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Review

Gap junction- and hemichannel-independent actions of connexins

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Abstract

Connexins have been known to be the protein building blocks of gap junctions and mediate cell–cell communication. In contrast to the conventional dogma, recent evidence suggests that in addition to forming gap junction channels, connexins possess gap junction-independent functions. One important gap junction-independent function for connexins is to serve as the major functional component for hemichannels, the un-apposed halves of gap junctions. Hemichannels, as independent functional units, play roles that are different from that of gap junctions in the cell. The other functions of connexins appear to be gap junction- and hemichannel-independent. Published studies implicate the latter functions of connexins in cell growth, differentiation, tumorigenicity, injury, and apoptosis, although the mechanistic aspects of these actions remain largely unknown. In this review, gap junction- and hemichannel-independent functions of connexins are summarized, and the molecular mechanisms underlying these connexin functions are speculated and discussed.

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Keywords: Gap junction- and hemichannel-independent; Connexin; Cell proliferation; Tumorigenicity; Cell differentiation

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1. Introduction

Gap junctions comprised of connexins form transmembrane channels connecting cytoplasm of adjacent cells, which allow ions, small metabolites, and second messengers to translocate from cell to cell (for review, see Refs. [1,2]). This type of intercellular communication is known to be

essential for various physiological and pathophysiological functions, such as cell growth, proliferation and differentiation, tissue homeostasis, tumorigenicity, wound healing, etc. Up to now, approximately 20 types of connexin molecules have been reported in the mouse and human [3]. Connexin mutations have been identified to be related to several diseases, such as X-linked Charcot–Marie–Tooth disease, congenital deafness and skin disorders, and congenital cataracts caused by mutations in Cx32, Cx26, Cx46 and 50, respectively. More than 160 mutations of Cx32 have been found [4], some of which lead to complete loss of functional

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channels, while others form functional channels with certain abnormalities in channel behavior. Recently, several reports revealed other unconventional functions of connexins. In addition to being a component of gap junctions, connexin molecules can form hemichannels, which are un-opposed halves of the gap junction channels. The actions of connexins that form hemichannels have been discussed in detail elsewhere (for review, see Ref. [5]). In this review, we focus on the roles of connexins that are gap junction- and hemichannel-independent.

A study indicating that connexins may have separate, non-gap junction-related functions was reported in 1995 [6]. Since then, the molecular mechanism of this novel, significant type of action by connexins has started being investigated and elucidated. Increasing evidence suggests gap junction-independent function of connexins in cell growth and proliferation, tumorigenicity, differentiation, injury, and apoptosis.

2. Gap junction-independent functions of connexins on cell growth and tumorigenicity

It has long been known that gap junction intercellular communication (GJIC) contributes to the maintenance of normal cell growth and that the rate of cell growth is inversely correlated with the extent of GJIC [7,8]. Disruption of communication often results in abnormal cell growth and tumors. Therefore, the role of gap junctions and connexins is suggested in tumor suppression [9–11]. Additionally, aberrant connexins are most common in tumor tissues, such as the reduction in connexin expression and/or aberrant localization of connexin. Despite some of the arguments as to whether GJIC is involved in the control of cell growth, there is unequivocal evidence to show that connexins can suppress cell growth both *in vitro* and *in vivo*.

There is increasing evidence for gap junction-independent roles of connexins in the control of cell growth and the suppression of tumorigenicity (for summary, see Table 1). Mesnil et al. [6] observed that among cells transfected with various connexin genes, there was no correlation between their GJIC and tumorigenicity. GJIC levels were significantly higher in tumors induced by injecting cells transfected with Cx26 in nude mice although all of the connexin genes (Cx43, Cx40, and Cx26) examined could establish GJIC in HeLa cells. The report by Huang et al. [12] shows that transfection of the Cx43 gene into human glioblastoma cells reversed the transformed phenotype of these tumor cells; however, the tumor suppression by Cx43 was unrelated to the activity of GJIC determined by Lucifer Yellow dye coupling. Moreover, tumor-suppressive effect of connexins is more connexin species-specific than their activity in cell coupling [12,13]. Two reports from Yamasaki's group further show that certain mutants of Cx26 and Cx43 exert dominant negative effects on cell growth and tumorigenicity, but such effects are independent of the actions on GJIC assessed by dye transfer of

Lucifer Yellow [14,15]. In one study, three mutated Cx26 genes (C60F, P87L and R143W) were expressed in HeLa cells that contained the wild-type Cx26 gene, and were GJIC-competent and non-tumorigenic. Interestingly, mutants, Cx26-P87L and Cx26-R143W, enhanced the tumorigenicity of the HeLa Cx26 cells in nude mice without any changes in GJIC. On the other hand, expression of the Cx26-C60F mutant reduced the GJIC level without affecting their growth *in vivo* [14]. In another study, two Cx43 mutants (L160M and A253V) were used to examine dominant-negative effects of Cx43 mutants on cell growth control exerted by wild-type Cx43 in C6 cells [15]. The mutant Cx43-L160M diminished *in vitro* growth-suppressive functions and GJIC capacity of wild-type Cx43 while it showed only weak dominant-negative effects on *in vivo* tumor-suppressive function. In contrast, the mutant Cx43-A253V presented a strong dominant-negative effect on both *in vitro* cell growth and *in vivo* tumor suppression exerted by wild-type Cx43 but not on GJIC. In the communication-proficient rat bladder carcinoma BC31 cell line, dominant negative mutants derived from the third transmembrane domain, Cx43-K162A, Cx43-E166E, and Cx43-T154Y, all blocked cell coupling; but only Cx43-T154Y substantially accelerated cell growth *in vivo* [16]. Interestingly, only cells transfected with the Cx43-T154Y mutant exhibited plasma membrane localization and allowed the transfer of the smaller molecules like neurobiotin, but not bigger molecules like Lucifer Yellow. The study by Moorby and Patel [17] provide direct evidence suggesting that growth regulation by Cx43 is independent of gap junction formation. They show that there was no correlation between the action of Cx43 mutants (S255A, S279A, and S282) on cell growth and Lucifer Yellow dye coupling in 3T3 A31 fibroblasts and that blockade of gap junction formation by either heptan-1-ol or culturing cells at low density had no effect on the ability of the Cx43 mutants to control cell growth. Moreover, wild-type Cx43 inhibited growth of Neuro2a cells independent of gap junction formation and the C-terminus of Cx43 that could not form gap junctions was as effective as the wild-type in suppressing the growth of Neuro2a cells. The second extracellular loop mutants of Cx43 (F199L, R202 E, and E205R) in another study were shown to inhibit cell growth despite their inability to form functional gap junction channels [18]. All these data suggest that certain mutant connexins exert dominant negative effects on connexin-regulated growth and that such effects are independent of the ability of GJIC.

Several studies show gap junction-dependent and -independent functions of connexins. Cx43 inhibited cardiomyocyte DNA synthesis irrespectively of cell–cell coupling; however, GJIC was required to reverse Cx43 inhibition of DNA synthesis by phosphorylation of Cx43 at S262, a phosphorylation site regulated by growth factor or protein kinase C [19]. In some cases, overexpression of connexins does not affect cell growth *in vitro*, but exerts the effect on tumor suppression. Bond et al. [13] found that Cx32-overexpressing C6 glioma cells did not have a

Table 1
Summary of the published studies of gap junction-independent functions of connexins in cell growth and tumorigenicity

References	Cell Systems	Connexin types	GJIC-independent functions in cell/tumor growth	GJIC	Connexin localization
Mesnil et al. [6]	HeLa cells	Cx26, Cx40, Cx43 (transfected)	Cx26—cell growth inhibition; Cx 40 and Cx43—no effect	GJIC for all three connexins	Cx40, Cx43—cell–cell contact; Cx26—cytosol
Huang et al. [12]	Human glioblastoma cells	Cx43 (transfected)	Inhibition on cell and tumor growth	No GJIC	Mainly in the nucleus and cytoplasm
Duflot-Dancer et al. [14]	HeLa cells expressing Cx26	Cx26 mutants: C60F, R87L, R143W (transfected)	P87L and R143W—increased tumorigenicity; C60F—no change	R87L and R143W—no change; C60F—reduced	Cell–cell contact area
Omori and Yamsaki [15]	C6 glioma cells expressing Cx43	Dominant negative Cx43 mutants: L160M and A253V	Both mutants inhibit the effect of wt Cx43 on suppression of tumor growth	A253V—no inhibition on GJIC; L160M—inhibition	No changes of distribution pattern
Krutovskikh et al. [16]	Rat bladder carcinoma BC31 cell	Dominant negative Cx43 mutants: K162A, E166G and T154Y	None of the mutants had effects on cell growth; T154Y accelerated tumor growth.	All three mutants blocked GJIC; T154Y—only transfer of neurobiotin	Wt and T154Y in the lateral membrane; K162A and E166G in the cytoplasm.
Moorby and Patel [17]	3T3 A31 fibroblasts expressing endogenous Cx43 and Neuro2a cells with no endogenous connexins	Cx43 mutants: S255A, S279A, S282A, C-terminal truncated (1–256) and C-terminal domain	In 3T3 cells, S279A and S282A grew slowly; S255A faster than control; mutants affected cell growth without cell contact and GJ; In Neuro2a cells, wt Cx43 and Cx43 C-terminus, but not Cx43(1–256), suppressed cell growth without GJ.	Cells expressing S255A, S279A, and S282A mutants were coupled similarly and prevent PDGF-induced loss of cell coupling in 3T3 cells.	N/A
Olbina and Eckhart [18]	HeLa and C6 glioma cells	Second extracellular mutants of Cx43: F199L, R202E, and E205R	Mutants had similar inhibition of cell growth as wt Cx43	Loss of GJIC by mutants	Wt—plasma membrane; Mutants—membrane and cytoplasm
Dang et al. [19]	Cardiomyocytes and HeLa cells	C-terminal domain of Cx43	Inhibition of cell growth	N/A	Cytoplasm and nucleus
Bond et al. [13]	C6 glioma cells	Cx32 (transfected)	Cell growth in vitro—no change; tumor growth—retarded	No correlation of the function with GJIC	N/A
Qin et al. [20]	MDA-MB-231	Cx43 and Cx26 (retroviral delivery)	Dramatic suppression of tumor growth	No substantial rescue of GJIC	Cytoplasm of cells and xenoplants

reduced growth rate *in vitro* but that tumor growth was retarded *in vivo*; however, GJIC was shown to be highly up-regulated. Retroviral delivery of Cx43 and Cx26 genes to MDA-MB-231 cells failed to exhibit significant differences in *in vitro* cell growth, but resulted in a dramatic suppression of tumor growth [20]. In this study, GJIC determined by Lucifer Yellow dye transfer was not substantially rescued and most of the Cx43 expressed was in cytoplasmic stores with no detectable gap junctions.

One possible argument for the above observations of gap junction-independent role of connexins is that most GJIC activities are analyzed by using mainly a single type of dye, such as Lucifer Yellow, which may not accurately reflect the ability of an intracellular substance to pass through gap junctions. However, there are several lines of evidence against the argument. First, Cx43-transfected cells that show tumor suppression predominantly express the unphosphorylated form of Cx43 [12], suggesting a lack of gap junction assembly on the cell surface [21]. Second, the connexins, which have the effect on cell growth inhibition and tumor suppression, localize in the cytoplasm or nucleus [12,20], excluding the possible involvement of gap junctions and hemichannels. Third, only the C-terminal domain of Cx43 displays similar or even more growth suppression as the wild-type Cx43 [17,19] and the C-terminus of Cx43 mainly localizes to the cytosol and nucleus [17,19]. These studies suggest that the expression of connexins *per se*, but not the extent of GJIC, corresponds to *in vitro* or *in vivo* growth control and suppression.

3. Gap junction-independent functions of connexins on cell differentiation

Recently, we and others have suggested the gap junction-independent effect of connexins on cell differentiation. The studies by Le and Musil [22] show that the inhibition of intercellular coupling with the chemical reagent, 18 β -glycyrrhetic acid (β -GA), had no effect on lens cell differentiation. Since β -GA is a blocker for gap junctions and hemichannels, this study implies the involvement of a GJIC- and hemichannel-independent process. However, it is not clear whether the process of lens cell differentiation requires connexin expression at all. In contrast to this observation, an earlier report shows that the inhibition of GJIC by Rous sarcoma virus blocked lens cell differentiation [23]. In these inhibitory studies, general channel inhibitors of gap junctions and hemichannels are used instead of specific reagents; thus, it is difficult to evaluate the inhibitory effect on the activities of individual connexins. In our study, primary cultures of chick lens, which display the similar process as *in vivo* epithelial-fiber differentiation, were used to explore the roles of connexins in lens cell differentiation [24]. Of the three lens connexins (Cx43, Cx45.6 and Cx56) overexpressed in these cultures, only Cx45.6 stimulated lens cell differentiation, but the other two did not. Moreover, over-

expression of Cx45.6 did not affect GJIC assessed by using two types of dye-transfer approaches and two different fluorescent dyes. Our study demonstrates a GJIC-independent effect of Cx45.6 on lens cell differentiation. In support of our observations, the expression levels of Cx45.6 orthologue also correspond to the degree of lens cell differentiation. Yang and Louis [25] show that in contrast to the level of Cx44, the ovine orthologue of Cx56, which remained low, the level of Cx49, the ovine orthologue of Cx45.6, was increased in association with the differentiation of lens primary culture. Recently, we show that the C-terminus of Cx45.6 is sufficient to promote lens cell differentiation to a similar extent as wild-type Cx45.6 [26]. This further reinforces the GJIC-independent role of Cx45.6 in lens cell differentiation.

4. Other GJIC-independent functions of connexins

Tumor cells expressing the herpes simplex virus type 1 thymidine kinase (HSV-tk) are killed by nucleoside analogues such as ganciclovir (GCV). GCV affects not only the cells expressing HSV-tk but also the neighboring cells that do not express the gene through a pathway known as the “bystander effect”. The “bystander effect” of tumor cells has been known to be mediated by GJIC. Cx43-mediated GJIC has been reported as a major mechanism for the transfer of GCV to neighboring cells [27–29]. Recently, a gap junction-independent mechanism has also been proposed. Human lung carcinoma cell lines [30], as well as a rat colon tumor line [31], exhibited a bystander effect that was not altered by gap junction inhibitors [30,31] or an enhancer [31]. However, these studies do not provide direct evidence if this process relies on the expression of connexins. Another report shows that a human colon tumor cell line was highly sensitive to HSV-TK/GCV bystander effect killing, even though these cells had both minimal gap junction dye transfer capability and diffuse Cx43 expression [32]. GCV metabolites have been suggested to be effluxed from cells and that some type of cellular uptake mechanism independent of gap junctions exists for nucleotide entry into neighboring cells [33]. However, the existence of connexins in these cells is not conclusive even though the expression of Cx43 is found to be minimal; the membrane components responsible for efflux/uptake cycle of GCV metabolites are also unknown. Recently, connexin-forming hemichannels are reported, which facilitate the transport of charged small molecules, such as ATP, NAD⁺, and PGE₂ [34–39]. The connexin-forming hemichannels may offer an alternative pathway for efflux/uptake of GCV metabolites.

A recent report shows that connexins mediate gap junction-independent resistance to astrocyte injury [40]. In this report, they found that connexin-expressing cells retained their resistance in cell cultures with few cell contacts. The expression of two connexin mutants that failed to establish functional GJIC efficiently protected the cells against injury. Moreover, pharmacological inhibition of

gap junction channels using α -GA only insignificantly reduced the injury resistance of connexin-expressing cells. Although they did not directly examine the involvement of connexin-forming hemichannels, the fact that α -GA can block both gap junctions and hemichannels excludes the functional participation of hemichannels. Gene array analysis showed that 10 genes were regulated by both gap junction forming- and non-forming-Cx26, including the genes related to angiogenesis [41]. This study suggests that Cx26 regulates angiogenesis-related molecules by mechanisms that are both GJIC-dependent and -independent.

5. Molecular mechanism of gap junction- and hemichannel-independent actions of connexins

The mechanistic aspects of the gap junction- and hemichannel-independent functions of connexins remain largely unknown. One of the important questions remains as to how connexin expression, in the absence of substantial increases in GJIC and hemichannels, induces a reduction in cell and tumor growth. Several possible mechanisms have been hypothesized. First, the carboxyl terminus of Cx43 can regulate cell growth, implying possible interactions with other proteins. Transcription factors are possible interactive candidates. The connexins that exhibit cell growth suppression function have been localized to the cytoplasm or nucleus [17,19]. Targeting of Cx43 to the nucleus is plausible, given the putative nuclear-targeting sequence encoded within the carboxyl terminus [17]. Indeed, overexpression of connexins appears to regulate gene expression. Overexpression of Cx43 or Cx26 in MDA-MB-231 cells resulted in down-regulation of fibroblast growth factor receptor (FGFR)-3, a member of the FGFR family consisting of potent angiogenic factors that promote tumor growth and invasion. [20]. Overexpression of Cx43 also suppressed the expression of milk fat globule epidermal growth factor 8 (MFG-E8) [42], a growth factor involved in cell anchorage and integrin signaling, and the expression of monocyte chemotactic protein, MCP-1 [43]. Second, the suppression of cell and tumor growth by connexins could be through the regulation of proteins responsible for cell cycle progression. Zhang et al. [44] have reported that overexpression of Cx43 is attributed to an accumulation of the hypophosphated retinoblastoma (Rb) protein and a marked increase in the level of cyclin-dependent kinase inhibitor p27, although the gap junction-dependent and the gap junction-independent mechanisms contributed to the increase of p27. This group also reported that Cx43 inhibited expression of S phase kinase-associated protein 2 (Skp2), the human F-box protein that regulates the ubiquitination of p27 [45]. This inhibition was independent of GJIC due to the fact that gap junction inhibitors did not affect the inhibitory effect and that the C-terminus domain that did not form the gap junction channels exhibited nearly the same inhibitory effects as that of the full-length Cx43 [46]. Both Skp2 and p27 were required for Cx43 to inhibit cell

proliferation, in that Cx43 barely inhibited cell proliferation of the SKP2 $-/-$ and p27 $-/-$ cells [45]. They postulated that cAMP was involved in the gap junction-dependent pathway for p27 synthesis [44], and a gap junction-independent inhibition of Skp2 expression was responsible for the reduced p27 degradation [45,46]. They suggested that the gap junction-independent pathway contributed much more than the gap junction-dependent one in the Cx43-induced cell growth suppression by targeting the proto-oncogene SKP2 and consequently increasing the level of the cell cycle controller p27. The exact mechanism of Cx43 in regulation of Skp2 is yet to be determined.

Recent findings suggest that connexins interact with other proteins including tight junction proteins, ZO-1, occludin, claudins, N-cadherin, and the cytoskeletons, microtubules, actin, and catenins [49–54]. Cx43 and N-cadherin are suggested to modulate neural crest cell mobility, by engaging in a dynamic cross-talk through p120 catenin signaling [50]. β -Catenin signaling, which was down-regulated by overexpression of N-cadherin, was found to inhibit activation of another cyclin-dependent kinase inhibitor p21 gene expression. The inhibitory effect of N-cadherin was augmented by co-expression of Cx43; however, this effect appears to correlate with the activity of GJIC [47]. In fact, α -catenin, a cadherin-associated protein, is shown to trigger trafficking and assembly of Cx43 and Cx32 into gap junctions in androgen-independent human prostate cancer cell lines [48]. Cell–cell and cell–extracellular matrix interactions are likely to induce cell differentiation and other cellular functions through the interactions of connexins with other junctional and/or cytoskeletal proteins, which could be a GJIC-dependent or independent process.

6. Perspectives

Connexins appear to at least possess three types of functions. The first function is its well-accepted role in forming gap junction channels and allowing the transfer of small molecules between cells [2]. The second function is related to the newly identified role of connexin as a component in formation of hemichannels [5]. The third type of functions appears to be independent of gap junctions and hemichannels. The multiple functions of connexin may in part explain why vertebrates have so many types of connexins [55]. Undoubtedly, the differences in the tissue distribution, substrate selectivity, and gating properties of gap junctions and hemichannels result in unique functions for individual connexins [1,5]. However, there are considerable overlaps, especially the expression of more than one type of connexin in a single type of cell, such as lens fibers and hepatocytes, although the formation of heteromeric connexons has been observed [56,57]. The lack of homology between the cytoplasmic carboxyl termini of connexins suggests that the C-terminus is likely to be functionally involved in the gap junction- and

hemichannel-independent function of connexins. Indeed, studies have shown that GJIC is independent of C-terminal domains [58,59]. The three-dimensional structures of gap junctions formed by wild-type and C-terminus truncated Cx43 are almost identical [60,61], further implying that the C-terminal tail may not be an essential structural component of gap junction channels. Unlike most of the structural proteins with slower turnover, connexins behave more or less like regulatory proteins with apparent half-lives around 1–3 h [1,2]. Together, all these evidences suggest novel gap junction-unrelated functions of connexins. Future work should be directed towards elucidating the molecular mechanism underlying the gap junction-independent roles of connexins in many aspects of cellular activities, such as cell growth and differentiation, cellular injury, and tumorigenicity.

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