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Biochemical and Biophysical Research Communications 333 (2005) 174–179
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Nontransformed cells can normalize gap junctional communication with transformed cells

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Received 11 May 2005
Available online 31 May 2005

Abstract

We demonstrate that the Src kinase can augment gap junctional communication between cells derived from homozygous null Cx43 knockout mice. The total conductance between Src transformed cells was nearly twice that of nontransformed cells. In addition, the unitary conductance of the majority of single channel events between transformed cells was about 35% greater than that of nontransformed cells. Analysis showed that both nontransformed and transformed cells expressed at least two populations of channels, suggesting that Src increased junctional conductance by up-regulating one population and/or by increasing the unitary conductance of another population of channels. Interestingly, the conductance displayed by heterologous pairs of transformed and nontransformed cells resembled that of nontransformed cells. The majority of single channel events between heterologous pairs shifted back to lower conductances that were exhibited by nontransformed cells. Thus, nontransformed cells can effectively "normalize" the conductance of gap junction channels expressed by adjacent tumor cells.

Keywords: Gap junctions; Intercellular communication; Connexin; Src kinase; Cell junctions; Transformation; Heterologous; Growth control

Gap junctions form channels between adjacent cells exclusive of the extracellular space. Each gap junction channel is formed by 12 connexin subunits with each cell of a pair contributing six subunits. Connexins are an ancient protein family that has evolved into over 20 members in humans and mice [1,2].

Gap junction channels are permeable to a host of cytoplasmic solutes from monovalent cations and anions to second messengers and metabolites. The permeability of these molecules is governed by size and charge of a solute and the specific connexins comprising the gap junction channel [3–7]. This form of communication allows cells within a syncytium to function properly in a coordinated fashion [8–10].

At the organism level the critical role of connexins is evidenced by deleterious phenotypes in connexin knockout mice. For example, both Cx43 and Cx45 are required for proper development and function of the heart [11]. The importance of connexins to human health is further underscored by many diseases that are associated with mutations that affect connexin function or protein expression [1,12].

Evidence indicates that connexins play important roles in cell growth control. In particular, experiments have identified Cx43 as a tumor suppressor gene [13–15]. It is of pivotal interest that Cx43 can be phosphorylated by the Src tyrosine kinase. Moreover, this event

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0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.05.104

reduces intercellular communication as revealed by dye transfer mediated by Cx43 between transformed cells [16–18].

We have previously shown that Cx45 is expressed in nontransformed and Src transformed cells derived from Cx43 homozygous null knockout mice, while Cx43, Cx40, and Cx30 are not [2]. In this study, we have investigated the effects of the Src tyrosine kinase on gap junctional communication between these cells. We examined the coupling between homologous pairs of transformed or nontransformed cells, as well as between heterologous pairs consisting of transformed and nontransformed cells.

A unitary conductance of about 28 pS was most frequently observed between homologous pairs of nontransformed cells, with less frequent events at 38 pS. Homologous pairs of Src transformed cells displayed a total conductance greater than nontransformed cells, with an increased occurrence of the 38 pS channel population. Heterologous cell pairs of transformed and nontransformed cells displayed reduced junctional coupling relative to homologous pairs, and the frequencies of unitary conductance resembled those of nontransformed cells at 28 pS.

These data demonstrate that neoplastic transformation can increase gap junctional communication between cells while heterologous coupling to nontransformed neighbors was reduced. However, the data also indicate that normal cells can modulate the gap junctional communication of adjacent tumor cells, particularly at the level of unitary conductance. These results may be applicable to in vivo situations where tumor cells meet surrounding tissue during growth and invasion.

Materials and methods

Cell culture. Nontransformed and v-Src transformed cells from homozygous null connexin 43 knockout mice (KoA and KoASrc) were used as described [2]. For all analyses, cells were maintained in DMEM supplemented with 10% FBS at 37 °C in 100% humidity. Src transformed cells were identified in coculture by staining with DiI or DiD as described, while nontransformed cells were stained with cell tracker green (5-chloromethyl-fluorescein diacetate; Molecular Probes) as described [2].

Evaluation of gap junctional communication. Electrophysiological measurements were performed on cell pairs cultured for 1–3 days. A dual voltage-clamp method and whole-cell recording was used to to control the membrane potential of both cells and to measure currents [3]. During experiments, cells were bathed in a solution containing: 110 mM CsCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM Hepes (pH 7.4). The patch pipettes were filled with solution containing: 110 mM CsCl, 0.1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 3 mM EGTA, and 10 mM Hepes (pH 7.2). Data acquisition and analysis was performed with pClamp 8 software [19]. Curve fitting and statistical analysis were done with SigmaPlot and SigmaStat, respectively (Jandel Scientific). Results are presented as means ± SD.

Detection of connexin gene expression. Connexin mRNA expression was analyzed by RT-PCR as described [2]. Briefly, RNA was extracted from cells by the phenol–chloroform–isoamyl alcohol method, pretreated with DNase, and 200 ng was reverse transcribed in a thermal cycler (Perkin-Elmer, Norwalk, CT) using SuperScript II (Invitrogen #18064-014) and oligo(dT) primers according to manufacturer's instructions for 1 h at 42 °C in a 20 μl reaction. 0.2 μl of resulting cDNA was amplified with *Taq* polymerase and one set of oligonucleotide primers. Samples were denatured for 5 min at 95 °C and then amplified for 30 cycles at 94 °C for 45 s, 58 °C for 1 min, and 72 °C for 1 min. Two microliter aliquots from each PCR sample were then analyzed by agarose gel electrophoresis. Forward and reverse primer sequences were was follows: for Cx26 (5′-AGGAAGGTGCCACTGAGAAA, 5′-ACGAGACGCTTCCAGTTTGT), Cx30 (5′-GGTACCTTCTACTAATTAGCTTGG, 5′-AGGTGGTACCCATTGTAGAGGAAG), Cx32 (5′-CCATAAGTCAGGTGTAAAGGAGC, 5′-AGATAAGCTGCAGGGACCATAGG), Cx36 (5′-GTAGGGGAGACGGTGTACGA, 5′-TCGAAACACCACTTGGATGA), Cx37 (5′-CACACCCACCCTGATCTACC, 5′-ACCCCTACCACCAACATGAA), Cx40 (5′-CTGTCCCCACCCAGTCAACT, 5′-CCGTTTGTCACTATGGTAGC),Cx43 (5′-CCCCACTCTCACCTATGTCTC, 5′-ACTTTTGCCGCCTAGCTATCC), Cx45 (5′-TTCCAAGTCCACCCATTTTAT, 5′-ATCGTTCCTGAGCCATTCTGA), and GAPDH (5′-AATGCATCCTGCACCACCAA, 5′-GTAGCCATATTCATTGTCATA). Superscript II was omitted from some cDNA preparations to rule out false-positive reactions. RNA from brain tissue (cortex and cerebellum) isolated from an adult CD-1 mouse was used as a positive control.

Results

We have previously shown that nontransformed and Src transformed cells from Cx43 knockout mice express low levels of Cx45 [2]. We have verified this by RT-PCR with primers specific for Cx26, Cx30, Cx32, Cx36, Cx37, Cx40, Cx43, and Cx45. As shown in Fig. 1, both cell

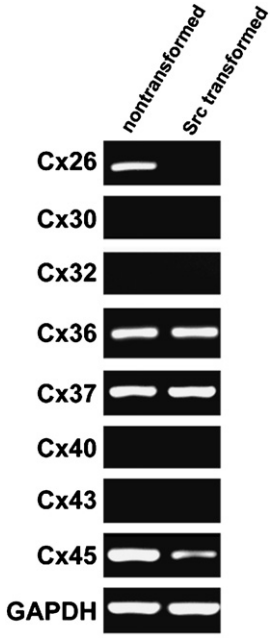

Fig. 1. Analysis of connexin mRNA. Expression of mRNA encoding several connexins was examined by RT-PCR. mRNA encoding Cx36, Cx37, and Cx45 was detected in both cell types, while Cx26 was only detected in nontransformed cells. Cx30, Cx32, Cx40, and Cx43 were not detected in either cell type.

Table 1
Total conductance between nontransformed and Src transformed cells

Cells	Pairs tested	Pairs coupled	Total conductance (pS)
Nontransformed	19	14	390 ± 70
Src transformed	10	9	710 ± 170
Heterologous	24	10	170 ± 50

Total conductance mediated by gap junctions channels between homologous and heterologous pairs of nontransformed and Src transformed cells. gj was calculated as the mean values obtained from coupled pairs (± SEM).

types expressed mRNA encoding Cx36, Cx37, and Cx45. Cx26 was expressed in nontransformed cells, but was not detected in Src transformed cells. Cx30, Cx32, Cx40, and Cx43 were not detected in either cell type.

As presented in Table 1, coupled pairs of nontransformed Cx43 cells displayed an average total conductance of 390 pS, while Src transformed cells displayed an average total conductance of 710 pS. This is an 82% increase and was statistically significant ($p < 0.001$). Heterologous pairs of these cells displayed an average total conductance of 170 pS. This is a 56% reduction relative to nontransformed cells and a 76% decline relative to Src transformed cells which was also statistically significant ($p < 0.001$).

The low coupling levels exhibited by these cells enabled unitary conductance measurements to be made without the aid of chemical uncouplers. The conductance values obtained from single channel recordings as shown in Figs. 3–5 were sampled in 2-pS bins and plotted as frequency histograms. Fig. 2 shows histograms for all three types of cell pairs investigated: nontransformed (upper panel, $n = 318$, 5 cell pairs), transformed (middle panel, $n = 695$, 9 cell pairs), and heterologous (lower panel, $n = 128$, 5 cell pairs). In all cases, the data were best fitted by four Gaussians using the analytical approach of Kullmann [20] with the following unitary conductances: nontransformed cell pairs: 28 ± 2, 42 ± 5, 54 ± 2, 70 ± 2 pS; transformed cell pairs: 28 ± 2, 38 ± 4, 52 ± 3, 73 ± 5 pS; and heterologous cell pairs: 28 ± 2, 36 ± 4, 50 ± 4, and 67 ± 4 pS.

The close resemblance of unitary conductances acquired from different cell pairs implied that the same channel types were present throughout. However, the channel relative frequency (f_{rel}) varied considerably. Nontransformed cells displayed a prominent unitary conductance of about 28 pS with f_{rel} of 0.73. In addition to these most frequent events, some relatively rare events ($f_{rel} = 0.18$) were seen at around 38 pS and 50 pS as indicated in the event histogram shown in Fig. 2, and current traces and all point histograms shown in Figs. 3A and B. The low frequency of channel openings in these records is indicative of Cx45 channel gating in other cell systems [3]. The unitary conductance of ~30 pS measured in this study using 110 mM CsCl is equivalent to

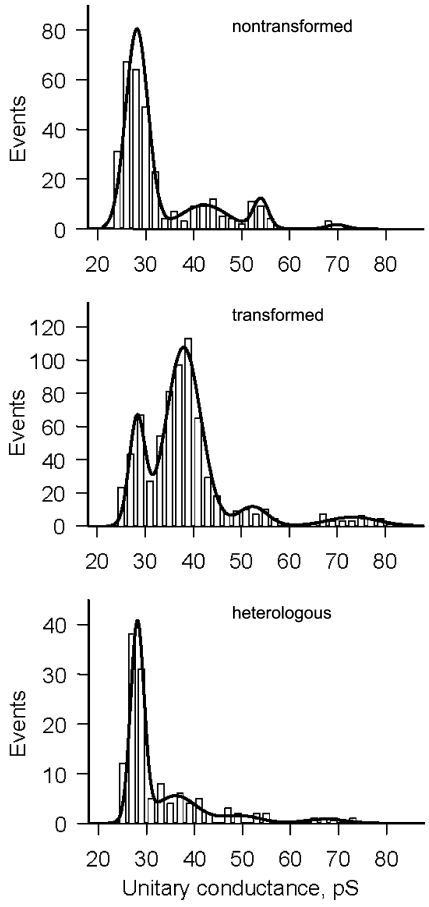

Fig. 2. Unitary conductances of gap junction channels. Histograms of single channel conductances between homologous pairs of nontransformed cells (top panel), homologous pairs of Src transformed cells (middle panel), or heterologous pairs of nontransformed and Src transformed cells (bottom panel). The smooth curves represent the fits of data to Gaussian distributions. For specific data see text.

the value reported by Van Veen et al. [21] where Cx45 single channel conductance was determined to be 39 pS using 135 mM CsCl.

Of particular interest is the heterotypic-like behavior occasionally observed in nontransformed cells. Fig. 3B shows a record of one operational single channel. Biphasic pulse of 70 mV applied to cell1 yielded polarity dependent currents. Unitary current (I_2) was smaller (outward current) when cell1 was hyperpolarized and increased (inward current) after inversion of voltage polarity by cell1 depolarization. The subsequent unitary channel conductances were 32 pS and 53 pS with a strong dependence on voltage polarity, a property of heterotypic channels [22,23].

Fig. 3C shows single channel I–V curves obtained using voltage ramp protocols. In the record shown on the right panel, the slope conductance was 28 pS and was insensitive to voltage polarity, indicative of homotypic channels. While in the other record shown on the left panel, slope conductance varies from 28 pS to 45 pS exhibiting voltage dependence. Again, this latter

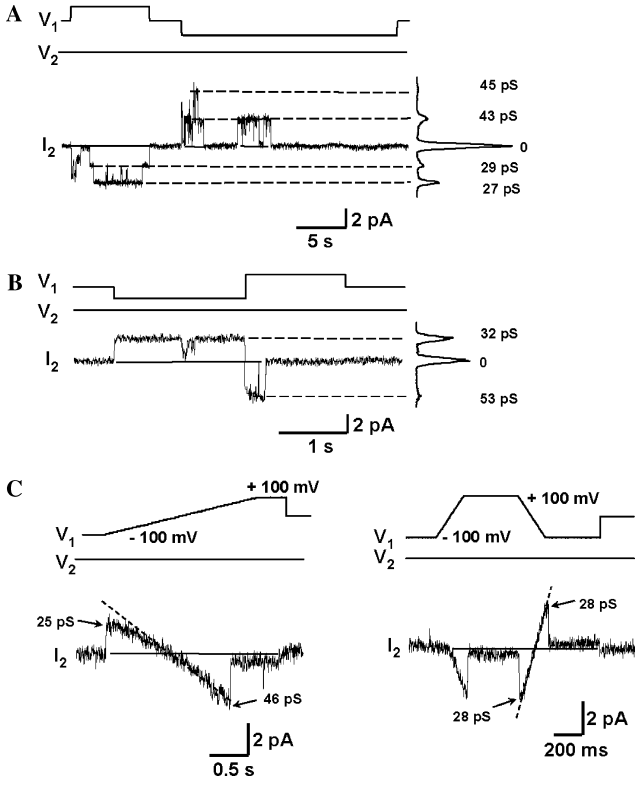

Fig. 3. Single channel properties of gap junctions between nontransformed cell pairs. (A,B) Bipolar pulse protocol (V_1 and V_2) and associated single channel currents (I_2) recorded at V_j of 70 mV. Solid line represents zero current level while dashed lines represent discrete current steps indicative of opening and closing channels. The current histograms indicate the operation of at least two different channels with conductances of 43–45 pS and 27–29 pS (A) and heterotypic-like channels with conductances of 32 pS and 52 pS for outward and inward currents, respectively (B). The left-hand side of (C): single channel currents elicited by voltage ramp protocols ($V_j = \pm 100$ mV) that show rectification (25 pS, outward current and 46 pS inward current). The right-hand side of (C): a voltage ramp evoking single channel currents with a slope conductance (dashed line) corresponding to a unitary conductance of 28 pS, which resembles homotypic Cx45 channels.

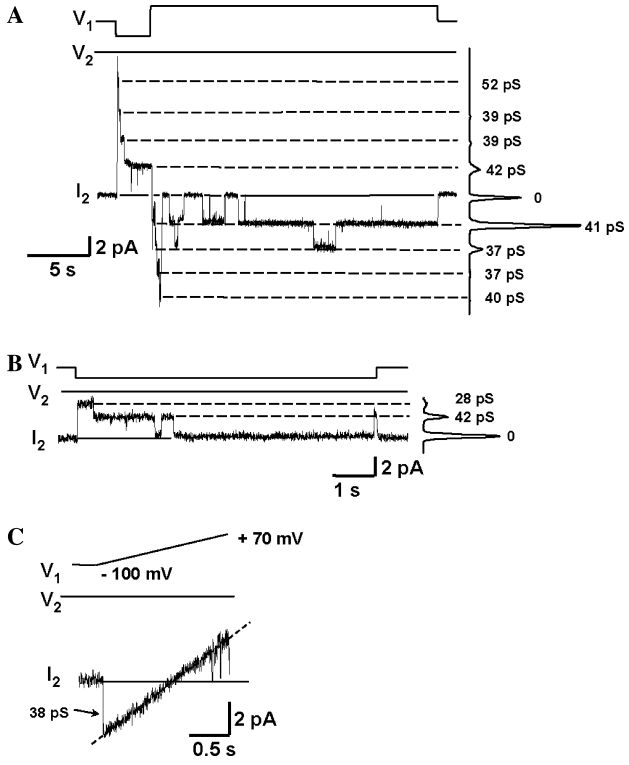

Fig. 4. Single channel properties of gap junctions between Src transformed cell pairs. (A) Multichannel recording during V_j of ±70 mV. Current histograms revealed similar conductances for positive and negative V_j in the 37–52 pS range. (B) Current recording during maintained V_j of 50 mV which illustrates the operation of two different channels with conductances of 28 pS and 42 pS, which may represent a homotypic Cx45 channel and channels formed by another endogenous connexin. (C) Voltage ramp from −100 to +70 mV that produced a linear single current with slope conductance events of 38 pS representing homotypic endogenous channel.

behavior is indicative of heterotypic channels but cannot be used as an unequivocal test because it has also been observed in homotypic channels. In homotypic channels, it is thought to arise from inside-out or plasma membrane voltage dependence [24–26].

In contrast to nontransformed cells, the most prominent unitary conductance between Src transformed cells shifted from 28 pS to 38 pS. In addition to the most common event of about 38 pS with f_{rel} of 0.7, Src transformed cells also exhibited events of ~28 pS ($f_{rel} = 0.19$), as well as events at about 50 pS ($f_{rel} = 0.06$) (Figs. 2 and 4). The event histograms illustrate the frequency of these events but they are not a direct measure of open probability. Both channel forms appear to have a low open probability indicative of the transformed cells shown in Fig. 3 and other Cx45 expressing cells [3].

Investigations of heterologous cell pairs of transformed and nontransformed cells revealed a unitary channel size of about 28 pS ($f_{rel} = 0.62$) typical of Cx45 along with fewer channel events of 38 to 50 pS (Figs. 2 and 5). Thus, coupling between these heterologous pairs more readily resembled nontransformed cells. In addition, as indicated in Table 1 the average junctional conductance was significantly less than Src transformed or nontransformed cells.

Discussion

The cells used in this study express Cx45 but not Cx43, Cx40, or Cx30 [2]. In addition to 28 pS unitary events arising from the activity of Cx45 in these cells, another unitary conductance of 38 pS was also observed. However, the frequency of occurrence (0.18 versus 0.70) for this channel type was significantly increased by transformation with the Src tyrosine kinase.

Our results indicate that the Src tyrosine kinase affects the activity of gap junction channels by either

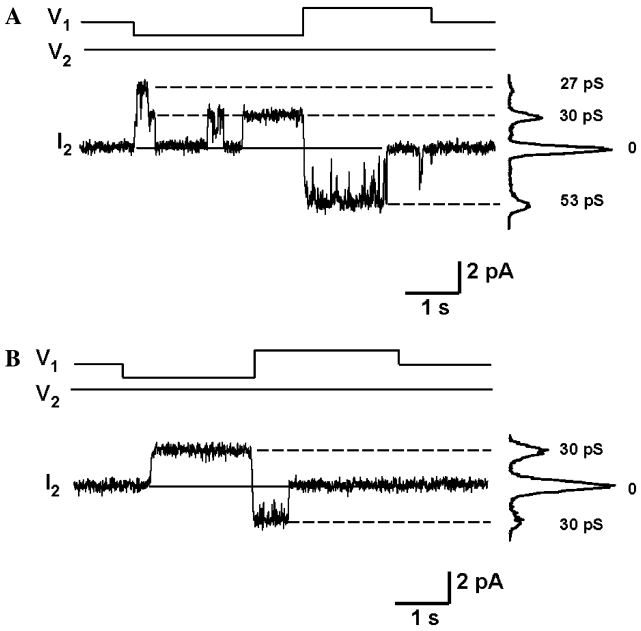

Fig. 5. Single channel properties of gap junctions between heterologous pairs of nontransformed and Src transformed cells. (A,B) Current recordings during biphasic V_j of ±70 mV that show the operation of nonhomotypic channels (A) and homotypic (Cx45 like) channels (B).

increasing the gating activity of a population of unidentified channel types (40–50) pS or by alteration of the unitary conductance of Cx45 gap junction channels. Indeed, protein kinases can modulate single channel conductance mediated by Cx43 [27–30]. However, this may not be the case with Cx45 as evidenced by van Veen et al. [21] where phosphorylation did not affect single channel conductance. This latter observation suggests that increased events induced by Src arise from a different channel type altogether.

In this study, Src may have modulated junctional conductance between transformed cells by elevating the expression and/or open probability of channels formed by another connexin. This view is consistent with our results from single channel analysis.

Possible expression of different connexins was investigated by RT-PCR with primers specific for Cx26, Cx30, Cx32, Cx36, Cx37, Cx40, Cx43, and Cx45 (Fig. 1). Nontransformed and transformed cells expressed mRNA encoding Cx36, Cx37, and Cx45. Interestingly, Cx26 was expressed in nontransformed cells, but seemed to be inhibited by Src in transformed cells. Cx36 channels have relatively small unitary conductances of about 10–15 pS [31,32]. Cx37 produces larger channels with a unitary conductance of about 340 pS [33], and Cx26 produces channels with a unitary conductance of about 100–130 pS [22,24]. However, single channel data in this study revealed (Fig. 2) that there were no single channel events recorded corresponding to connexins detected by RT-PCR except Cx45.

The majority of channels presented in nontransformed cell pairs showed a conductance of about 28 pS ($f_{rel} = 0.73$, see Figs. 2 and 3). The presence of channels with higher unitary conductances in the 40–50 pS range (Fig. 3) suggests the expression of a connexin other than Cx45. Src transformation increased the frequency of occurrence of these channels with a unitary conductance in the 40 pS range, suggesting that Src can increase the expression of the connexin that forms them (Fig. 4C). It has been reported that mouse brain also expresses Cx47, which produces channels with a unitary conductance of about 55 pS [34], which resembles conductances in the 40–50 pS range keeping in mind different pipette solutions used.

Data shown in Figs. 3B and C suggest the operation of homotypic and nonhomotypic channels. Evidently, channel formations involving Cx45 remain in Src transformed cells. Moreover, recordings between heterologous pairs of transformed and nontransformed cells revealed a majority of channels ($f_{rel} = 0.62$) to be homotypic Cx45 with a unitary conductance at 28 pS (Figs. 5A and B). However, a fraction ($f_{rel} = 0.34$) of channels displayed conductances in the 40–50 pS range, which may represent channels formed by another connexin that is induced by Src and/or heterotypic/heteromeric combination with Cx45.

Data summarized in Table 1 demonstrate that Src-transformation results in an increase in junctional conductance. In these experiments, the increase was 82%. Of particular interest are heterologous pairs between Src transformed and nontransformed cells which exhibited not only reduced junctional conductance (44% of nontransformed and 24% of transformed cell pairs), but also reduced incidence of coupling (42% of investigated pairs). The simplest explanation of this phenomenon could be that homotypic docking of Cx45 hemichannels occurs more readily than heterotypic or heteromeric docking with other connexins that may be expressed in Src transformed cells. Thus, more homotypic Cx45–Cx45 channels may be formed by these pairs than heterotypic or heteromeric channels (Fig. 2, lower panel).

A number of studies have suggested that gap junctional communication can play a significant role in the suppression of tumor cell growth by the coupling of transformed and nontransformed cells [35–39]. The macroscopic data of this study suggest that neoplastic transformation can augment homologous coupling between tumor cells, while reducing coupling between tumor cells with their nontransformed neighbors. This reduced heterologous coupling might help eliminate tumor suppressive effects thought to be mediated by nontransformed cells. Whether such a process is connexin specific, Cx45 or otherwise, is open to speculation.

It should be stressed that the cells examined here were transformed by the Src tyrosine kinase and expressed

Cx45. Some serine/threonine protein kinases can increase gap junctional communication by Cx45 without significantly altering unitary conductance between HeLa cells [21]. This finding is consistent with Src transformation not affecting Cx45 unitary conductance via phosphorylation but rather initiating the up-regulation of another connexin that forms channels with a unitary conductance of about 40pS. This study cannot rigorously distinguish between affecting the conductance of an existing channel population and the up-regulation of another, but it does illustrate the dynamic effects of connexins on cell communication and provides evidence for cellular modulation of gap junction mediated coupling between transformed and nontransformed cells.

Acknowledgments

This work was supported by grants from the United States National Institutes of Health CA88805 and EY014479 to G.S.G. and GM555263 to P.R.B., the Canadian Institutes of Health Research MOP-14358 to C.C.N., and the American Heart Association AHA0335236N to V.V.

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