Connexin 43 (Cx43) in Cancer: Implications for Therapeutic Approaches via Gap Junctions

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Abstract

Gap junctions are membrane channels found in all cells of the human body that are essential to cellular physiology. Gap junctions are formed from connexin proteins and are responsible for transfer of biologically active molecules, metabolites, and salts between neighboring cells or cells and their extracellular environment. Over the last few years, aberrant connexin 43 (Cx43) expression has been associated with cancer recurrence, metastatic spread, and poor survival. Here we provide an overview of the general structure and function of gap junctions and review their roles in different cancer types. We discuss new therapeutic approaches targeting Cx43 and potential new ways of exploiting gap junction transfer for drug delivery and anti-cancer treatment. The permeability of Cx43 channels to small molecules and macromolecules makes them highly attractive targets for delivering drugs directly into the cytoplasm. Cancer cells overexpressing Cx43 may be more permeable and sensitive to chemotherapeutics. Because Cx43 can either act as a tumor suppressor or oncogene, biomarker analysis and a better understanding of how Cx43 contextually mediates cancer phenotypes will be required to develop clinically viable Cx43-based therapies.

Keywords: Cancer, connexins, gap junctions, Cx43

Introduction

Connexins are transmembrane proteins that allow the exchange of molecules with the extracellular environment or adjacent cells via channels in the plasma membrane. Intercellular communication and exchange of biologically active molecules, salts, and nutrients to the extracellular environment are important for cell survival, and aberrant expression (up- or downregulation) of transmembrane proteins is often associated with cancerous phenotypes [1]. Gap junction signaling is responsible for a significant proportion of cellular communication, with cells using gap junctions formed from two opposing hemichannels on adjacent cells to deliver and exchange small molecules, peptides, ions, endogenous nucleic acids, and other cellular metabolites to neighboring cells. Thus, aberrant gap junction expression can alter cellular metabolism and contribute to cancer development and progression [2-5].

Gap junctions are found in all cells in the human body and are formed from proteins called connexins [6]. At least twenty-one connexin isoforms exist in humans, and each can form homomeric channels with the same connexin isoform or heteromeric hemichannels with different connexin isoforms [7]. The most abundant and extensively studied connexin is connexin 43 (Cx43), named according to its molecular weight of 43 kDa [6]. Cx43 expression patterns have been studied in various cancer types and vary depending on the cancer type and stage [3, 4, 8]. To fully understand how Cx43 expression affects cancer progression, however, Cx43 expression patterns and levels must be quantified and characterized in cancer tissues from patients.

In this review we provide an overview of the general structure and function of gap junctions in different cancers. We further discuss current pre-clinical therapeutic approaches and potential new ways of exploiting gap junction transfer for drug delivery and anti-cancer therapy.

2. Connexin structure and their role in compound transfer between cells

Gap junctions are composed of two hexameric hemi-channels aligned 2-4 nm apart on neighboring cells [1]. These channels are built from proteins called connexins and have four transmembrane domains, two extracellular loops, and three intracellular domains comprising a cytoplasmic loop and the N-terminal and C-terminal domains (**Figure 1**) [9]. Initially, the transfer of molecules via gap junctions was thought to be non-specific for any molecule with a molecular weight of less than 1.5 kDa [10]. However, structural studies of connexin channels, in particular Cx43, strongly indicate specificity with regard to molecular transport and exchange. This review primarily focuses on Cx43 due to its documented role in cancer progression and metastasis and because it has greater capacity to transport macromolecules than other connexin proteins.

Of the hemi-channel isoforms studied in their homomeric form, it is evident that there exists some substrate preference according to size, charge, and shape. Weber et al. investigated the ability of fluorescent probes with molecular weights between 350 and 760 kDa to move through six different homomeric channels: Cx26, Cx32, Cx37, Cx40, Cx43, and Cx45 [11]. All connexins could transfer the low molecular weight dye, the mid-weight dye was transferred efficiently across all channels (Cx37 and Cx45 pairs displayed a decrease in transfer compared to the low molecular weight dye), and Cx43 could transfer the mid-weight dye more efficiently than its counterparts. When tested with the high molecular weight probe (Mw = 760 Da), Cx32 and Cx43 transferred the probe much more efficiently than the other connexins (Cx26, Cx32, Cx37, Cx40). Cx43, Cx45, Cx40, and Cx26 all had similar permeability for cationic solutes [12]. Cx40 and Cx26 had low permeability for anionic solutes, and Cx43 and Cx45 discriminated the molecules mostly by size and independent of charge [11].

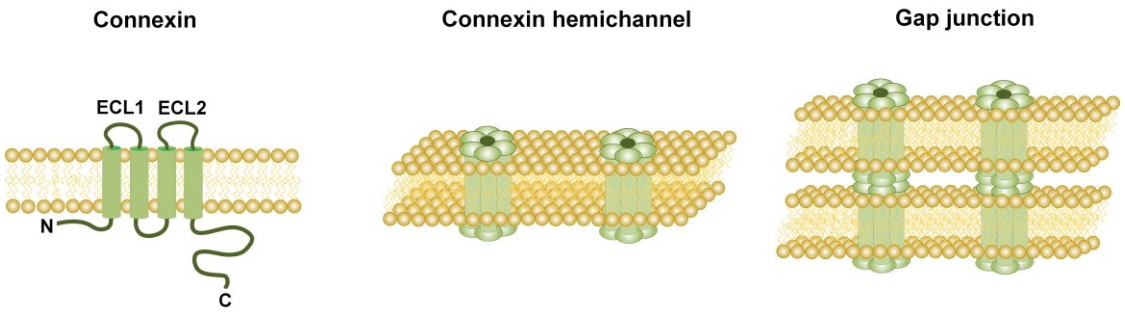

Figure 1. The connexin protein has four transmembrane domains, two extracellular loops, and three intracellular domains comprising a cytoplasmic loop and the N-terminal and C-terminal domains (left). Connexin hemichannels are formed from six connexin proteins (middle). Opposing connexin hemichannels form gap junctions (right).

3. Cx43 expression in cancer

Aberrant Cx43 expression, both up- and downregulation, can contribute to cancer development and progression. As shown in **Table 1**, Cx43 expression patterns are cancer specific and can change according to cancer stage. Although there is an overall consensus that aberrant Cx43 expression correlates with tumor growth and/or metastasis, there is not always a clear correlation between Cx43 expression, cancer stage, and cancer type.

In an immunohistochemical analysis of 117 gastric cancer samples, reduced Cx43 and E-cadherin expression were shown to contribute to the development of primary gastric cancer; however, increased Cx43 and E-cadherin expression contributed to lymph nodes metastasis [13]. In primary urothelial bladder cancer, enhanced Cx43 expression was associated with poor patient prognosis [3]. The study examined tissue samples from 174 patients in tissue microarray format, and Cx43 expression was evaluated semi-quantitatively (0, 1+, and 2+) by immunohistochemical analysis. Of 174 patient samples, 31 samples (17.8%) showed high (2+) Cx43 expression, which was associated with higher tumor grade, increased proliferation, and shorter progression-free survival [3]. In an *in silico* analysis of the gene expression profiles of breast cancer tissues, increased Cx43 and Cx26 expression in primary breast cancers was associated with recurrence and poor patient survival. In contrast, in a tissue microarray study of 483 cases of invasive breast cancer, none of the connexin markers (Cx26, Cx32, and Cx43) correlated with patient survival or tumor grade [14].

In melanoma patients, increased Cx43 and Cx26 expression correlated with metastasis and poor patient survival. Furthermore, in brain metastases from primary breast cancers, Cx43 was only overexpressed in the metastases but not in healthy brain tissue [4]. These data strongly suggest that breast cancer and melanoma cells use Cx43 to initiate metastasis formation at distant sites. In support of this, Cx43

knockdown in 4T-1 cells decreased micro-tumor formation by approximately three-fold, and Cx43 was required to form functional gap junctions with endothelial cells necessary for microtumor formation [4]. Human breast cancer cells expressing Twist and with increased Cx43 expression rapidly extravasated and formed more microtumors compared with controls. Consistent with these findings, Tsai et al. over-expressed Cx43 in breast cancer cell lines and observed that transfected cells migrated at a significantly higher rate than their non-transfected counterparts [5], in keeping with Cx43 contributing to metastatic disease and cancer progression.

In a study of oral squamous cell carcinoma tissue biopsies from 35 patients analyzed for Cx26, Cx43, and Cx45 expression, high tumor cell membrane Cx43 expression was prognostic for short overall survival. There was no association between overall survival and Cx26 and Cx45 expression [2]. Similar results were found when biopsies from 98 patients with esophageal squamous cell carcinoma were analyzed. Patients with a higher degree of Cx43 expression had a survival rate of 40% at 60 months. In contrast, patients with low Cx43 expression had an 80% survival rate at 60 months [8].

An immunohistochemical analysis of Cx43 expression in colorectal adenomas was performed using a two point scale. Samples were considered negative when less than 10% of cells were Cx43 positive and positive when more than 10% of cells were Cx43 positive. Cx43 expression was correlated with patient characteristics and histopathological features. Increased Cx43 expression was found in the mucosa surrounding adenomas with high-grade dysplasia [15].

In the case of thyroid cancer, metastasis seemed to be associated with low Cx43 expression [16] in a Cx43 mRNA study of 120 patients (60 with benign thyroid disease and 60 with malignant thyroid cancer). Cx43 and cadherin expression was decreased in 78.3% of primary gastric cancers [17] and was associated with advanced pathological stage and lymph node metastasis. Similarly, Cx43 was significantly downregulated in chronic B cell leukemia patients (n = 113) compared to cells from healthy donors [18].

In the case of astrocytic brain tumors, two independent studies reported that Cx43 protein expression decreased with increasing tumor grade. Pu et al. noted a decrease in Cx43 expression from 100% Cx43-positive samples in grade I glioma to 14.3% Cx43-positive samples in grade IV glioma, suggesting that loss of functional Cx43 results in more malignant phenotypes [19]. However, while Caltabiano at el. also reported that Cx43 protein expression decreased in grade III and IV tumors, Cx43 mRNA levels remain high [20].

Table 1. Clinical studies assessing Cx43 expression in different types of cancer

Type of cancer	***Sample/Model***	***Methodology***	***Clinical observation***
***Cx43 upregulation* – Clinical Studies**			
Gastric cancer	Tumor biopsy	Immunohistochemical analysis: 117 patients	- Reduced Cx43 and E-cadherin contributes to occurrence of primary gastric cancer. - Increased Cx43 and E-cadherin expression contributes to metastasis to lymph nodes [13].
Urothelial bladder cancer	Tissue/tumor biopsy of primary bladder cancer	Immunohistochemical analysis: 174 patients	High Cx43 associated with poor prognosis in patients with non-muscle invasive bladder cancer [3].
Breast cancer	Oncomine cancer database: primary breast cancer tumors	Gene expression profiles	Increased Cx43 and Cx26 expression in primary breast cancers associated with recurrence and poor patient survival [4].
	Tissue microarray of invasive breast carcinomas	Tissue microarray analysis: 438 patients	Cx43, Cx26, and Cx32 not reliable markers for breast cancer [14].
Melanoma	Oncomine cancer database	Gene expression profiles	Increased Cx43 and Cx26 expression in melanoma associated with metastasis and poor patient survival [4].
Breast cancer metastasis to the brain	Human brain metastasis tumor biopsy	Immunofluorescence analysis: 4 patients	Increased Cx43 expression in metastatic nodules compared to non-cancerous brain tissue [4].
Leiomyosarcoma	Tumor biopsy	Microarray/GeneChip expression profiles and immunohistochemical analysis: 28 patients	*GJA1* (gene encoding Cx43) found to be overexpressed in metastases compared to primary urinary tumors [21].

Oral squamous cell carcinoma	Tumor biopsy	Immunohistochemical analysis: 35 patients	High Cx43 membrane expression associated with overall short survival time [2].
Esophageal squamous cell carcinoma	Tumor biopsy	Immunohistochemical analysis: 98 patients	High Cx43 expression associated with significantly reduced survival rates when compared to those with low Cx43 expression [8].
Colonic adenomas	Tumor biopsy	Immunohistochemical analysis: 87 patients	Increased Cx43 expression seen in mucosa surrounding high-grade dysplasia, large, and villous adenomas [15].
***Cx43 downregulation* – Clinical Studies**			
***Thyroid* cancer**	Aspiration biopsy	Gene expression: 60 patients with thyroid cancer and 60 patients with benign tumors	Cx43 expression was significantly decreased in cancer patients, 40% compared to 86.67% in benign tumors. Low Cx43 expression linked to distant metastasis, tumor growth, and malignancy [16].
Astrocytomas	Tumor biopsy	Northern blot and immunohistochemical analyses: 44 resected astrocytic tumors, 8 normal brain tissue samples	Cx43 expression decreased with increasing tumor grade. Cx43 downregulation linked to progression of astrocytic tumors to malignant stages [19].
	Tumor biopsy	Immunohistochemical analysis of Cx43 protein and quantification of Cx43 mRNA by *in situ* hybridization: 32 samples	Decreased Cx43 protein expression in high grade tumors. Persistent high Cx43 mRNA levels in high grade tumors [20].
Laryngeal squamous cell carcinomas	Tumor biopsy	Immunohistochemical analysis: 87 patients	Low or negative Cx43 expression in poor and moderately expressed carcinomas resulted in a worse patient prognosis and short survival time [22].
Chronic B cell leukemia	Blood sample analysis of lymphocytes	Western blot and flow cytometry: 113 patients	Reduced expression of Cx43 compared to healthy donors [18].

4. Therapeutic approaches exploiting Cx43 function

Due to their potential role in cancer metastasis, attempts have been made to alter connexin function to inhibit cancer growth. Therapeutic approaches include Cx43 peptide mimetics, Cx43 inhibitors, chemical agents capable of enhancing Cx43 function, and nanocarriers surface-decorated with Cx43-targeting ligands (**Table 2)**.

4.1 Cx43 mimetic peptide

Cx43 has been shown to control the response of glioblastoma (GBM) cells to temozolomide, a DNA-alkylating chemotherapeutic agent. GBM cells overexpressing Cx43 displayed chemoresistance to temozolomide and downregulation of Cx43 restored chemosensitivity. Based on these findings, Murphy et al. developed αCT1, a 25mer peptide composed of the ZO-1 PDZ-binding domain of Cx43 fused to the cell-penetrating peptide antennapedia. Treatment of O-6 methylguanine-DNA methyltransferase (MGMT)-deficient GBM cells with αCT1 blocked the AKT/AMPK/mTOR signaling pathway and induced apoptosis in GBM cells [23].

In another study, the same 25mer peptide impaired proliferation and enhanced apoptosis of MCF-7 breast cancer cells [24]. The authors found that αCT1 enhanced the sensitivity of MCF-7 and HER2+ breast cancer cells to chemotherapeutics. The authors proposed that αCT1 enhances the activity of Cx43 and that it increases cell permeability to cytotoxic agents such as tamoxifen and lapatinib in MCF-7 and HER2+ breast cancer cells [24].

4.2 Cx43 inhibitors to prevent cell-to-cell communication

Cells, including cancer cells, rely on gap junctions to exchange ions, metabolites, solutes, and second messengers, which are necessary for cell survival. Blocking these gap junctions prevents the transfer of essential compounds between cells and leads to cellular apoptosis. To further potentiate the anti-tumor therapeutic efficacy of their mesenchymal stem cells expressing tumor necrosis factor-related apoptosis-

inducing ligand (TRAIL), Yulyana et al. added a Cx43 gap junction inhibitor, carbenoxolone, and found that the combination therapy enhanced glioma cell death and survival of treated mice by 27% [25].

In tumor tissues from thyroid cancer patients, Jensen et al. found that thyroid cancer cells that had infiltrated blood vessels showed a high degree of Cx43 translocation to the cellular membrane [26]. Those cells also more readily formed spheroids than adherent thyroid cancer cells that had predominantly cytoplasmic Cx43 expression. It was proposed that membranous Cx43 promotes gap junctional intercellular transfer (GJIT), AKT activation, cancer cell survival under low adherent cellular conditions, and metastasis. As a result, treatment with carbenoxolone, a Cx43 inhibitor, only led to apoptosis of thyroid cancer cells growing in spheroids and hardly affected adherent thyroid cancer cells [26]. The authors proposed that GJIT could be a potential target to prevent cancer metastasis.

Table 2. Current approaches to target Cx43 with nucleic acids, peptides, and small molecules

Type of Molecule	Relevance to Cx43	Therapeutic effect
CX43 peptide mimetic		
αCT1	25mer peptide composed of the ZO-1 PDZ-binding domain of Cx43 that is fused to the cell-penetrating peptide antennapedia.	αCT1 restored temozolomide sensitivity in MGMT-deficient, high Cx43-expressing glioblastoma cells [23].
		αCT1 increased functional activity of Cx43 and enhanced chemosensitivity of MCF-7 and HER2+ breast cancer cells to tamoxifen and lapatinib [24].
Cx43 inhibitor		
Carbenoxolone	Cx43 inhibitor	Carbenoxolone enhanced the anti-tumor therapeutic efficacy of MSCs expressing TRAIL by inhibiting gap junctional exchange of molecules between cells [25].
		In non-adherent thyroid cancer cells, treatment with carbenoxolone inhibited Cx43-mediated signaling and decreased cell-viability [26].

Cx43 expression upregulators		
All-trans retinoic acid (ATRA)	Upregulates Cx43 mRNA and protein expression.	ATRA was given in conjunction with suicide gene therapies and increased apoptosis in MCF-7 cells six-fold compared to the control group [27].
Dioscin (glucoside saponin)	Upregulates Cx43 and Cx26 protein expression.	Dioscin enhanced ganciclovir-induced bystander killing of B16 melanoma cells [28].
Nanocarriers targeting Cx43		
Cisplatin-loaded nanogel	mAbs conjugated to the cisplatin-loaded nanogel targeted the extracellular loop of Cx43 and BSAT1	mAb Cx43 and BSAT-1 conjugated nanogels saw a decrease in tumor growth and an increase in overall survival when compared to existing formulations [29].
Liposomal nanocarriers	mAbs conjugated to liposomes targeted the extracellular loop of Cx43	mAb Cx43 accumulated in the peritumoral zone, proximal to where invasive gliomas reside [30].

4.3 Cx43 increases cellular permeability to chemotherapeutics

Cx43 expression on cancer cells has been reported to increase cell permeability to chemotherapeutics. For example, human glioblastoma cells overexpressing Cx43 showed significantly greater permeability to paclitaxel and doxorubicin than cells with low Cx43 expression [31]. In this study, Cx43 significantly increased cancer cell sensitivity to chemotherapeutics including VP16 (a topoisomerase II inhibitor), paclitaxel, and doxorubicin at clinically relevant doses. This is significant because most patients with glioblastoma also develop a high degree of drug resistance.

Similarly, when Cx43 expression in MCF-7 cells was increased with all-*trans* retinoic acid (ATRA), greater drug permeability and higher chemo-sensitivity were observed. ATRA is known to increase the expression of Cx43 by binding to the retinoic acid response element (RARE) upstream of Cx43 and resulting in transcriptional activation. This so-called bystander effect is caused by an increase in GJIC caused by Cx43 upregulation. When ATRA was given in conjunction with previously developed suicide gene therapies (GCV, FC), apoptosis in MCF-7 cells increased six-fold compared to the control group [27]. Thus, Cx43 overexpression may be exploited for targeted drug delivery to cancer cells via enhanced drug permeability.

Dioscin, a glucoside saponin analogue, is another chemical agent capable of increasing cellular Cx43 and Cx26 expression levels. When B16 melanoma cells were treated with dioscin, the bystander effects of herpes simplex virus thymidine kinase/ganciclovir (HSV-tk/GCV) were enhanced through increased gap junction formation and GJIC in B16 melanoma cells. The combination of dioscin and GCV reduced tumor volume by 70% compared to 33% when only GCV was used [28].

4.4 Cx43-targeting nanocarriers

Novel treatments for gliomas have included cisplatin-loaded nanogels conjugated to monoclonal antibodies targeting BSAT1 and Cx43. Targeting Cx43 in glioma offers the advantage of exploiting one of the few mechanisms by which therapeutics reach an intracranial tumor. Baklaushev et al. found that rats treated with BSAT1 and Cx43-targeting nanogels lived longer than controls treated with 5% dextrose. These rats also had significantly smaller tumor volumes at day 30 compared to controls. The Cx43-targeting nanogels successfully inhibited tumor growth and increased survival when tested in conjunction with a current glioma treatment, indicating that Cx43 is a promising practical target in this type of glioma [29]. It should be noted that Cx43 overexpression was required to increase drug permeability in a different form of glioblastoma [29], further highlighting the complexity of Cx43 regulation pathways even in similar cancers.

Further studies on targeting Cx43 in glioma have been conducted using PEGylated liposomes conjugated to an anti-Cx43 antibody targeting the second extracellular loop. When mice expressing intracranial C6 glioma were dosed intravenously with the Cx43-targeted liposomes, a significant amount of the injected dose accumulated at the periphery of the glioma, where Cx43 was expressed [30].

4.5 Gap junction transport of nucleic acids using Cx43-expressing cells and Cx43-positive exosomes

Since Cx43 is upregulated in different cancer types, it represents an attractive target for drug and nucleic acid delivery. Furthermore, Cx43 also facilitates the direct cytoplasmic delivery of drugs and nucleic acids whilst completely bypassing the degradative environment of the endocytotic pathways [32]. Thus, drug

bioavailability at the site of action could be significantly increased and drug doses could be decreased if Cx43 is exploited as a therapeutic target.

An emerging area of drug delivery is through the use of whole cells as delivery vehicles. Cells exploit gap junctions for the direct transfer of molecules into the cytoplasm of neighboring cells. Connexin hemi-channels allow direct cytoplasmic delivery of small molecules *and* macromolecules whilst bypassing the harsh degradative environments of the endocytic and lysosomal pathways [33]. Using the direct cytoplasmic delivery capacity of connexin hemichannels is a highly attractive delivery approach for small RNAs or other nucleic acid cargoes, as 99% of the cargo delivered using conventional carriers does not reach the intended site of action [33, 34].

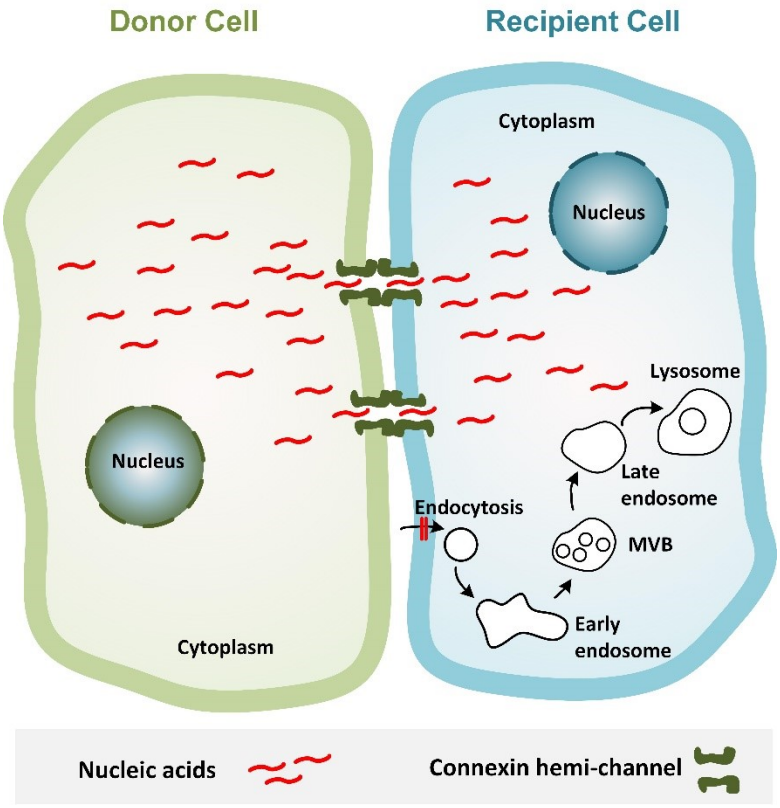

Figure 2. Model for direct cytoplasmic delivery of nucleic acids via connexin hemi-channels. The nucleic acid cargoes from either cell are able to be transported into the cytoplasm of adjacent cells without being subjected to the harsh environment of the endosomal and lysosomal pathway.

Apart from being permeable to small molecular weight solutes, Cx43 can also transport nucleic acids such as small RNAs. Valiunas et al. showed that the rate and extent of transport decreases with increasing length of single-stranded oliognucleotide (24-mer < 16-mer < 12-mer). The single-stranded oligonucleotides had a diameter of ~1 nm and widths ranging from 3.8 (12-mer) to 7.6 nm (24-mer). Furthermore, the hybridized double-stranded 12-mer had significantly lower permeability than the single-stranded variant. The rate and extent of nucleic acid transport appears to be a function of the length and diameter of the oligonucleotides. The siRNA delivered via Cx43 was functional and successfully mediated pol-β knockdown in recipient HeLa cells. Notably, only cells expressing Cx43 delivered oligonucleotides to recipient cells while cells without Cx43 but expressing Cx26 and Cx32 could not transport oligonucleotides [35]. Similar findings were reported when GFP-expressing human embryonic stem cells (hESCs) were co-cultured with hESCs stably expressing shRNA directed against GFP. The authors found that GFP shRNA-expressing hESCs inhibited GFP expression in co-cultured GFP-expressing hESCs in a dose-dependent manner. No decrease in knockdown was observed when the GFP-targeting shRNA was mutated to become non-functional or when the cells were treated with α-glycyrrhetinic acid, a gap junctional inhibitor [36].

The exact mechanisms of how Cx43 transports nucleic acids is not fully understood. However, a study by Varela-Eirin et al. proposed that connexin RNA-binding motifs could be responsible for the recruitment of RNA molecules to the hemichannels, thereby facilitating transport through the gap junctions [9]. Whether and how these predicted connexin RNA-binding motifs contribute to the gap junction transport of RNA needs further investigation.

Similar to how cells use Cx43 for genetic material transfer, nanosized Cx43-positive exosomes have been shown to use the same mechanism. Soares et al. investigated the presence of Cx43 hemi-channels in different cell lines and body fluids and found that exosomes are enriched in Cx43 compared to parental cells [37]. HEK cell lines stably transfected to express Cx43 showed that homomeric Cx43 hemi-channels localized to the exosomal lipid membrane and that Cx43-positive exosomes could deliver heterologous

DNA to recipient cells. This group also demonstrated that exosomes lacking Cx43 had decreased delivery of luciferin compared to their Cx43-positive counterparts [37].

Endosomal degradation is currently a substantial barrier to gene delivery methods [33, 38-41], and with the large number of endogenous miRNAs being utilized by a cell it is not unlikely that these obstacles are overcome through gap junctional transfer. For tumors with Cx43 upregulation, dosing exosomes or other nanoparticles equipped with gap junctions loaded with small-molecule chemotherapy or therapeutic macromolecules could exploit this increased Cx43 gap junction expression.

5. Conclusion

In conclusion, the permeability of Cx43 channels to small molecules and macromolecules makes them highly attractive targets for delivering drugs directly into the cytoplasm of cancer cells. Cx43 hemichannels have the potential to decrease drug resistance by increasing the cancer cell's permeability to chemotherapeutics. Further, gap junctional nucleic acid delivery is desirable because it allows direct cytoplasmic transfer whilst bypassing the harsh degradative environments of the endocytic and lysosomal pathways. Aberrant expression of connexins especially that of Cx43, has been shown to be involved in cancer formation and progression. Depending on the cancer stage and cancer type, connexin can either act as a tumor suppressor or oncogene. Thus, to advance Cx43-based and/or Cx43-targeted therapy into the clinic, a careful quantitative biomarker analysis and a better understanding of how Cx43 mediates cancer phenotypes in different contexts will be required.

Conflict of interest statement

None to declare

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