattern (yellow, arrowheads). B, human epidermis, BCC or SCC biopsies were colabeled with Cx43 (green) and Cav-1 (red). In normal epidermis, Cx43 and Cav-1 were detected in the vital layers (between dashed and dotted lines) and absent in the cornified layer (between dashed lines). Cx43 was found partially colocalized with Cav-1 in the epidermal vital layers (yellow, arrowheads). In the BCC cores ("C", underneath the dotted lines) and SCCs, Cx43 and Cav-1 could be detected but their expression was lower than that found in normal epidermis (B and C; *, P < 0.05). Cx43 levels were higher than that found in normal keratinocytes in the epidermis in the periphery (P; above the dashed lines) of the BCC cores, whereas Cav-1 was lower (B and C; *, P < 0.05). Overlay images indicate that the colocalization of Cx43 with Cav-1 was reduced in keratinocyte tumors (D; **, P < 0.05). Blue, nuclei; bar, 50 μm.
Figure 6. Overexpression of the Δ244-GFP Cx43 mutant, as well as the reduction of Cav-1 in Cx43-GFP overexpressing cells, do not protect against induction of cell invasion. The Δ244-GFP Cx43 mutant was localized at the plasma membrane (arrows) and within the intracellular compartment (arrowheads), which is similar to Cx43-GFP and Cx43 endogenously expressed in wild-type (WT) REKs and REKs that express the empty vector (V; A). Overexpression of this mutant did not affect Cx43 expression or phosphorylation (A). Δ244-GFP–overexpressing REKs were more invasive in the presence of serum, TPA, and EGF when compared with REKs that overexpress Cx43-GFP (B). Cx43-GFP localization was not altered in Cav-1–reduced REKs, as Cx43-GFP was still found at the plasma membrane (C, arrows) and within intracellular compartments (C, arrowheads). REKs that overexpressed Cx43-GFP, but are reduced in Cav-1, were more invasive than the control cells in the presence of EGF, but not TPA (D, *, P < 0.05). Blue, nuclei; bar, 20 μm.
through its interaction with key regulatory binding partners (36, 37). Previously, we showed that the recombinant COOH-terminal domain of Cx43 interacts directly with Cav-1 and that the Δ244-GFP Cx43 mutant does not associate with this protein (15). Now we extend these findings to show that invasion could be induced in cells that overexpress the Δ244-GFP mutant and that the overexpression of Cx43-GFP no longer protects REKs against invasion when Cav-1 is reduced. Thus, we are the first to suggest the importance of the Cx43 COOH-terminal tail interaction with Cav-1 in cell transformation. The Δ244-GFP mutant did not alter endogenous Cx43 levels and/or phosphorylation status (Fig. 6A), nor did it inhibit GJIC in REKs (data not shown). However, it could intermix with endogenous Cx43 to reduce the overall ability of Cx43 oligomers to interact with Cav-1, promoting the increase in cell invasion seen when the truncated mutant was expressed in unstimulated cells, not unlike the dominant-negative action of other Cx43 mutants when coexpressed with endogenous Cx43 (38). All the evidence indicates that Cx43/Cav-1 interactions play a protective role in EGF-induced REK invasion. However, our findings showing that the overexpression of the Δ244-GFP mutant does not protect against TPA-induced invasion and that REKs that overexpress Cx43-GFP, but that are reduced in Cav-1, are not invasive in the presence of TPA suggest, as one would expect, that other molecules aside from Cav-1 are likely involved in the Cx43-linked regulation of carcinogenesis.

A large body of evidence has been accumulated supporting a role for Cav-1 as a negative regulator of cell transformation and tumorigenesis (16, 39–42). Results suggest that caveolins are important signaling modulators within the context of caveolae, in which Cav-1 binding would serve to inhibit downstream signaling events (16). Furthermore, a role for Cx43 in signal transduction, such as the regulation of TGF-β signaling (43, 44), well-known for its involvement in human cancer (45), has also been suggested. Interestingly, some proteins are known to bind both Cx43 and Cav-1, and to be present in caveolae. For example, a previous study using lens epithelial cells showed that activated PKCγ is recruited into Cav-1–containing lipid, which stimulated its interaction with Cx43 and Cav-1 and a possible reduction in Cx43 gap junction plaques (46). This raises the interesting possibility that as part of a multiprotein complex, Cav-1 might direct Cx43 into caveolae and allow its transient association with, or dissociation from, critical molecules that regulate carcinogenesis. Alternatively, Cav-1 may directly stimulate or inhibit Cx43 or Cx43-associated proteins and thus regulate signaling events downstream of Cx43. Conversely, the increase in Cx43 levels might also change the dynamic balance of interacting proteins by sequestering more Cav-1 from a site of action or from interacting with other proteins involved in carcinogenesis.

Although our current findings indicate that Cx43 protects against TPA- and EGF-induced invasion through a mechanism that is GJIC-independent, we cannot rule out the regulation of GJIC by Cav-1 (15) in other tumor-suppressive processes mediated by Cx43.

Although many groups have investigated the expression pattern of Cx43 in mouse models of skin cancer, studies on Cx expression performed on human biopsies are rare. In keeping with a previous report (47), we found that the Cx43 level was significantly reduced in human keratinocyte skin tumors. Our finding that Cx43 is increased in the epidermis surrounding the BCC core might reflect a compensation mechanism to protect the surrounding tissue not unlike the previously described effect of UV on Cx43 expression in human epidermis in vivo (48). In addition to Cx43, we also looked at Cav-1 levels in skin tumors, which have not been assessed until now. Our current findings indicate that Cav-1 expression is significantly reduced in both BCCs and SCCs, which is in accordance with a previous study showing that Cav-1 is greatly reduced or absent in the hyperproliferative basal cell layer of the skin of patients with chronic plaque psoriasis (49). In keeping with our in vitro data using REKs and human skin cell lines, our current findings reveal that the colocalization of Cx43 with Cav-1 is significantly dysregulated in human keratinocyte skin tumors. Collectively, these findings suggest a role for Cx43/Cav-1 interaction in processes inhibiting keratinocyte transformation in vivo.

In summary, we showed that Cx43 possesses tumor-suppressive functions in keratinocytes through a GJIC-independent mechanism that involves its COOH-terminal domain. Our data provide the first evidence that the interaction of Cx43 with Cav-1 is lost in keratinocyte transformation processes in vitro, as well as in human keratinocyte tumors in vivo. Collectively, our current findings suggest that Cx43 possesses tumor-suppressive functions, beyond its well-known role in regulating GJIC, that involve its association with Cav-1.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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