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Non-canonical roles of connexins

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Connexin, cell growth, cell death.

List of abbreviations

ASK1	Apoptosis signal-regulating kinase 1
Bcl-2	B-cell lymphoma 2
Cx	Connexin
Dlgh1	Discs-Large homolog 1 protein
GJIC	Gap junctional intercellular communication
MAGUK	Membrane-associated guanylate kinase
MAPKs	Mitogen-activated protein kinases
NOV	Nephroblastoma overexpressed
Wnt	Wingless-Int
ZONAB	ZO-1-associated nucleic acid binding protein
ZO-1/2	Zonula occludens-1/2

Textual abstract

Gap junctions mediate cellular communication and homeostasis by controlling the intercellular exchange of small and hydrophilic molecules and ions. Gap junction channels are formed by the docking of 2 hemichannels of adjacent cells, which in turn are composed of 6 connexin subunits. Connexin proteins as such can also control the cellular life cycle independent of their channel activities. This has been most demonstrated in the context of cell growth and cell death. Different mechanisms are involved mainly related to direct interaction with cell growth or cell death regulators, but also implying effects on the expression of cell growth and cell death regulators. The present paper focuses on these atypical roles of connexin proteins.

1. Introduction

Tissue homeostasis is controlled by a variety of communication mechanisms. Among those, gap junctions are communicating cell junctions that typically gather in plaques at the plasma membrane surface (Cooreman et al., 2019; Vinken, 2011). These cell junctions arise from the interaction of 2 hemichannels of adjacent cells, in turn composed of 6 connexin (Cx) units. At present, more than 20 connexin isoforms have been identified. The connexin family members all share the same structural properties, consisting of 4 transmembrane domains, 2 extracellular loops, 1 cytoplasmic loop, 1 cytoplasmic *N*-terminal tail and 1 cytoplasmic *C*-terminal tail (Figure 1). Variation between connexins is mainly located within the cytoplasmic domains. Connexins are named based upon their predicted molecular weight. Thus, Cx43, which is the most widespread connexin family member, has a molecular mass of 43 kDa (Cooreman et al., 2019; Vinken et al., 2006).

Connexins generally follow a classical secretory pathway during the formation of gap junctions. As such, connexin proteins are synthesized by membrane-bound ribosomes and co-translationally integrated into the endoplasmic reticulum. Subsequently, connexins oligomerize

and form homomeric or heteromeric hexamers, composed of a single type or different types of connexin species, respectively. Localization of oligomerization is specific for the different connexin isoforms. Thus, Cx26 and Cx32 oligomerize in the endoplasmic reticulum, while Cx43 assembles in the trans-Golgi apparatus. Following oligomerization, connexins are transported to the plasma membrane to form hemichannels and gap junctions (Laird, 2006; Martin et al., 2001; Vinken et al., 2008).

Gap junctions govern direct intercellular communication by mediating passive intercellular diffusion of small (1-1.5 kDa) and hydrophilic molecules as well as ions. A plethora of physiological processes is driven by substances that are transported *via* these channels. Consequently, gap junctional intercellular communication (GJIC) is regarded as a key determinant in the control of tissue homeostasis. GJIC can be controlled at many levels, including at the posttranslational level of connexin expression. In fact, phosphorylation of connexins monitors different processes, such as trafficking, degradation and gating of gap junction channels (Laird, 2005; Solan and Lampe, 2005; Vinken et al., 2008).

Over the past few years, a large body of evidence has been generated showing that connexin hemichannels can also establish a pathway for communication independent of their role as structural precursors of gap junction channels. Unlike GJIC, connexin hemichannels support exchange of small messengers between the cytosol of an individual cell and its extracellular environment, and typically become active in pathological conditions (Vinken et al., 2006). Furthermore, it has now become clear that connexin proteins as such may affect tissue homeostasis by actions that are not related to GJIC or connexin hemichannel communication (Vinken, 2011). In the present paper, these non-canonical roles of connexins are reviewed, whereby particular attention is paid to their relevance in cell growth and cell death. Focus is put on 2 major mechanisms elucidated thus far, namely direct interaction of connexin proteins

with cell growth or cell death regulators, and effects of connexin proteins on the expression of cell growth and cell death regulators.

2. Direct interaction of connexins with cell growth and cell death regulators

2.1. β-catenin

β-catenin is a protein present in the cytosol and at the plasma membrane surface. Membrane bound β-catenin is involved in the regulation and coordination of cell-cell adhesion (Valenta et al., 2012). β-catenin has a structural and mechanistic role by acting as a building block for adherens junctions. Adherens junctions form bridges that connect the cytoskeleton of adjacent cells, which is critical for the maintenance of tissue and cellular architecture (Meng and Takeichi, 2009). β-catenin also participates in intracellular signaling by regulating the canonical Wingless-Int (Wnt) signaling pathway. As a result, cellular processes, such as cell renewal and regeneration, are controlled by the interplay between Wnt signaling and cytosolic β-catenin (Nusse and Clevers, 2017; Valenta et al., 2012). Absence of any Wnt stimulus leads to formation of a complex composed of cytosolic β-catenin, adenomatous polyposis coli tumor suppressor, axin and glycogen synthase kinase-3β (Kimelman and Xu, 2006; Valenta et al., 2012). This complex phosphorylates β-catenin, which downregulates cytosolic β-catenin levels (Valenta et al., 2012). Furthermore, activation of the Wnt signaling pathway results in translocation of degradation complex components to the cell plasma membrane surface. This translocation prevents phosphorylation of β-catenin and triggers an increase of cytosolic β-catenin levels (Upadhyay et al., 2008). Accumulation of cytosolic β-catenin ensures the transfer of β-catenin to the nucleus and initiates transcription of genes that regulate the G1/S transition of the cell cycle (Bryja et al., 2017; Orford et al., 1999).

Cx43 is known to interact with β-catenin at cell-cell contact areas, thereby participating in Wnt-mediated regulation of cell growth and cell death (Ai et al., 2000; Kanczuga-Koda et al., 2014;

Moorer et al., 2017; Rinaldi et al., 2014; Sirnes et al., 2012; Spagnol et al., 2018). Augmented expression of Cx43 in cardiomyocytes induces sequestration of β-catenin in the cell plasma membrane and downregulation of transcriptional activity of β-catenin (Ai et al., 2000). In MDA-MB-231 cells and MCF-7 cells, highly and moderately invasive human breast cancer cells, respectively, overexpression of Cx43 affects β-catenin. Downregulation of nuclear β-catenin levels has been observed in these cancer cells transfected with Cx43 along with reduced extravasation capacity. This suggests a tumor suppressive effect of the Cx43-β-catenin interaction, since removing β-catenin from the nucleus may result in reduced cell growth and in diminished invasiveness as well as an alleviated malignant phenotype (Talhouk et al., 2013). Cx43-β-catenin interactions are controlled by the *C*-terminal tail of Cx43 and deletion of this domain even affects osteoblast proliferation in mice (Moorer et al., 2017). Furthermore, in bone marrow derived mesenchymal stem cells, upregulation of Cx43 expression was reported to positively modulate osteogenic differentiation. In particular, Cx43 controls osteogenic gene levels and cell growth *via* proteasomal degradation of β-catenin (Lin et al., 2018).

2.2. E-cadherin

E-cadherin is the most prominent cadherin protein expressed by epithelial cells (Niessen et al., 2011; Yang et al., 2018). Cadherins bind to cytoskeletal components *via* catenins to form adherens junctions (Hartsock and Nelson, 2008; Meng and Takeichi, 2009; Niessen et al., 2011). Crosstalk between adherens junctions and connexins is crucial for the proper formation, localization and functioning of connexins (Dbouk et al., 2009). In this respect, cadherin-mediated cell-cell adhesion is considered as a prerequisite for the establishment of gap junctions in epithelial cells (Fujimoto et al., 1997).

Studies carried out on human colorectal cancer tissues showed a link between the expression of connexins, particularly Cx26 and Cx32, and E-cadherin. Loss of membrane-bound E-

cadherin in colorectal cancer tissues is related to a disturbed cell differentiation process in tumors leading to metastases. The direct interaction between connexins and cadherins, and its correlation to the carcinogenic process is still a matter of debate, yet interaction of Cx43 with members of the cadherin family also influences cell migration and controls cell signaling (Kanczuga-Koda et al., 2014). In human lung cancer LH7 cells, overexpression of Cx43 significantly upregulates E-cadherin expression and inhibits cell proliferation. This is associated with a reduction of cells in the S and G2 phases of the cell cycle, suggesting that Cx43 and E-cadherin may act as tumor suppressors (Xu et al., 2008).

2.3 Zonula occludens 1-associated nucleic acid binding protein

Zonula occludens 1 (ZO-1)-associated nucleic acid binding protein (ZONAB), which is a transcription factor, regulates cell growth. By accumulating in the cell nucleus, ZONAB controls the expression of cell cycle regulators such as cyclin D1 and proliferating cell nuclear antigen (Ikari et al., 2014; Sourisseau et al., 2006). ErbB-2 is another ZONAB target gene that has been linked to cell growth processes (Balda et al., 2003; Sourisseau et al., 2006). ZONAB, as the name suggests, binds with ZO-1. Additionally, the interaction between ZO-1, a tight junction component, and ZONAB is involved in cell growth processes. ZONAB-ZO-1 interaction causes cytoplasmic sequestration of ZONAB and inactivation of its proliferation activity (Sourisseau et al., 2006).

Colocalization of ZONAB with oligodendrocytic Cx32 and Cx47 as well as with astrocytic Cx43 has been observed in mouse brain. Furthermore, ablation of Cx47 in mice oligodendrocytes leads to loss of ZONAB (Li et al., 2008; Penes et al., 2005).

2.4 Zonula occludens proteins

Besides their role as components of tight junctions, ZO proteins also regulate cell growth. ZO-2 indeed limits cyclin D2 expression in Madin-Darby canine kidney cells. Following translocation of ZO-2 to the cell nucleus, cyclin D2 protein levels are downregulated and consequently cell proliferation is inhibited (Huerta et al., 2007; Tapia et al., 2009).

Direct interactions between connexin isoforms and ZO proteins has been well established (Chen et al., 2008; Giepmans, 2004; Giepmans and Moolenaar, 1998; Hervé et al., 2014; Toyofuku et al., 1998). Curiously, Cx43 interacts with ZO-1 and ZO-2 proteins in a cell cycle stage-specific manner. Cx43 prefers binding with ZO-1 during the G0 phase, whereas interaction between Cx43 and ZO-2 occurs equally during G0 and S phases in rat kidney epithelial cells (Singh et al., 2005).

2.5 Nephroblastoma overexpressed protein

Nephroblastoma overexpressed (NOV) protein, previously denoted as CCN3, belongs to the group of CCN proteins. Members of the CCN family are matricellular proteins, which trigger signal transduction events in several biological processes, such as cell proliferation. Furthermore, CCN proteins modulate the activities of different growth factors and cytokines and are able to induce apoptosis (Chen and Lau, 2010; Leask and Abraham, 2006; Perbal, 2001).

NOV itself has an antiproliferative effect (Benini et al., 2005). NOV colocalizes with Cx43 plaques in human glioma cells (Fu et al., 2004). Overexpression of Cx43 upregulates NOV levels, which causes inhibition of cell growth (Fu et al., 2004; Gupta et al., 2001; McLeod et al., 2000). Furthermore, different growth phases are characterised with altered Cx43 and NOV levels (Wun et al., 2008). Moreover, *N*-truncated NOV proteins shuttle to the cell nucleus as transcriptional regulators in certain human cancers (Planque et al., 2006). Cell growth

processes may also be controlled by nuclear localization of NOV. It has been suggested that Cx43 could change cellular localization of NOV and regulate cell growth (Gellhaus et al., 2004).

2.6 Discs-Large homolog 1 protein

Membrane-associated guanylate kinase (MAGUK) proteins are constituents of tight junctions (Kim, 1995). Discs-Large homolog 1 protein (Dlgh1) is a scaffolding protein and member of the MAGUK protein family (Ishidate et al., 2000). Dlgh1 is located at intercellular contact sites and contains different protein interaction domains (Ishidate et al., 2000; Kim, 1995; Macdonald et al., 2012). Cx32 is known to interact with these tight junction components. Moreover, knockout and downregulation of Cx32 in mouse is associated with reduced protein expression of Dlgh1. In Cx32-null livers, Dlgh1 levels are no longer detected at the cell plasma membrane. Dlgh1 proteins move to the cell nuclei, where they promote cell proliferation. These findings suggest that Cx32 is responsible for maintaining Dlgh1 at the cell plasma membrane surface and for controlling hepatocyte cell growth (Duffy et al., 2007). Cx43 also interacts with Dlgh1. In human cervical epithelial cells, relocation of Cx43 and Dlgh1 from the cell plasma membrane to the cytoplasm has been observed upon tumorigenic transformation (Macdonald et al., 2012).

2.*7 B-cell lymphoma-2 proteins*

B-cell lymphoma-2 (Bcl-2) family proteins are key regulators of programmed cell death (Tsujimoto, 1998; Zhang et al., 2017). More specifically, Bcl-2 proteins control the intrinsic apoptotic pathway. Members of the Bcl-2 family can either have a pro-apoptotic or an anti-apoptotic effect. Anti-apoptotic Bcl-2 proteins include Bcl-2, Bcl-xL, Bcl-w and Bfl-1 (Kanczuga-Koda et al., 2005b). Representative pro-apoptotic Bcl-2 proteins are Bax and Bak.

They control cell death by affecting mitochondrial membrane permeability. Apoptotic signals, like temperature variations, exposure to hydrogen peroxide and pH perturbations, induce mitochondrial outer-membrane permeabilization by triggering Bax. Consequently, cytochrome C is released leading to apoptotic cell death by activating cysteine proteases (Li et al., 1997; Zhang et al., 2017).

In human glioma cells, a positive correlation exists between expression levels of connexins and apoptosis-related proteins. Overexpression of Cx43 leads to increased production of Bcl-2 and decreased expression of Bax, resulting in suppression of apoptosis (Lu et al., 2017). Furthermore, colocalization between Bak and cytoplasmic Cx26 expression as well as between Bak and cytoplasmic Cx43 has been reported in human breast cancer cells (Kanczuga-Koda et al., 2005b). In addition, aberrant cytoplasmic Cx26 production and association with Bax and Bcl-xL has been shown in human colorectal cancer cells (Kanczuga-Koda et al., 2005a).

2.8 Apoptosis signal-regulating kinase 1

Apoptosis signal-regulating kinase 1 (ASK1) belongs to the superfamily of the mitogen-activated protein kinases (MAPKs). MAPKs control cell proliferation and differentiation, and are regulators of cellular functions related to stress. In fact, ASK1 plays a role in diverse stress responses, including hydrogen peroxide-induced apoptosis (Dhanasekaran and Reddy, 2008; Hayakawa et al., 2006; Iriyama et al., 2009). For this reason, the regulation of ASK1 signaling is suggested to be a key event in apoptotic cell death (Nishitoh et al., 2002; Sakauchi et al., 2017).

Cx43 expression controls ASK1 activity and protects rat glial cells against hydrogen peroxide-induced apoptosis. Therefore, Cx43 proteins interact with MAPKs and mediate an anti-apoptotic effect (Giardina et al., 2007).

3. Connexin-mediated modulation of cell growth and cell death regulator expression

Evidence is accumulating showing that connexin proteins as such, independent of their channel forming capacities, can affect cell growth and cell death processes (Chen et al., 1995; Matsuyama and Kawahara, 2009; Qiu et al., 2016; Zhang et al., 2001). In this respect, the phosphorylation status of connexin proteins may influence cell growth mechanisms not related to gap junction or hemichannel functionality (Dang et al., 2006; Johnstone et al., 2012). Genetic approaches based on ablation or overexpression of connexins have unveiled many of these channel-independent functions of connexins. Connexin proteins, as single entities, control the cellular life cycle by mediating the expression of cell growth and cell death regulators. In this respect, Cx43 controls expression levels of cell death-related genes. Microarray analysis of Cx43-null mouse astrocytes showed altered patterns in both pro-apoptotic and anti-apoptotic genes (Iacobas et al., 2003). Similarly, microarray analysis of heart tissue extracted from Cx43 knockout mice revealed transcriptional modifications of many apoptotic genes (Walker et al., 2005). Absence of Cx43 is therefore associated with both inhibition and induction of apoptotic cell death.

The role of connexin proteins in the control of homeostasis remains to be elucidated. It has been suggested that specific parts of connexin proteins directly affect gene transcription. Thus, the region between amino acids 266 and 283 within the *C*-terminal tail of Cx43 inhibits c-Src, a proto-oncogene, activity in glioma cells and astrocytes (González-Sánchez et al., 2016). In this regard, TAT-$Cx43_{266-283}$, a peptide mimicking the effect of Cx43 on c-Src, impairs malignant growth of glioma cells in mouse (Jaraíz-Rodríguez et al., 2019). Moreover, upon forced Cx43 expression in human cervical cancer cells, human embryonic kidney cells and mouse neuroblastoma cells, parts of the *C*-terminal Cx43 tail are found to reside in the cell nucleus. Consequently, relocation of Cx43 provokes inhibition of cell growth (Dang et al., 2003; Moorby and Patel, 2001). However, truncated Cx43, lacking the *C*-terminal tail, inhibits

cell growth of human cervical cancer cells as well (Omori and Yamasaki, 1999). Other interactions between connexins and transcription regulators may therefore regulate cell growth processes.

Connexins may also control gene transcription of key tissue homeostasis determinants through connexin response elements. The promotor region of at least 2 rat osteoblasts genes contains a connexin response element (Stains et al., 2003; Stains and Civitelli, 2005a). Transcription of these connexin response elements is controlled by gap junctions. Disruption of GJIC limits gene transcription of osteocalcin and collagen (Stains et al., 2003; Stains and Civitelli, 2005a, 2005b).

4. Conclusions and perspectives

Connexin proteins can participate in the modulation of cell growth and cell death, independent of their role as building blocks of gap junctions and hemichannels. Different mechanisms are involved in such non-channel functions of connexins (Table 1 and Table 2). Connexin-mediated modulation of cell growth and cell death regulator expression has been most frequently revealed in connexin knockout animals through genetic approaches. However, few reports have been published in this research domain over the years, whereby groundbreaking innovation is still missing. Connexin-mediated modulation of tissue homeostasis determinants might be explained by connexin response elements. Future studies should answer whether connexin response elements indeed play an important role in the regulation of the expression of mediators of cell growth and cell death. Thus far, the non-junction functions of other cell junction components, such as β-catenin, E-cadherin and ZO-1/2, in the management of cell growth and cell death processes are better established than those of connexins. The exact function of connexins in cell growth and cell death pathways needs to be clarified. The channel-independent roles of connexins are often linked with the connexin cytoplasmic *C*-terminal tail,

in particular of Cx43. Besides affecting gene transcription, the *C*-terminal tail of Cx43 is critical for mediating protein-protein interactions (Dang et al., 2003; Giepmans, 2004; Moorby and Patel, 2001). ACT1, a 25-amino acid synthetic peptide containing the *C*-terminal tail of Cx43, has been found to exhibit cutaneous wound healing properties for improved patient outcomes across a variety of injuries (Ghatnekar et al., 2015; Montgomery et al., 2018). Research of this as well as of the abovementioned aspects deserves full scrutiny in the upcoming years and may reveal additional yet unidentified functions of connexins.

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Figures and Tables

Figure 1: Gap junction, hemichannel and connexin structure. Gap junctions arise from the docking of 2 hemichannels of adjacent cells (left panel). A hemichannel is composed of 6 connexins. Connexins share an identical structure, consisting of 4 transmembrane domains, 2 extracellular loops, 1 cytoplasmic *N*-terminal tail and 1 cytoplasmic *C*-terminal tail (right panel).

Table 1: Interaction of connexins with cell growth and cell death regulators.

Table 2: Connexin-mediated modulation of cell growth and cell death regulator expression.

Figure 1:

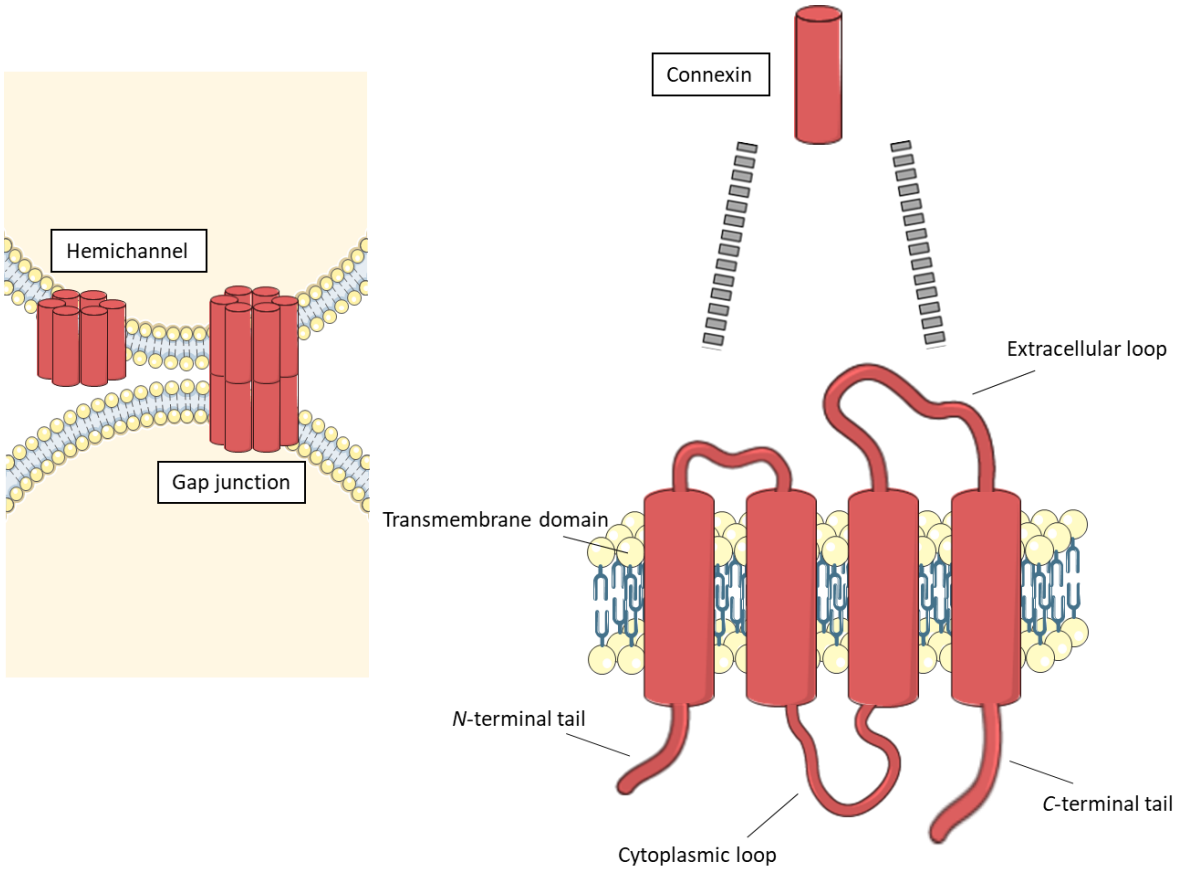
Hemichannel
Gap junction
Connexin
Extracellular loop
Transmembrane domain
N-terminal tail
C-terminal tail
Cytoplasmic loop

Table 1:

Cell type (species)	Cell growth and/or cell death regulator	Interaction with connexin species	Effect	Reference
Myocytes (rat)	β-catenin	Cx43	Cx43-β-catenin interaction downregulates transcriptional activity of β-catenin	(Ai et al., 2000)
Bone marrow derived mesenchymal stem cells (mouse)		Cx43	Deletion of the Cx43 *C*-terminal tail domain accelerates the rate of proliferation	(Moorer et al., 2017)
Colorectal cancer cells (human)		Cx43	Cx43 colocalize with β-catenin and expression of Cx43 is associated with increased levels of apoptosis	(Sirnes et al., 2012)
Breast cancer cells (human)		Cx43	Overexpression of Cx43 affects nuclear β-catenin levels and reduces extravasation capacity	(Talhouk et al., 2013)
Bone marrow derived mesenchymal stem cells (rat)		Cx43	Cx43 controls cell growth *via* proteasomal degradation of β-catenin	(Lin et al., 2018)
Lung cancer cells (human)	E-cadherin	Cx43	Overexpression of Cx43 upregulates E-cadherin expression and inhibits cell proliferation	(Xu et al., 2008)
Oligodendrocytes (mouse)	ZONAB	Cx32, Cx47	ZONAB colocalizes with Cx32 and Cx47	(Li et al., 2008; Penes et al., 2005)
Astrocytes (mouse)		Cx43	ZONAB colocalizes with Cx43	(Penes et al., 2005)
Kidney epithelial cells (rat)	ZO-1, ZO-2	Cx43	Cx43 interacts with ZO-1 and ZO-2 in a cell cycle stage-specific manner	(Singh et al., 2005)
Glioma cells (human)	NOV	Cx43	Overexpression of Cx43 upregulates NOV levels and causes inhibition of cell growth	(Fu et al., 2004; Gupta et al., 2001; McLeod et al., 2000; Wun et al., 2008)

Hepatoma cells (mouse)	Dlgh1	Cx32	Cx32 is responsible for maintaining Dlgh1 at the cell plasma membrane surface and for controlling cell growth	(Duffy et al., 2007)
Breast cancer cells (human)	Bak	Cx26, Cx43	Bak colocalizes with Cx26 and Cx43	(Kanczuga-Koda et al., 2005b)
Colorectal cancer cells (human)	Bax, Bcl-xL	Cx26	Cx26 associates with Bax and Bcl-xL	(Kanczuga-Koda et al., 2005a)
Glioma cells (rat)	ASK1	Cx43	Cx43 interacts with ASK1 and mediates an anti-apoptotic effect	(Giardina et al., 2007)

Table 2:

Cell type (species)	Connexin-mediated modulation	Effect	Reference
Epithelial kidney cells (dog)	Cx43	Overexpression of Cx43 alters expression levels of genes involved in cell cycle regulation, including cyclin A, cyclin D1, cyclin D2, CDK5 and CDK6	(Chen et al., 1995)
Cardiomyocytes (rat)	Cx43	Cx43-knockdown increases proliferative activity	(Matsuyama and Kawahara, 2009)
Ovarian cancer cells (human)	Cx43	Cx43 negative regulates epidermal growth factor-induced cell proliferation	(Qiu et al., 2016)
Osteosarcoma cells (human)	Cx43	Cx43 expression inhibits cell proliferation	(Zhang et al., 2001)
Embryonic kidney cells (human)	Cx43	The phosphorylation status of Cx43 regulates growth inhibition	(Dang et al., 2006)
Vascular smooth muscle cells (mouse)	Cx43	Phosphorylation of Cx43 is critical for cell proliferation	(Johnstone et al., 2012)
Astrocytes (mouse)	Cx43	Cx43 controls the expression levels of cell death-related genes, including upregulation of Nfkb2, Map4k5, $BclX_2$ and Bax	(Iacobas et al., 2003)
Heart tissue (mouse)	Cx43	Knockout of Cx43 reveals expression modifications of many apoptotic genes, including Bok, Bax, Nix, Dapk1, Pdcd10, Casp9, Diablo, Bcl2-Rambo, Bid, Casp6 and Dad1	(Walker et al., 2005)
Glioma cells (mouse)	Cx43	The region between residues 266 and 283 within the *C*-terminal Cx43 tail controls c-Src activity and TAT-$Cx43_{266-283}$ impairs malignant growth	(Jaraíz-Rodríguez et al., 2019)
Cervical cancer cells (human)	Cx43	Relocation of Cx43 provokes inhibition of cell growth	(Dang et al., 2003)
Embryonic kidney cells (human)	Cx43	Relocation of Cx43 provokes inhibition of cell growth	(Dang et al., 2003)
Neuroblastoma cells (mouse)	Cx43	The cytoplasmic carboxyl domain of Cx43 suppresses cell growth	(Moorby and Patel, 2001)
Osteoblasts (rat)	Cx43, Cx45	Transcription of connexin response elements is controlled by gap junctions	(Stains et al., 2003; Stains and Civitelli, 2005b, 2005a)