



## The pro- and anti-tumoral properties of gap junctions in cancer and their role in therapeutic strategies

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### ABSTRACT

Gap junctions (GJs), essential structures for cell-cell communication, are made of two hemichannels (commonly called connexons), one on each adjacent cell. Found in almost all cells, GJs play a pivotal role in many physiological and cellular processes, and have even been linked to the progression of diseases, such as cancer. Modulation of GJs is under investigation as a therapeutic strategy to kill tumor cells. Furthermore, GJs have also been studied for their key role in activating anti-cancer immunity and propagating radiation- and oxidative stress-induced cell death to neighboring cells, a process known as the bystander effect. While, gap junction (GJ)-based therapeutic strategies are being developed, one major challenge has been the paradoxical role of GJs in both tumor progression and suppression, based on GJ composition, cancer factors, and tumoral context. Therefore, understanding the mechanisms of action, regulation, and the dual characteristics of GJs in cancer is critical for developing effective therapeutics. In this review, we provide an overview of the current understanding of GJs structure, function, and paradoxical pro- and anti-tumoral role in cancer. We also discuss the treatment strategies to target these GJs properties for anti-cancer responses, via modulation of GJ function.

### 1. Introduction

Gap junctions (GJs) are protein channels that enable direct intercellular communication (Fig. 1) [1], thus allowing cells to exchange signals and molecules directly from the inside of one cell to a neighboring cell. As such, they provide an essential way for the maintenance of physiological functions, e.g., cell growth, differentiation, homeostasis [2], angiogenesis [3], neural migration [4], and stem cell development [5]. Recently, the importance of GJs for disease induction and progression is becoming more appreciated, especially in the context of oncology, and is therefore seen as a novel target for therapy development. Studies into solid tumor cells revealed a lack of communication through GJs in certain tumor types, resulting in abnormal cell growth [6–8]. Moreover, restoration of gap junction intercellular communication (GJIC) in tumor cell lines reduced tumor growth and proliferation

[9–11], suggesting that GJs have anti-tumorigenic properties. However, interestingly, GJIC was also able to facilitate the sharing of cancer cell metabolites with normal (healthy) cells, which in turn led to a gain of malignant properties in normal cells [12,13]. These reports suggest a pro-tumorigenic role of GJs properties. Hence, the current understanding of GJs in cancer cells is paradoxical, as GJs have both tumor-suppressing and promoting properties which depend on gap junction (GJ) type, cancer stage, and tumoral factors [14]. In fact, GJs are often reduced or lost completely in early cancer stages and upregulated in later stages and metastatic lesions, which contribute to tumor aggressiveness [14,15]. Therefore, therapeutic strategies to enhance GJs in early tumor development [16–18] or to inhibit them in advanced stages [19–21] have emerged.

Peptides [17,22,23], antibodies [24,25], and chemotherapeutic agents [26,27] have been used to inhibit GJ functions in cancer cells, targeting the pro-tumorigenic properties of GJs. They have been proven

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**Abbreviations**

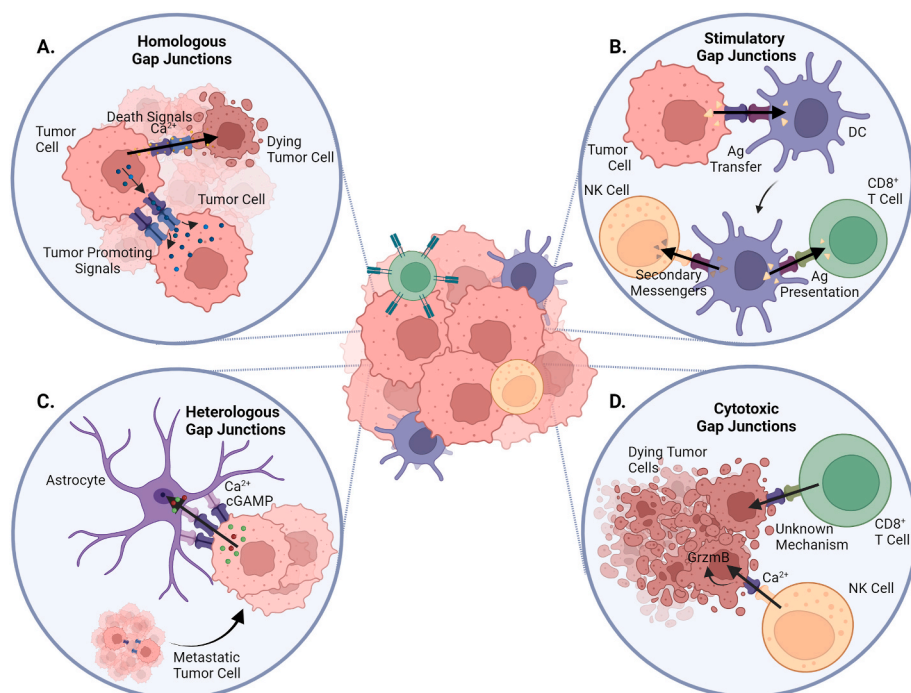
GJ	Gap junction
Ca <sup>2+</sup>	Calcium ions
Ag	Antigen
DC	Dendritic cell
CD8 <sup>+</sup>	Cluster of differentiation 8 <sup>+</sup>
NK	Natural killer
cGAMP	Cyclic guanosine monophosphate–adenosine monophosphate
GrzmB	Granzyme B
GJIC	Gap junction intercellular communication
RONS	Reactive oxygen and nitrogen species
PDT	Photodynamic therapy
NTP	Non-thermal plasma
Cx	Connexin
TM	Transmembrane
EL-1	Extracellular loop 1
EL-2	Extracellular loop 2
CL	Cytoplasmic loop
NT	Amino terminus
CT	Carboxyl terminus

NAD <sup>+</sup>	Nicotinamide adenine dinucleotide
ATP	Adenosine triphosphate
MD	Molecular dynamics
IP3	Inositol 1,4,5-trisphosphate
cAMP	Cyclic adenosine monophosphate
miR-125b	MicroRNA-125b
Cx26-GJs	Cx26 proteins-composed GJs
Cx32-GJs	Cx32 proteins-composed GJs
SCC	Squamous cell carcinoma
Cx43-GJs	Cx43 proteins-composed GJs
LUAD	lung adenocarcinoma
G <sub>1</sub>	Gap 1
S	Synthesis
G <sub>2</sub>	Gap 2
HCC	Hepatocellular carcinoma
EMT	Epithelial-to-mesenchymal transition
KIRC	kidney renal clear cell carcinoma
miRNAs	MicroRNAs
αCT1	Alpha connexin carboxy-terminus 1
IS	Immunological synapse
APCs	Antigen presenting cells
CTL	Cytotoxic T lymphocyte

useful to restore the sensitivity of cancer cells to cytotoxic drugs [28] and reduce tumor growth [24]. On the other hand, GJs have also been explored to kill cancerous cells by stimulating their anti-tumorigenic property. Strategies that leverage GJs ability to facilitate the transport of reactive oxygen and nitrogen species (RONS) between adjacent cells can cause cellular stress and death via oxidative damage to proteins and membrane lipids [29–31]. An increasing body of experimental evidence demonstrates that oxidative stress modulates channel gating (pore opening/closing) of GJs, and that in turn facilitates the traffic of RONS to the cell interior [32,33]. Therefore, oxidative stress-inducing therapies as novel anti-cancer modality for modulating GJs, are gaining attention, which include photodynamic therapy (PDT) [34,35] and non-thermal plasma (NTP) [36,37].

GJs have been shown to propagate oxidative stress-induced cell death [38,39], apoptotic cell death [40,41], and radiation-induced cell death [42,43] in cancer cells. This phenomenon is named the “bystander effect”, and refers to the transmission of responses from cells exposed to certain stimuli, to non-targeted neighboring or more distant cells by means of intercellular communication. Therefore, the development of therapeutic strategies to improve this propagation can contribute to tumor suppression in cancer cells. Furthermore, GJs are also able to transport antigenic peptides between cancer cells and dendritic cells (DCs), which supports activation and tumor-specific killing by cytotoxic lymphocytes [44,45]. Modulation of GJs is therefore also an emerging target in immunotherapeutic research.

However, a better understanding of GJ structures and their function



**Fig. 1.** Schematic representation of GJs and effector functions between different cell types. GJs are key mediators of intercellular communication. Moreover, a previously underestimated role of GJs in alternative pathways for immune regulation and activation has been recently described (see section 6 for explanation). (A) Tumor cell transfers tumor promoting signals to another tumor cell via homologous GJs, increasing tumoral effects. Besides, homologous GJs also transfer death signals (Ca<sup>2+</sup>) between tumor cells, inducing tumor cell death. (B) Tumor cell transfers Ag peptides to DC via GJs, leading to antigen cross-presentation by DC. Further, DC presents Ag peptides and transfers secondary messengers to CD8<sup>+</sup> T cell and NK cell, respectively, killing target cells by cytotoxic lymphocytes. (C) Metastatic tumor cell transfers Ca<sup>2+</sup> and cGAMP to astrocyte via heterologous GJs, inducing further tumor spreading and therapy resistance. (D) NK cell transfers Ca<sup>2+</sup> to tumor cells via Cx43 GJs, for induction of GrzmB-mediated cell death. A rise in the intracellular Ca<sup>2+</sup> concentration in the target cell is needed for efficient killing by cytotoxic T lymphocytes or NK cells.

are required to elucidate in which conditions GJs act as pro- or anti-tumorigenic agents. Here, computer simulations can be a powerful tool, to investigate the interaction mechanisms at the atomic and molecular level. Moreover, computer simulations can also provide insights into the structural and dynamic properties of GJs, which is sometimes inaccessible with experimental methods. For these simulations to accurately represent the physiological conditions, a detailed understanding of GJ structures is needed. In this way, we can design better GJ promoters or inhibitors for improved cancer treatment.

In this review, we provide an overview of the in-depth knowledge on the structure and functions of GJs, and cover the current understanding of their paradoxical pro- and anti-tumoral properties, together with the therapeutic opportunities of GJ function modulation.

## 2. GJs structure and composition

The first time that GJs were isolated and characterized by X-ray diffraction analysis was in 1972 [46], and since then, it is known that three families of GJs proteins make up this structure: innexins, pannexins, and connexins [47,48]. Of these three, the connexin (Cx) family, first described in 1974 [49], is the most abundant in vertebrate animals [50].

The human Cx protein family contains 21 members, named according to their relative molecular mass [51,52]. Each Cx is a transmembrane (TM) protein with four TM domains in the  $\alpha$ -helical conformation (TM1 to TM4) (Fig. 2A). These domains are connected by two extracellular loops (EL-1 and EL-2) and one cytoplasmic loop (CL), containing an amino (NT) and carboxyl terminus (CT) in the cytoplasm (Fig. 2B) [53]. Whereas the size of the CT domain is the major determinant of Cx molecular mass, which can range from 23 to 62 kDa, the NT domain is of similar length in all Cxs (first 22–23 amino acids), with their first part present in an  $\alpha$ -helical conformation. Although present on almost all human cells [54,55], Cx isotype expression is usually restricted to a certain organ, tissue, or cell type. However, certain Cx proteins, such as Cx43, are more ubiquitously expressed.

Cx proteins are able to come together to form a large diversity of strictly organized assemblies. For instance, six Cx protein monomers oligomerize to form a hemichannel usually referred to as “connexon” (Fig. 3A), i.e., a hydrophilic pore that enables the passage of molecules for direct cytoplasm-to-extracellular communication [51,56]. Connexons are normally closed, but when activated, they can release autocrine and paracrine signals, such as nicotinamide adenine dinucleotide (NAD<sup>+</sup>), glutamate, and adenosine triphosphate (ATP). These signals can affect cell proliferation and survival [57]. The NT domain of a Cx is critical for holding the connexon open, and the pore diameter can differ depending on the Cx they are composed of. For example, a connexon made up of Cx26 proteins has a pore diameter of  $\sim 10$  Å, as estimated by molecular dynamics (MD) simulations [58] and  $\sim 14$  Å by crystallography [59] (Fig. 3A). Likewise, a connexon made up of Cx46 or Cx50 proteins has a pore diameter of  $\sim 14$  Å, as visualized with single-particle cryo-electron microscopy [60].

Once at the plasma membrane, two opposing connexons from

adjacent cells can interact with each other to form a GJ channel, facilitating GJIC. Depending on the type of GJIC, various biophysical properties will be impacted [61,62]. The GJIC can be either homomeric, i.e., when they are composed of Cxs within the same class (Fig. 3B), or heteromeric, i.e., when they are composed of Cxs from different classes (Fig. 3C). Thus, GJIC could be classified as (1) homotypic-homomeric, which consists of two identical connexons formed by only one class of Cx; (2) heterotypic-homomeric, which consists of different connexons, each one formed of the same class of Cx; (3) heterotypic-heteromeric, which consists of different connexons, each one formed with two or more non-symmetric isotypes of Cxs; (4) homotypic-heteromeric, which consists of identical connexons, both similar formed with two or more non-symmetric isotypes of Cxs (Fig. 3D).

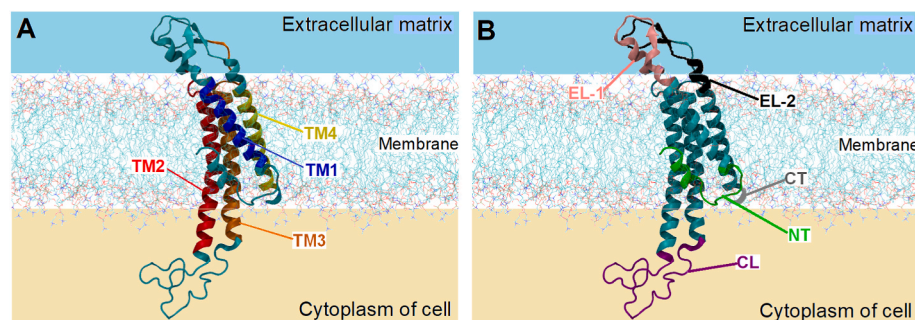
It has already been reported that homotypic and heterotypic GJIC exhibit different electrical potentials between the inside and outside ( $V_{i-o}$ ) of the cells and different voltage differences between the interiors of communicating cells, known as the *trans*-junctional voltage ( $V_j$ ). For example, homotypic Cx26 proteins-composed GJs (Cx26-GJs) are sensitive to both  $V_{i-o}$  and  $V_j$ , while homotypic Cx32 proteins-composed GJs (Cx32-GJs) are sensitive to only  $V_{i-o}$  [63]. Conversely, heterotypic Cx26/Cx32 proteins-composed GJs (Cx26/Cx32-GJs) are more sensitive to  $V_j$  than to  $V_{i-o}$  [63]. These changes in electrical properties suggest that there are differences between the molecular conformations of connexons that depend on the Cx type-composed connexons. Interestingly, changes on GJs electrical properties have been linked to pathological conditions [64].

The GJ is stabilized by non-covalent interactions via H-bonds between EL-1 and EL-2 of their Cxs, which are responsible for docking processes [65,66], while the NT domain participates in the oligomerization, trafficking, and channel gating of Cxs [67,68]. The CL and CT domains are responsible for specific channel properties of different Cxs, including unitary conductance, pH and voltage dependence, and selective permeability to small molecules ( $< \sim 1.2$  kDa) [69,70] (Fig. 3D). In addition, the CT domain plays a role in the phosphorylation of some Cxs (e.g. Cx43 and Cx47 proteins) [71,72] and regulates intercellular Ca<sup>2+</sup> flow [73,74], cell growth, and cell mobility. Recently, Ray and Mehta demonstrated experimentally the importance of the two CT cysteine residues of Cx32 proteins in regulating the trafficking and stability of Cx32 proteins from the endoplasmic reticulum to the Golgi apparatus, and hence its ability to assemble into GJs [75].

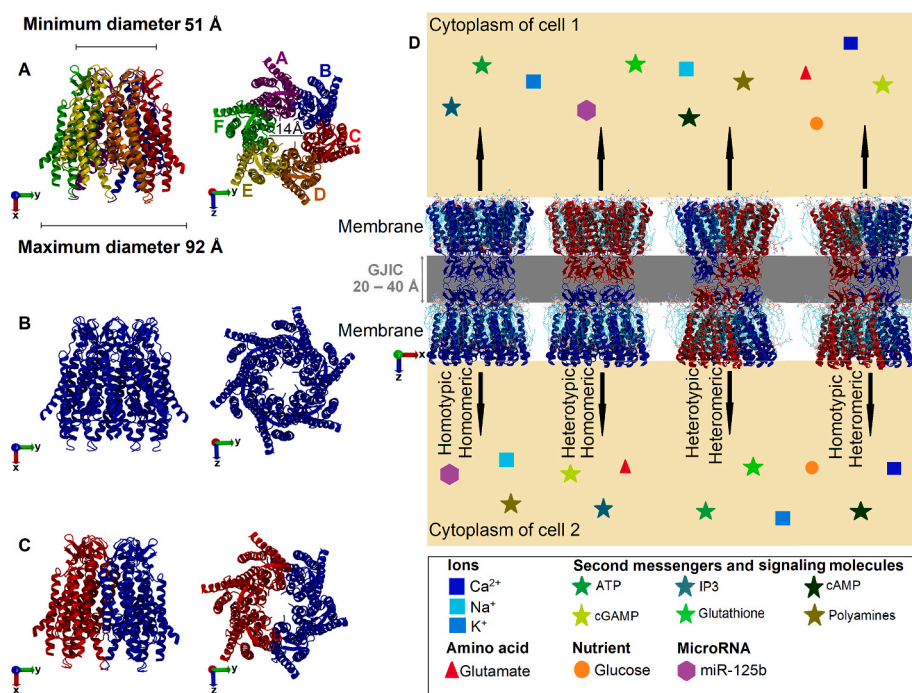
Structural, electrical and dynamic properties of GJs channel can facilitate the exchange of various molecules such as RONS, ions, and different cytokines between adjacent cells [29–31] (Fig. 3D), but these properties can be altered during malignant cellular transformations.

## 3. Pro-tumorigenic properties of Cxs and GJs

An increasing body of experimental work has demonstrated the pro-tumorigenic properties of Cxs and GJs in normal and cancer cells (Fig. 1A). Several Cx subtypes (e.g. Cx26, Cx32, and Cx43) are linked to malignant transformation and tumor progression, mainly in the advanced cancer stages. Increased expression of Cx26 proteins was



**Fig. 2.** Schematic representation of a Cx43 protein in a lipid bilayer membrane. (A) The transmembrane domains TM1, TM2, TM3, and TM4 are represented in blue, red, orange and yellow colors, respectively. (B) The extracellular (EL-1 and EL-2) and cytoplasmic (CL) loops are represented in pink, black, and purple colors, respectively. The amino (NT) and carboxyl (CT) terminus are represented in green and gray colors, respectively. For the sake of clarity, only a small part of the CT domain is represented. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)



**Fig. 3.** Representations of the 3D structure of Cx26 proteins-composed connexons. The Cx26 structure can be obtained from the Protein Data Bank website (<https://www.rcsb.org/>) (accession no. 2ZW3). Side view (on the left) and top view (on the right). (A) Each color represents a Cx monomer. (B) Homomeric connexon. (C) Heteromeric connexon. (D) Schematic representation of GJIC (or only GJ) between Cx46 proteins-composed connexons (4 possibilities are shown). The Cx46 structure can be obtained from the Protein Data Bank website (<https://www.rcsb.org/>) (accession no. 6MHQ). Exchange of possible types of ions, amino acids, secondary messengers, cancer-associated signaling molecules, nutrients and microRNAs between two cells is illustrated as geometrical shapes of different colors. For simplicity only a few examples for each class are shown. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

found in lung squamous cell carcinomas (SCC), which facilitated invasion and metastasis [76]. The authors found that Cx26-positive lung SCC cells were specifically located facing the tumor stroma or fibrous capsule, and the ratio of Cx26-positive over Cx26-negative cancer cells was significantly higher in metastatic lesions, compared to the corresponding primary tumor. Moreover, in these cancer cells, Cx26 proteins were preferentially localized on the plasma membrane, and could form Cx26-GJs between lung SCC cells and normal lung cells [76]. This heterologous communication between malignant and normal cells via GJs was also reported by others; Zhang et al. demonstrated dye transfer between lung cancer cells and normal lung fibroblasts via GJs formation. GJs formation allowed the sharing of metabolites to initiate metastasis, although coupling levels may need to exceed a certain threshold to allow propagation of signals over a sufficient distance to affect the behavior of a cell population [77]. Likewise, more in-depth studies detected coupling between melanoma and endothelial cells via homologous [78] and heterologous [79] Cx26-GJs, which contributed to the intravasation and extravasation of melanoma cells during the metastatic process [78, 79]. Conversely, inhibition of Cx26 proteins rendered the tumor cells deficient in Cx26-GJs formation and reduced their metastatic potential [79].

Overexpression of Cx32 proteins in normal and metastatic breast cancer cells led to a more mesenchymal-like phenotype [80]. Adak and co-workers reported an increased migratory capacity of healthy breast cells, while mesenchymal markers, including vimentin, were further upregulated in the metastatic counterpart, thus presenting, for the first time, the metastasis-stimulating properties of the Cx32 protein in breast cancer [80]. Heterologous Cx43 protein-composed GJs (Cx43-GJs) have also been linked to the initiation of brain metastatic lesions from both melanoma and breast cancer. Depletion of Cx43 proteins or pharmacological blocking of the Cx43-GJ coupling inhibited brain colonization via blocking of tumor cell extravasation and blood vessel co-option, a non-angiogenic mechanism of tumor vascularization in which cancer cells utilize pre-existing blood vessels instead of inducing new blood vessel formation [81]. Taken together, these results suggest an important pro-tumoral role of Cxs in advanced cancer stages, and more specifically points at GJ formation between cancer and normal cells as an important facilitator of cancer cells growth, tumor progression and

metastasis.

In addition to cancer stage, Aasen et al. dictate the importance of Cx isotype and histological tumor subtypes for pro-tumorigenic properties of GJs and patient prognosis [82]. The team compared the mRNA expression levels of different Cxs between healthy lung tissue and lung tumors, and found that Cx26, Cx30.3, and Cx30 proteins were upregulated in lung adenocarcinoma (LUAD) and lung SCC. However, Cx32 proteins were slightly upregulated in LUAD and downregulated in lung SCC, thus highlighting the importance of Cx isotype and cancer subtype-specific [82]. Yamasaki et al. also observed that in many tumor cells, Cxs were normally expressed but aberrantly localized, possibly due to a lack of an appropriate cell-cell recognition apparatus, and phosphorylation of Cxs [83]. Cx phosphorylation is described to influence GJs formation, connexon function, and GJs/Cxs degradation [84]. Thus, the pro-tumorigenic properties of Cxs do not only depend on the pathological overexpression of specific Cx isotypes and cancer stage, but also on tumor subtype and Cxs delocalization and phosphorylation.

It is critical to acknowledge that the role of Cxs and GJs in cancer development and progression is highly complex, nuanced, and far from being fully understood. A comprehensive view must be taken to develop strategies that modulate GJs during cancer metastasis, and in-depth studies are required to reveal under which conditions Cxs and GJs may act as tumor promoter.

#### 4. Anti-tumorigenic properties of Cxs and GJs

Apart from the pro-tumorigenic properties, studies have demonstrated that Cxs and GJs can also have anti-tumorigenic properties in specific structural and tumoral context. Transfection of Cx30 proteins into rat glioma cell lines, which have lost their Cx expression, reduced tumor cell growth and proliferation [85]. Likewise, transfection of Cx26 proteins into human hepatoma cells [86] and breast cancer cells [87] reduced their malignant potential, by inhibiting dedifferentiation, suppressing cell proliferation and tumor growth, and inducing apoptosis [86,87]. Mesnil et al. reported that out of several transfected Cx subtypes (e.g. Cx26, Cx40, Cx43), only the Cx26 proteins inhibited tumor growth and proliferation in HeLa cells, both *in vitro* and *in vivo* [88]. Moreover, different Cx species or combinations of them are expressed in

different tumor types, suggesting that Cxs have cancer type-specific roles. For example, Cx43 proteins had no effect on the proliferation of tumorigenic rat insulinoma cells [89], while they were able to reduce tumor growth and proliferation in human breast cancer tumors [90]. Taken together, these results suggest that Cxs can also act as tumor suppressors, dependent on Cx isotype, Cx expression levels, and cancer type.

Interestingly, GJs in solid tumors are frequently decreased or missing in different cell populations within a tumor, suggesting that the loss of GJ coupling and may be another characteristic of malignant transformation. Jamakosmanovic and Loewenstein observed that the electrical coupling found in normal thyroid cells was lost in thyroid cancer [91]. This phenomenon was also observed in cancerous and non-cancerous epithelial cells [92]. These results suggest that inhibition of intercellular communication through GJs in cancer cells likely affect tissue growth and differentiation. Restoration of GJs have therefore, resulted in a decrease in tumor progression. Daniel-Wójcik et al. demonstrated a direct link between Cx43-GJ coupling intensity and cellular motility, a crucial aspect in cancer progression. An increase in Cx43 proteins at the cell-to-cell contact site and an enhancement of Cx43-GJs inhibited cancer cell motility in both prostate carcinoma and melanoma cells [93]. Another aspect to consider is that Cx-mediated tumor growth suppression can occur in a manner independent of GJ formation. Although Cx26, Cx40, and Cx43 protein-associated GJs increased in HeLa cells, only Cx26 proteins inhibited tumor growth and proliferation [88]. The authors observed that all three transfected Cxs communicated to a similar extent via GJ coupling, so the difference in their tumorigenicity may be related, at least in part, to the pattern of Cxs localization in the cells [88]. Altogether, these results demonstrated that GJs also have anti-tumorigenic properties, GJs-independent mechanisms of tumor suppression must be considered in order to find adequate therapeutic targets.

Tumor-suppressing properties are also described to be specific to Cx subtypes. Sirnes et al. demonstrated a mechanism by which Cx43 proteins specifically were able to reduce tumor growth and induce apoptosis in colon cancer cells. Cx43 proteins were found to be partly colocalized with  $\beta$ -catenin at the plasma membrane and inhibited Wnt signaling [94]. Wnt signaling has a key role in disease development and deregulation of the pathway connected to cancer and metastasis [95].  $\beta$ -catenin is one of the proteins which regulate Wnt signaling [95], and thus inhibition of Wnt signaling by  $\beta$ -catenin targeting via Cx43 upregulation may suppress tumor development. Another mechanism by which Cxs and GJs may act as tumor suppressor has been proposed for the Cx37 subtype. Burt and co-workers reported that Cx37 protein expression suppressed cell proliferation in tumorigenic rat insulinoma cells by significantly extending the duration of some phases of the cell cycle (gap 1 (G<sub>1</sub>), synthesis (S), and gap 2 (G<sub>2</sub>)) and accumulating cells at the G<sub>1</sub>/S checkpoint [89]. Since cancer cells affect the normal interphase processes [96], extension of several cell cycle phases may slow down the progression of malignant cells. In addition, cell proliferation was also suppressed via the CT domain and pore-forming domains of Cx37 proteins, independently of connexon or GJ formation [97]. Therefore, Cx37 proteins can suppress tumor proliferation and growth by different mechanisms, such as affecting the interphase processes of the cell cycle and modulation of both the CT domain and pore-forming domains of Cx37 proteins. Lastly, the Cx32 protein also demonstrated tumoricidal effects in human gastric cancer cells, inhibiting cancer cell proliferation through G<sub>1</sub> phase arrest and upregulation of p21 (Cip1) and p27 (Kip1) proteins, i.e., stoichiometric cyclin-dependent kinase inhibitors [98]. Yang et al. also reported that the Cx32 protein inhibits the highly-invasive malignant phenotype of hepatocellular carcinoma (HCC) both *in vitro* and *in vivo* by negatively regulating epithelial-to-mesenchymal transition (EMT), via downregulation of Snail signaling through the Wnt/ $\beta$ -catenin pathway [99]. EMT refers to the transformation to a more mesenchymal-like phenotypic, resulting in increased cellular motility and invasiveness. Since EMT plays a crucial

role in liver cancer invasion and metastasis [100], negative regulation of EMT by Cx32 proteins is very appealing to inhibit cancer progression in HCC. Thus, Cx32 proteins can suppress cancer proliferation by affecting the G<sub>1</sub> phase of cell cycle, upregulation of p21 and p27 proteins, and downregulation of the EMT.

Taken together, these findings support the growing concept that Cxs and GJs have several significant tumor-suppressive functions, but also highlight that the GJ-mediated tumor-suppressive function depends on Cx isotypes and cancer type. In fact, higher Cx expression levels are linked to a better cancer prognosis, while lower Cx expression levels correspond to a worse prognosis. This is summarized in Table 1 for various Cx up- and down regulations and various cancer types, based on available literature.

One of the mechanisms to explain why Cx expression and GJs are reduced in cancer cells is based on alterations in the activity of post-transcriptional factors of Cx-targeting microRNAs (miRNAs). Fukuda and co-workers reported for the first time the capability of miRNAs,

**Table 1**  
Relationship between Cx expression and cancer prognosis. Arrow direction corresponds to up- or down-regulation of Cxs. *p*-values correspond to the probability of obtaining that prognosis result.

Connexin (gene)	Cancer type	Prognosis	<i>p</i> -value	Type of test
Cx26 ( <i>GJB2</i> ) ↓	Breast <sup>a</sup>	Unfavorable	$<1.00 \times 10^{-4}$	Mice
	Glioma <sup>b</sup>	Unfavorable	$4.45 \times 10^{-4}$	Clinical
	Lung <sup>b</sup>	Unfavorable	$6.25 \times 10^{-4}$	Clinical
	Pancreatic <sup>b</sup>	Unfavorable	$2.90 \times 10^{-4}$	Clinical
Cx31 ( <i>GJB3</i> ) ↓	Renal <sup>b</sup>	Unfavorable	$2.53 \times 10^{-6}$	Clinical
	LUAD <sup>b</sup>	Unfavorable	$3.54 \times 10^{-8}$	Clinical
	Lung <sup>b</sup>	Unfavorable	$4.56 \times 10^{-4}$	Clinical
Cx32 ( <i>GJB1</i> ) ↑	Pancreatic <sup>b</sup>	Unfavorable	$7.56 \times 10^{-5}$	Clinical
	KIRC <sup>b</sup>	Favorable	$4.88 \times 10^{-8}$	Clinical
	Renal <sup>b</sup>	Favorable	$2.85 \times 10^{-6}$	Clinical
Cx32 ( <i>GJB1</i> ) ↓	Lung <sup>c</sup>	Favorable	$\leq 5.00 \times 10^{-2}$	Mice
	HCC <sup>d</sup>	Unfavorable	$<5.00 \times 10^{-2}$	Clinical
	Intestinal <sup>e</sup>	Unfavorable	$6.60 \times 10^{-2}$	Mice
Cx43 ( <i>GJA1</i> ) ↑	Liver <sup>f</sup>	Unfavorable	$<5.00 \times 10^{-2}$	Mice
	Ovarian <sup>g</sup>	Unfavorable	$<5.00 \times 10^{-2}$	Clinical
	Bladder <sup>h</sup>	Favorable	$1.00 \times 10^{-3}$	Clinical
Cx43 ( <i>GJA1</i> ) ↓	KIRC <sup>b</sup>	Favorable	$2.60 \times 10^{-4}$	Clinical
	Melanoma <sup>i</sup>	Favorable	$5.00 \times 10^{-2}$	Mice
	Bone metastases <sup>j</sup>	Unfavorable	$1.00 \times 10^{-8}$	Mice
Cx45 ( <i>GJC1</i> ) ↓	Colorectal <sup>k</sup>	Unfavorable	$<5.00 \times 10^{-2}$	Mice
	Glioma <sup>l</sup>	Unfavorable	Not available	Clinical
	LUAD <sup>m</sup>	Unfavorable	$2.00 \times 10^{-2}$	Mice
	Lung <sup>n</sup>	Unfavorable	$3.50 \times 10^{-2}$	Clinical
	Stomach <sup>b</sup>	Unfavorable	$4.99 \times 10^{-5}$	Clinical
Cx45 ( <i>GJC1</i> ) ↓	Renal <sup>b</sup>	Unfavorable	$3.55 \times 10^{-8}$	Clinical
	Urothelial <sup>b</sup>	Unfavorable	$7.83 \times 10^{-4}$	Clinical

<sup>a</sup> [101].

<sup>b</sup> Data were taken from “The Human Protein Atlas” website, after analysis by Uhlen et al. [102].

<sup>c</sup> [103].

<sup>d</sup> [104].

<sup>e</sup> [105].

<sup>f</sup> [106].

<sup>g</sup> [107].

<sup>h</sup> [108].

<sup>i</sup> [109].

<sup>j</sup> [110].

<sup>k</sup> [111].

<sup>l</sup> [112].

<sup>m</sup> [113].

<sup>n</sup> [114].

produced by normal cells, to attenuate tumor progression after transfer to malignant cells through a Cx-dependent mechanism [115]. Another explanation for the depletion of Cxs in cancer cells is based on transcriptional and post-translational modifications, such as phosphorylation, acetylation and palmitoylation [116], and methylation [117]. It was demonstrated that many growth factors, oncogenes, and tumor-promoting chemicals were potent inducers of Cx phosphorylation, which is often associated with the inhibition of GJs [84].

The mechanism underlying the tumor-suppressive functions of Cxs and GJs are highly complex, involving several properties intrinsic to Cxs proteins and other proteins surrounding it. Different electrical potential of GJIC, pore diameters in the connexons, and post-translational modifications of the Cxs conformations may also be responsible for changes in anti-tumorigenic properties of GJs. Such changes must be considered at both cellular and molecular levels for novel therapy development. To summarize, Cxs and GJs can act as pro- and anti-tumorigenic agents, depending on many factors such as GJs, cancer and tumor (Fig. 4).

## 5. Therapeutic strategies applied to Cxs and GJs

Novel combination strategies to restore GJs and Cx properties as tumor-suppressors in early cancer stages, or inhibit these structures in advanced stages when they display a tumor-supportive role have been studied to improve standard-of-care treatments such as chemotherapy and radiotherapy.

While chemotherapy is limited by drug toxicity and development of therapy resistance [118], novel multi-modal approaches to improve treatment response and tolerance are under investigation. Herein, stimulation of Cxs and GJs expression, via gene therapy, has been explored to potentialize anti-cancer drug activity in cancer cells. For instance, Cx43 gene therapy, in which the Cx43 gene is transfected into target cells to promote expression, has been demonstrated to increase cell sensitivity to several chemotherapeutic agents, in different cancer types [16,119,120]. In prostate cancer, the combination of Cx43-expressing plasmid DNA and the chemotherapeutic agent docetaxel had significantly stronger anti-cancer effects compared to docetaxel alone, both *in vitro* and *in vivo* [16]. Notably, transfection of Cx43

proteins into the cells without docetaxel neither inhibited tumor growth nor increased GJs. However, combination therapy of Cx43 protein upregulation and docetaxel significantly inhibited cell growth and induced apoptotic cell death by downregulation of Bcl-2 expression, a protein that regulates cell apoptosis; and upregulation of caspase-3 activity, a protein which induces apoptosis, compared to docetaxel alone [16]. Later, gene therapy in colorectal cancer showed that Cx43 protein upregulation improves sensitivity for the chemotherapeutic drug paclitaxel. Transfection of Cx43 into the cells increased GJ function and subsequently the mitotic arrest, tubulin polymerization, and apoptotic effects of paclitaxel, compared to cells treated with paclitaxel or Cx43 proteins alone [119]. Cell death was induced by activation of the caspase-3 apoptotic pathway [119]. More recently, similar results were found in breast cancer, in which Cx43 overexpression could attenuate EMT and improve the sensitivity of cancer cells to the chemotherapeutic drug tamoxifen [120]. EMT is an important event to confer tamoxifen resistance, and overexpression of Cx43 proteins was sufficient to inhibit TGF- $\beta$ 1-induced EMT activation, and to retard PI3K/Akt activation, a signaling pathway that plays a vital role in initiating EMT and drug resistance in different malignancies [120]. Altogether, these results show that enhancing the tumor-suppressive functions of Cx43 proteins and GJs has the potential to be combined with chemotherapeutic agents in order to overcome chemoresistance.

Due to the paradoxical anti- and pro-tumorigenic role of Cxs and GJs in cancer cells, therapeutic strategies to inhibit Cxs and GJs when they may act as tumor promoter have also emerged [20,21]. Considering that the CT domain of Cxs plays a pivotal role in the regulation of GJ function [22,121,122], manipulation of its secondary structure can help in the regulation of GJ function. For instance, peptides that mimic the CT domain of Cxs have been used to block GJs function [22,23,28]. An example of a clinically tested therapeutic peptide is the alpha connexin carboxy-terminus 1 ( $\alpha$ CT1), a selective inhibitor of Cx43-GJs that mimics the CT domain of Cx43 proteins. Administration of  $\alpha$ CT1 restored the sensitivity of resistant glioblastoma cells to temozolomide chemotherapy [28]. The combination of  $\alpha$ CT1 and temozolomide induced autophagy and apoptosis in those tumor cells, through attenuation of Akt/MTOR activity, signaling pathway known to induce temozolomide-resistance [28]. Due to the tumor-sensitizing capacities, multiple cell-penetrating mimetic peptides targeting different Cx domains and Cx types are currently developed in an attempt to improve remaining shortcomings, like target specificity and selectivity [123]. Once these problems are overcome, Cx manipulation - and in specific Cx43 proteins - via mimetic peptides is a very promising combination strategy for tumoral management with clinical applications.

The use of Cxs-targeting antibodies has been another strategy to inhibit pathological GJ function and improve cancer therapies. Monoclonal antibodies to the EL-2 loop of Cx43 proteins (MABE2Cx43) are intensively studied for human glioblastoma. Using a human glioblastoma rat model, MABE2Cx43 monotherapy led to significant tumor reduction and prolonged animal survival, presumably via inhibition of specific functions of Cx43 proteins in the peritumoral zone [24]. Treatment with MABE2Cx43 in combination with radiotherapy further inhibited tumor development and prolonged the median survival, likely due to the increase in blood-brain barrier permeability for antibodies after irradiation of the brain and inhibition of migration and/or signaling pathways [18]. Interestingly, MABE2Cx43-temozolomide combination therapy attenuated the tumor-suppressive activity of both monotherapies. Since a portion of the cytotoxic drugs penetrate into the cell through connexon gating, MABE2Cx43 binding and blocking of Cx43-GJ formation could affect the permeation of drugs like temozolomide into the cells [18]. These results highlight that combinatorial strategies using antibodies can be used to improve standard-of-care therapies like chemotherapy and radiation, however competitive inhibition of binding sites by MABE2Cx43 needs to be circumvented to overcome antagonistic treatment effects.

Heterologous GJs established between cancer cells and healthy cell

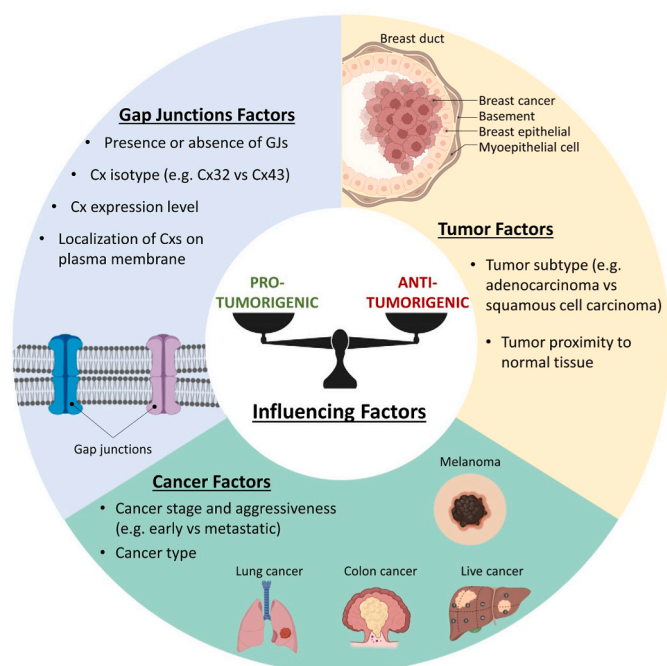


Fig. 4. Factors influencing the tumor-promoting and tumor-suppressing properties of Cxs and GJs.

populations are reported to promote tumor spreading and treatment resistance, making them interesting targets for therapeutic intervention (Fig. 1C, see figure caption for more details) [26,124]. Chen et al. demonstrated that breast and lung cancer cells were able to establish Cx43-GJs with astrocytes, promoting brain metastasis. Once the heterologous GJs were formed, cancer cells transferred the secondary messenger cGAMP to the healthy brain cells, thereby triggering paracrine signaling to promote tumor growth and chemoresistance [26]. Two modulators of GJs (i.e. meclofenamate and tonabersat) broke this paracrine loop, shown by inhibiting dye transfer from astrocytes to cancer cells and brain metastases [26]. This result suggests a chemoprotective mechanism mediated by heterologous Cx43-GJs in advanced cancer stages, and inhibition of this interaction has therapeutic potential.

In addition to the role of GJs to improve chemotherapy, Vance and Wiley suggested that ionizing radiation destroys not only targeted cells but also cells that have not been directly irradiated (the bystander effect) [125], and this effect is partially regulated by GJs [42], prompting GJIC as an appealing therapeutic target in combinatorial strategies with radiotherapy [126–128]. Zhang et al. found that iodide-induced upregulation of Cx43 protein expression and Cx43-GJ activity in genetically-modified non-small cell lung cancer cells significantly increased the bystander tumoricidal effects generated by ionizing radiation, thereby enhancing tumor cell killing both *in vitro* and *in vivo* [43]. Furthermore, the authors suggested that iodide could also modulate a cascade of molecular pathways including RONS signaling through Cx43-GJs, to further sensitize non-small cell lung cancer cells to ionizing radiation and chemotherapies like paclitaxel [43]. In concordance, experimental evidence suggested that GJs enhance the intercellular propagation of “death signals”, thereby expanding therapeutical cytotoxicity (Fig. 1A) [126–128]. Krutovskikh et al. observed that GJs propagate and increase cell death in rat bladder carcinoma cells, a cellular model that is predisposed to spontaneous apoptosis upon achieving confluency, by spreading cell-killing signals initially generated by a single apoptotic cell into healthy (non-apoptotic) surrounding cells [40]. In depth studies with a neuropeptide (oleamide) that selectively restricted GJs permeability to  $\text{Ca}^{2+}$  ions showed that the spreading of cell death was not prevented upon administration while Lucifer yellow dye transfer was blocked, suggesting that  $\text{Ca}^{2+}$  ions were the most probable cell-killing signals spread through GJs [40].

In summary, therapies that modulate Cxs and GJs could be a promising anti-cancer strategy, especially in combination with other conventional treatments such as chemotherapy and radiotherapy. However, further delineation of the conditions in which Cxs and GJs can act as anti- or pro-tumorigenic agents; and treatment-intrinsic difficulties like target selectivity and competitive inhibition are important issues to solve in order to fully optimize and implement them as cancer treatment.

## 6. Cxs and GJs in immune activation and immunotherapy

Engagement of the patient’s own immunity to recognize and eradicate malignant cells is a very promising anti-tumor strategy, which is highlighted by the prominent role of immunotherapy in the clinical management of cancer and development of new combination strategies. The formation of a stable immunological synapse (IS) enabling intercellular communication is one of the fundamental steps in the immune cell priming and activation process. This includes direct crosstalk between antigen presenting cells (APCs), and T cells and natural killer (NK) cells, or between target (e.g. malignant) cells with cytotoxic T lymphocytes (CTLs) and NK cells (Fig. 1B and D, see figure caption for more details) [129].

Several studies described a role of GJs in the antigenic peptide transfer and cross-presentation mechanism between target cells and APCs, whereby GJs are able to facilitate effective cell coupling and transport of antigenic peptides with lengths up to 16 amino acids when in extended formation (Fig. 1B, see figure caption for more details) [44,

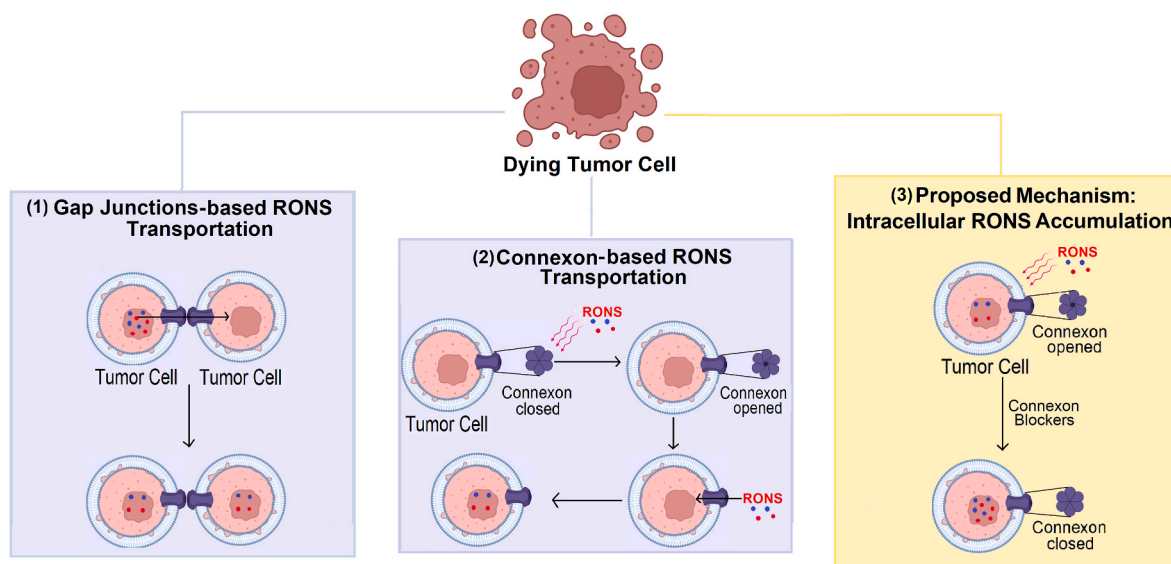
45]. Furthermore, functional GJs between DCs and cancer cells were reported in an *ex vivo* human melanoma model wherein antigen transfer between DCs led to activation and maturation of naive DCs, and subsequently specific CTLs engagement [10]. It was also found that GJs are required for DCs transfer secondary messengers to NK cells for subsequent NK cell activation, although the nature of these molecules is yet to unravel [130]. Mendoza-Naranjo et al. and others identified Cx43 proteins as the key Cx type mediating bidirectional GJs between DCs-DCs and DCs-T cells at the stimulatory IS, leading to antigen-dependent T cell activation, in both murine and human models (Fig. 1B, see figure caption for more details) [131–133]. It was found that efficient polarization of Cx43 proteins and subsequent functional Cx43-GJs in the cytotoxic IS between CTLs (or NK cells) and cancer cells are required for induction of granzyme B-mediated cell death in these target cells (Fig. 1D, see figure caption for more details) [134]. Further investigation into the underlying mechanisms revealed that Cx43 protein accumulation at different IS is antigen specific, time dependent, and requires an intact actin cytoskeleton. This process precedes a polarized  $\text{Ca}^{2+}$  influx, causing the granzyme B activity in the target cell via the NK cell/target cell lytic IS, while this mechanism is yet to be unraveled in the Cytotoxic T lymphocyte (CTL)-target cell synapse [130,135].

These data enlighten a previously underestimated role of GJs in alternative pathways for immune regulation and activation, and prompt these intercellular structures as potential targets for immunomodulating anti-cancer therapies. Illustrative of this potential is the recent finding that undifferentiated monocytes were able to elicit competent therapeutic CTL responses, solely when Cx43-GJs were established between tumor antigen-loaded monocytes and endogenous DCs in multiple *in vivo* mouse models [136]. In addition, a novel immunotherapeutic approach, based on immunogenic peptide release in the tumor microenvironment, pointed out that Cx43 protein overexpression and Cx43-GJs opening via post-translational modifications on target cells are required for the release of tumor-derived peptides and adequate anti-tumor responses in several model systems [137]. This research sheds light on the fact that besides mediating direct cell-cell contact, GJs have also a rather unexplored contribution in immunological processes. In addition, a role of other members of the Cx protein family cannot be ruled out, as research into this area is still very limited.

## 7. Oxidative stress on GJs as a cancer therapeutic strategy

One of the major roles of GJs, is the exchange of ions and small molecules between the cytoplasm of adjacently connected cells [51,56]. In this way, GJs may mediate RONS transfer between adjacent cells to cause cell death via oxidative stress [29–31] (Fig. 5 (1)). RONS (e.g.,  $\text{H}_2\text{O}_2$ ,  $\text{HO}^\bullet$ ,  $\text{HO}_2^\bullet$ ,  $\text{O}_2^{\bullet-}$ ,  $^1\text{O}_2$ ,  $^\bullet\text{NO}$ ) are products of normal cellular metabolism, generated within the mitochondria and cytoplasm. They are involved in cellular responses at physiological state [138], but elevated levels of RONS may lead to injurious oxidative stress; trigger damage to membrane lipids, proteins, and DNA; and ultimately can cause cell death [139]. When RONS permeate cell membranes, they can oxidize embedded proteins by direct reactions or indirect reactions with secondary products of oxidative stress, thus affecting membrane structure and dynamics. In particular, cysteine and methionine protein residues are more susceptible to oxidation, due to high reaction susceptibility of the sulfur group in those amino acids [140]. Oxidative modifications of proteins can change their physical and chemical properties, including conformation, structure, solubility, susceptibility to proteolysis, and enzyme activities. These modifications may represent the first step involved in mutagenesis and carcinogenesis [139]. On the other hand, protein oxidation has also been used as a therapeutic strategy to kill cancer cells, via inhibition of proliferative signaling pathways inside tumor cells [141] and reducing immunosuppressive signals on the surface of cancer cells [142].

Experimental reports have demonstrated that RONS can modulate GJs and connexons either via direct effects on channel gating (pore



**Fig. 5.** Schematic representation of the effects of RONS-induced oxidative stress on GJs and connexons. **(1)** GJs are able to transport RONS between cells. **(2)** RONS induce connexon opening to allow the entry of RONS to the cell interior. **(3)** Proposed mechanism to increase the intracellular RONS accumulation: Connexon blockers induce connexon close state and increase the intracellular accumulation of RONS, by entrance of them through the membrane and by natural intracellular production (e.g., within the mitochondria and cytoplasm). Taken together, these events induce cell death.

opening/closing) [32] or by affecting the intracellular trafficking of Cxs, impacting the number and stability of the GJ channel at the plasma membrane [143]. For instance, Ramachandran et al. demonstrated that  $H_2O_2$ -induced oxidative stress opens Cx43 proteins-composed connexons in fibroblastoid rat mammary tumor cell lines, via depolarization of the membrane potential. Consequently, the open connexons facilitated the entry of  $H_2O_2$  in the cell interior to cause cell death, probably through apoptosis [32]. This result stresses an impressive property of the GJ channel under oxidation stress, where an open state can improve cancer cell death through a higher entrance of RONS to the cell interior (Fig. 5 (2)).

Lowering intracellular redox potential has also been suggested to increase the opening of Cx43 proteins-composed connexons. Retamal et al. transfected Cx43 proteins into HeLa cells to evaluate the effect of reducing agents on dye uptake and connexon opening, and found that the redox molecule dithiothreitol increases the rate of dye uptake at the resting redox potential in cells expressing Cx43 proteins. Moreover, dithiothreitol increased the open probability of connexons at positive potentials. They found that the opening of connexons is regulated by the intracellular redox potential, which may act through the cysteine residues in the CT domain of Cx43 proteins [144]. Connexons can also be opened in  $Ca^{2+}$  ions deficient medium and dephosphorylation of critical residues [145,146].  $Ca^{2+}$  signaling is strongly intertwined with RONS signaling, and both play crucial roles in cell death; the entry of extracellular  $Ca^{2+}$  into the cell promotes RONS production [147,148]. Thus, molecules that increase intracellular  $Ca^{2+}$  may induce connexon activation and enhance intracellular RONS, increasing membrane permeability and eventually apoptotic cell death. Intracellular protein kinases can also affect the connexon opening probability [149,150], so small molecules that can trigger the activation of intracellular protein kinases could also indirectly modulate connexon activity. Hence, a potential approach in cancer therapy would be to enhance the connexon open state to improve oxidative stress-mediated cell death, though more studies focusing on the biophysical properties of potential connexon activators are necessary to improve their selectivity, solubility, permeability, and pharmacodynamics.

An open state of connexons can also contribute to the release of RONS and/or the activation of other signaling pathways which have a protective mechanism against cell death [33,151,152]. For example,  $H_2O_2$ -induced oxidative stress opened Cx43 proteins-composed

connexons in lens epithelial cells, mediating the exchange of oxidants and antioxidants in these cells undergoing oxidative stress [33]. These transporting activities facilitated a reduction of intracellular RONS accumulation and maintained the intracellular glutathione level, protecting lens against oxidative stress to prevent cataract formation during aging [33]. One therapeutic strategy to avoid this protective mechanism in cancer cells could be to design inhibitors that block connexons from opening during RONS-mediated oxidative stress, to increase intracellular accumulation of RONS (Fig. 5 (3)). In this way, monoclonal antibodies to the EL-2 loop of Cx43 proteins (173–208 amino acid residues) were developed, and they were demonstrated to block connexons from opening in glioma cells [153]. Moreover, these antibodies inhibited GJs formation, indicating that they react with target connexon solely [153]. Furthermore, it was shown that glioma cells presenting Cx43 proteins were more resistant to  $H_2O_2$ -induced oxidative stress, due to inhibition of caspase-3 activation; Cx43 proteins interacted with the upstream apoptosis signal-regulating kinase 1, known to mediate  $H_2O_2$ -induced apoptosis, providing a possible mechanism for the anti-apoptotic effect [151]. Interestingly, reducing the expression of Cx43 proteins with siRNA in cultured astrocytes sensitized these resistant cells to  $H_2O_2$ -mediated apoptosis, indicating that Cx43 proteins have an anti-apoptotic effect in normal astrocytes [151]. Thus, monoclonal antibody inhibitors of Cx43 proteins-composed connexon opening can be combined with oxidative stress-based cancer treatment, to improve cancer cell death. Therefore, the use of connexon blockers such as antibodies are also a promising therapeutic strategy during oxidative stress. However, further studies suggested that the use of antibodies should be treated carefully, as depending on the model, they may be considered anti- or pro-metastatic agents [154–156].

Considering the ability of GJs to enhance the intracellular accumulation of RONS, Wu et al. demonstrated that after PDT, the level of intracellular RONS was higher in HeLa cells with Cx32-GJs compared to those without. Hence, Cx32-GJs increased the efficacy of the treatment and this highlights the potential of GJs to transfer RONS to the cell interior [30] (Fig. 5 (1)). The same research group also observed that when Cx26 proteins were not expressed or if the Cx26-GJs were blocked, the phototoxicity of photofrin-mediated PDT in high-density cultures substantially reduced, emphasizing the importance of Cx26-GJs [157]. The GJs-mediated increase in PDT phototoxicity was associated with oxidative stress by RONS,  $Ca^{2+}$  ions, and lipid peroxide [157]. GJs have



been shown to propagate localized oxidative insults in endothelial cells, while stimulating de-novo generation of RONS in bystander cells [38]. Interestingly, the oxidative insult propagated through GJs for many hours, over hundreds of microns from the primary photogeneration site [38]. These results highlight an impressive property of GJs to propagate oxidative stress-induced cell death.

Cell exposure to ionizing radiation may affect mitochondrial and membrane oxidases, leading to oxidative stress. Thus, Autsavapornporn et al. studied the involvement of oxidative stress and GJs in enhancing toxicity in  $\alpha$ -particle-irradiated human fibroblast cells, and found that GJs were also able to propagate to neighboring cells the damaging effects of oxidative stress induced by  $\alpha$ -particles [158]. Inhibition of GJs or downregulation of Cx43 proteins protected the cells against the toxic effects, suggesting that GJs contribute to propagate radiation-induced cell death [158]. Therefore, designing strategies to increase GJs in cancer cells could improve the extension of cell death to neighboring cells, enhancing the efficiency of PDT or any other treatment based on oxidative stress, such as irradiation and NTP.

Overall, oxidative stress has a crucial effect on the function of GJs by inducing connexon opening to allow the entrance of RONS to cause cell injury and death. Afterwards, the use of inhibitors/blockers of connexons opening can increase the accumulation of intracellular RONS during oxidative stress, to enhance cell damage. To summarize, the oxidative damage caused by RONS on GJs can be used as a therapeutic strategy to induce cancer cell death, but their effects are dependent on the treatment type and may vary among different cancer types.

A promisor therapeutic strategy based on oxidative stress to overcome the resistance of various cancer types to traditional treatments such as radiotherapy, chemotherapy, and surgery [159] is the NTP, a promising therapeutic strategy being explored as a cancer (immuno-) therapy. NTP is a partially ionized gas composed of neutral gas molecules, positive and negative ions, free electrons, excited species and radicals. Of major importance for biomedical applications, including cancer therapy, is the multitude of short-lived and persistent RONS generated by NTP [36]. The observed anti-cancer effects of these RONS have been attributed to the therapeutic response of NTP on cancer cells [160], with a particular emphasis on the short-lived species (e.g.  $\text{HO}^\bullet$ ,  $\text{O}_2^{\bullet-}$ ,  $\bullet\text{NO}$ ) [161].

Despite advances in understanding the effect of RONS on GJs, some relevant questions pertaining to NTP treatment effect remain open. For instance: 1) How can RONS be transported through GJs? 2) Can RONS chemically react with amino acids present in connexons and affect the function of GJs? One method to investigate these questions is through the use of computational simulations. Xu et al. demonstrated the possible interaction of  $\text{HO}^\bullet$  and  $\text{HO}_2^\bullet$  with the NT domain of a Cx26 proteins-composed connexon via reactive MD simulations. They found that these radicals chemically react with the amino acids within the NT domain of Cx26 proteins, and can structurally damage them [162]. Considering the negative effects of Cxs and/or GJs upregulation in later cancer stages, structural damage induced by RONS can influence the effectiveness of GJs-mediated tumoricidal activities. In addition, Xu et al. also supported the hypothesis that NTP-generated RONS trigger the bystander effect based on GJs, highlighting again the potential role of GJs to propagate oxidative stress-mediated cell death [162]. However, additional studies will be necessary to elucidate the effects of connexon oxidation on GJs properties, as well as the mechanism of RONS transportation through GJs.

Bagati et al. reported additive-to-synergistic effects of a NTP combination therapy with the DNA-damaging agent tirapazamine in *in vitro* and *in vivo* metastatic melanoma cells, which underline the potential of NTP to improve cancer therapy via GJ modulation [163]. The authors observed that when high Cx26-GJs expression was induced in these cells, the combinatorial effects of NTP + tirapazamine therapy was augmented, spreading cell death. The presence of Cx26-GJs facilitated RONS cell penetration and signaling, while increased Cx26 protein expression and amplified tumoricidal activity [163]. Furthermore, they

also observed an immune response through differential regulation of cytokines and chemokines, suggesting potential for this therapy to induce a cytotoxic immune response [163]. Using a modified non-thermal helium plasma torch, the same research group showed that Cx43-GJs also contributes to NTP-induced cell death in melanoma cells [164]. They observed a higher sensitivity of these cells to RONS and a 6-fold increase in cell death by apoptosis compared with human keratinocytes [164]. Moreover, they observed an increased area of cell death, likely due to the bystander effect of passing apoptotic signals between cells [164].

Therefore, NTP therapy, and specifically NTP-generated RONS, have a great potential impact on the function of GJs via oxidative stress. A full understanding of RONS-GJs interactions can help in the modulation of their protecting and damaging mechanisms under oxidative stress, to improve NTP-based cell death. Computer simulations can be a useful and powerful tool to provide insights into these mechanisms, as well as their effects on membrane properties and the function of GJs.

To summarize, modulation of GJs can help inhibit or improve the pro- and anti-tumorigenic properties of GJs. Peptides, antibodies, and chemotherapeutic agents can be used to inhibit the pro-tumorigenic property of GJs, restoring the sensitivity of cancer cells for chemotherapeutic drugs and reducing tumor growth. Oxidative stress and the role of GJs to mediate the propagation of cell death and activation of the immune system can improve the anti-tumorigenic property of GJs, increasing cancer cell death. But their effects are dependent on the treatment type and may vary among different cancer types. The pro- and anti-tumorigenic properties of GJs have been explored to increase cancer cell death not only in traditional treatments, such as chemotherapy and radiotherapy, but as well as in novel treatments, such as PDT and NTP.

## 8. Conclusion

Overall, clinical, experimental, and modeling work performed up to now highlight the profound impact of GJs in the context of tumor development and progression. These studies confirm emerging concepts that GJs have pro- and anti-tumorigenic properties in cancer cells, which depend on GJ composition, cancer factors, and tumoral context. Thus, modulation of GJs can be used to inhibit or improve the pro- and anti-tumorigenic properties of GJs for anti-cancer responses. Peptides, antibodies, and chemotherapeutic agents have shown to be very useful in inhibiting the pro-tumorigenic properties of GJs, restoring the sensitivity of cancer cells to chemotherapeutic drugs and reducing tumor growth. The role of GJs to mediate the transport of RONS between cells, the propagation of cell death, and the activation of the immune system, opens possibilities to modulate the function of GJs to improve the anti-tumorigenic property of GJs. Indeed, GJs have been advantageous for inducing cancer cell death via the transport of RONS to the cell interior and via the propagation of cell death induced by oxidative stress, apoptosis, and radiation.

The success of future cancer treatments may be improved by understanding the underlying mechanisms involving Cxs and the function of GJs in cancer cells, which if accurately determined would lead to better therapeutic targets and strategies for each specific treatment cases. Although significant progress has been made towards understanding these topics, challenges remain to be addressed, such as when Cxs and GJs are pro- and anti-tumorigenic, how cancer therapies can modulate these properties, how RONS are transported through GJs to mediate oxidative stress-induced cell death, and how GJs propagate cell death. Thus, additional studies are necessary to elucidate the underlying mechanisms of the pro- and anti-tumorigenic properties of GJs. This will contribute to designing better GJs inhibitors and/or activators to improve traditional cancer treatments, such as chemotherapy and radiotherapy, as well novel cancer treatments based on oxidative stress, such as PDT and NTP.

## Conflicts of interest

None Declared.

## Data availability

Data will be made available on request.

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