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REVIEW

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Connexins in melanoma: Potential role of Cx46 in its aggressiveness

Viviana P. Orellana[1,2] | Andrés Tittarelli[3] | Mauricio A. Retamal[1,2]

[1]Universidad del Desarrollo. Centro de Fisiología Celular e Integrativa, Clinica Alemana Facultad de Medicina, Santiago, Chile

[2]Universidad del Desarrollo. Programa de Comunicación Celular en Cáncer, Clínica Alemana Facultad de Medicina, Santiago, Chile

[3]Programa Institucional de Fomento a la Investigación, Desarrollo e Innovación (PIDi), Universidad Tecnológica Metropolitana (UTEM), Santiago, Chile

Correspondence
Mauricio A. Retamal, Centro de Fisiología Celular e Integrativa and Programa de Comunicación Celular en Cáncer, Universidad del Desarrollo, Facultad de Medicina Clínica Alemana, Av. Las Condes 12438, Lo Barnechea, Santiago, Chile.
Email: mretamal@udd.cl

Andrés Tittarelli, atittarelli@utem.cl. Programa Institucional de Fomento a la Investigación, Desarrollo e Innovación (PIDi), Universidad Tecnológica Metropolitana (UTEM), Santiago, Chile. Ignacio Valdivieso 2409, San Joaquín, Santiago, Chile.

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Abstract

Melanoma is the most aggressive skin cancer, and in metastatic advanced states, it is completely refractory to chemotherapy. Therefore, it is relevant to understand the molecular bases that rule their aggressiveness. Connexins (Cxs) are proteins that under normal physiological conditions participate in intercellular communication, via the exchange of signaling molecules between the cytoplasm and extracellular milieu and the exchange of ions/second messengers between the cytoplasm of contacting cells. These proteins have shown important roles in cancer progression, chemo- and radiotherapy resistance, and metastasis. Accordingly, Cx26 and Cx43 seem to play important roles in melanoma progression and metastasis. On the other hand, Cx46 is typically expressed in the eye lens, where it seems to be associated with oxidative stress protection in fiber lens cells. However, in the last decade, Cx46 expression has been associated with breast and brain cancers, due to its role in potentiation of both extracellular vesicle release and cancer stem cell-like properties. In this review, we analyzed a potential role of Cx46 as a new biomarker and therapeutic target in melanoma.

KEYWORDS

connexins, Cx46, gap junctions, melanocytes, melanoma, skin

1 | CELLULAR ORGANIZATION OF THE SKIN

The skin is the largest organ in the human body, and it is constantly exposed to environmental insults, such as bacteria, viruses, air/water pollution, sunrays (Rodrigues et al., 2019). Three layers compose the skin: the epidermis, the dermis, and the hypodermis (Debeer et al., 2013). The epidermis is the outer layer of the skin, and it is the first barrier that protects the organism from external elements. Keratinocytes, melanocytes, Langerhans cells, and Merkel cells, arranged in five cellular layers, compose the epidermis. The deepest layer of the epidermis, the stratum basale, is constantly producing new cells that differentiate and migrate to the most external layer, named as stratum corneum, where they lose their nucleus and merge. In the normal skin, the most external cells detach constantly, taking around 2 weeks from birth in the Stratum basale to their loss in the Stratum corneum. The dermis is below the Stratum basale and has several functions, such as to feed the epidermis, to

give extra protection against physical trauma, body thermoregulation, and sensory and structural functions. Finally, the hypodermis, which is mostly conformed by fibroblasts, adipose, and immune cells, is highly vascularized and it is the skin area where the fat is stored (Debeer et al., 2013).

Melanocytes are specialized pigmented cells derived from the neural crest and normally are located at the Stratum basale of the epidermis. The main function of melanocytes is to produce melanin, a pigment that protects the organism from ultraviolet (UV) ray-induced damage. A single melanocyte can interact as much as 36 epidermal keratinocytes through their dendrites, which are distributed from the base to the top of the epidermal layers forming the "epidermal melanin unit" (Haass & Herlyn, 2005). Melanosomes are melanin pigment-containing organelles that are transported along with melanocyte dendrites toward keratinocytes for protecting these cells from UV radiation-induced damage. Under physiological conditions, undifferentiated keratinocytes from the Stratum basale regulate melanocyte homeostasis by secreting numerous factors in response to UV radiation, promoting proliferation, differentiation, survival, and melanin production in melanocytes (Kaidbey et al., 1979). Cell division is relatively infrequent in melanocytes but when it occurs, compresses a series of processes such as decoupling from keratinocytes, dendrite retraction, cell division and recoupling with neighboring keratinocytes to form a new epidermal melanin unit (Shain & Bastian, 2016).

2 | THE FUNCTION OF CONNEXINS IN THE SKIN

Connexins (Cxs) are a family of transmembrane proteins that exhibits four transmembrane domains, two extracellular loops, one cytoplasmic loop, and N-terminal and C-terminal portions at the cytoplasmic region (Retamal et al., 2015) (Figure 1a). Cxs are called by their predicted molecular weight (MW); for instance, Cx26 has a predicted MW of 26 kDa. The C-terminal portions of Cxs have the highest amino acidic variability and also present several sites for post-translational modifications (Beyer et al., 1990; Pogoda et al., 2016). Cxs oligomerize in hexamers in the endoplasmic reticulum or in the transit to the Golgi apparatus to form a hemichannel (Sáez et al., 2003). Once at the plasma membrane, hemichannels can dock to other hemichannels from neighboring cells forming a gap junction channel (GJC). The packing of GJCs in a restricted membrane zone is known as gap junction plaque or just gap junction (GJ) (Sáez et al., 2003). Because of their differential dispositions at the plasma membrane, hemichannels allow the bidirectional exchange of ions and small molecules between the intra and extracellular milieu, whereas GJCs allow the bidirectional exchange of ions and small molecules between the cytoplasm of adjacent cells (Retamal et al., 2015; Sáez et al., 2003). In general, these two types of Cx-formed channels present a permeability to molecules with a cutoff around 1.0–1.2 kDa (Sáez et al., 2010; Yeager & Nicholson, 1996);

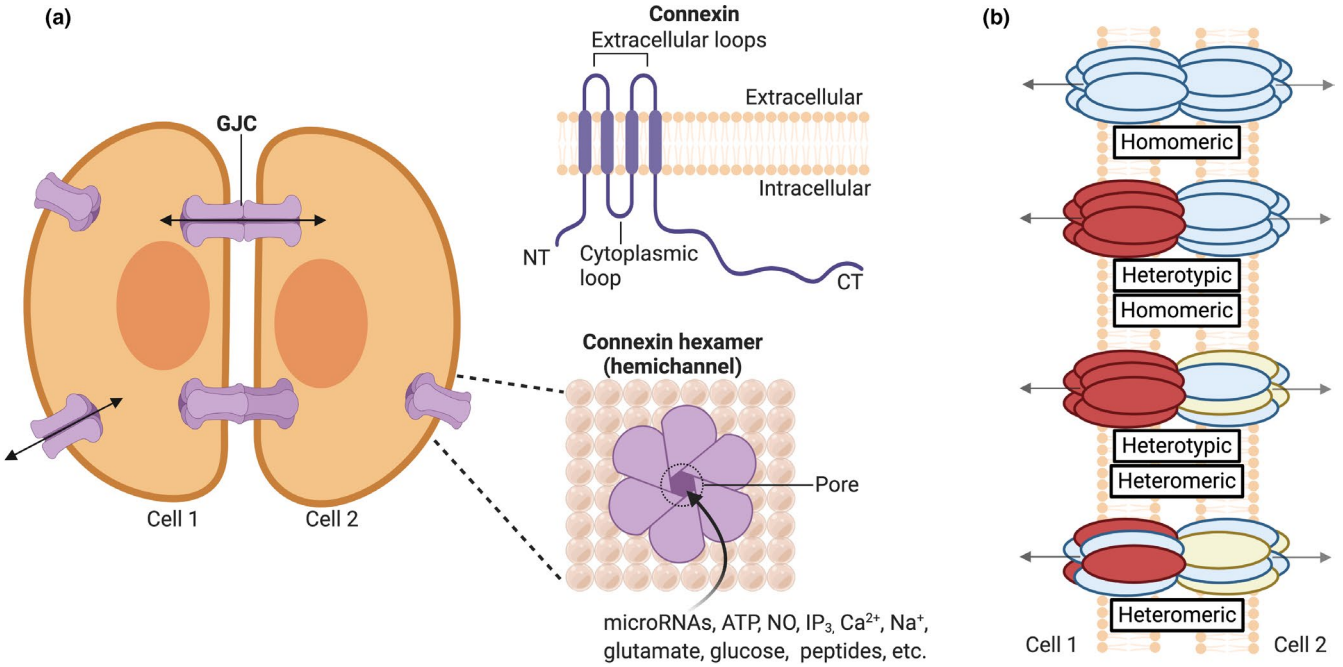

FIGURE 1 Connexins, hemichannels, and gap junctions. (a) Connexins (Cxs) are integral membrane proteins with cytosolic C-terminal (CT) and N-terminal (NT) domains. Six Cxs oligomerize to form a connexon or hemichannel. At the plasma membrane, the Cx-hemichannel can dock head to head with a Cx-hemichannel in an adjacent cell to form a gap junction intercellular channel (GJC). Through the pore formed by Cx-channels, small molecules of different nature can pass in a bidirectional manner. (b) Cxs can form four types of GJC: (i) homomeric, the hemichannels of the two connected cells are composed of the same type of Cx; (ii) heterotypic/homomeric, each hemichannel is composed of a type of Cx, which is different from the other cell hemichannel; (iii) heterotypic/heteromeric, one hemichannel is formed by a type of Cx, whereas the connected hemichannel from the adjacent cell is composed by a mix of different Cxs; and (iv) heteromeric, the hemichannels of the two connected cells are composed by mixed Cx types

consequently, molecules such as ATP, glutamate, glucose, microRNAs, peptides, and second messengers can pass through them (Figure 1a) (Fiori et al., 2012; Retamal et al., 2007; Sáez et al., 1989; Stout et al., 2002; Ye et al., 2003). For these reasons, hemichannels, as well as GJCs, participate in the regulation of multiple physiological cell functions, for example cell growth and adhesion, differentiation, migration, and apoptosis (Goodenough, 1992; Retamal et al., 2015; Sáez et al., 2003; Vinken et al., 2008; Zhou & Jiang, 2014).

Interestingly, Cxs can form hemichannels and GJCs made only by one type of Cx or by a mix of them with unique properties in terms of permeability and gating regulation (Cottrell & Burt, 2005; Koval et al., 2014) (Figure 1b). Formation of mixed hemichannels and GJCs is not a random mechanism, and on the contrary, it depends on a specific sequence in the intracellular loop—transmembrane loop 3 segment—and on the extracellular loop 2 (for more details, see Koval et al., 2014). In healthy human skin, several types of Cxs are expressed in keratinocytes (Cx26, Cx30, Cx30.3, Cx31, and Cx43), fibroblasts (Cx43), and melanocytes (Cx23, Cx26, Cx32, and Cx43) (Liovic et al., 2009; Wong et al., 2016). Therefore, there is a high possibility to find mixed hemichannels and/or GJCs in the skin. For instance, Cx26 and Cx30, which are expressed in the mice`s inner ear, co-localize and form heteromeric GJCs (Forge et al., 2003). So, there is no reason to suspect that Cx26 and Cx30 in the skin do not form heteromeric GJCs. Interestingly, Cx26 and Cx43 normally do not form heteromeric channels (Gemel et al., 2004); however, Cx26 H73R mutation causes palmoplantar keratoderma (PPK) and deafness, can form heterotypic hemichannels with an enhanced activity (Shuja et al., 2016). Similar results have been observed with Cx26 G12R, N14Y, and S17F mutations (García et al., 2015). We think that future experiments directed to clarify heteromeric/heterotypic hemichannels and GJCs properties and regulation in the skin are much needed.

Consistent with the expression of numerous Cxs, Lucifer yellow (GJ-permeable fluorescent molecule of ~457 g/mol) injected in a single keratinocyte can be transferred to 25–50 neighboring keratinocytes, confirming the presence of functional GJCs between these cells (Salomon et al., 1988). The role of Cxs in melanocyte biology is far to be understood. In co-cultures of keratinocytes with fibroblasts or melanocytes, only functional Cx43 GJCs between keratinocytes and melanocytes but not between keratinocytes and fibroblasts were observed (Hsu et al., 2000; Padma et al., 2015). The close contact between keratinocytes and melanocytes by E-cadherin and Cxs is associated with keratinocyte control over growth, morphology, melanin synthesis, and gene expression of melanocytes (Bogenrieder & Herlyn, 2002; Padma et al., 2015). On the other hand, the Cx mutant 41.8 (the Cx 40 zebrafish orthologous) impairs the communication between pigment cells, affecting cell shape, spatial organization, and cell-to-cell metabolic exchange, all inducing changes in zebrafish skin pigment pattern (Watanabe et al., 2006; Watanabe & Kondo, 2012). A similar role of GJs in skin pigment pattern has been shown in trout and avian, where GJs are required by pigment cells for shape transitions or regulation of the size and periodic pigment pattern, respectively (Djurdjevič et al., 2019; Inaba et al., 2019; Mahalwar et al., 2016). Taking into count the scarce knowledge about Cxs in melanocyte biology, we encourage the research community to study intensely this field to find new strategies to understand melanocyte malignant transformation.

The role of Cxs in the skin has been shown through the study of naturally occurred Cx mutations that affect skin function. Thus, for example, mutations in Cx26 (D50A, A88V, and G45E) are responsible for keratitis–ichthyosis–deafness (KID) syndrome, through an increase in hemichannel activity (leaky hemichannels) (Gerido et al., 2007; Mhaske et al., 2013; Retamal et al., 2015). Similarly, Cx43 mutations associated with leaky hemichannel formation also have been associated with skin diseases, such as Erythrokeratodermia Variabilis et Progressiva (EKVP) and Palmoplantar Keratoderma Congenital Alopecia-1 (PPKCA1) (Cocozzelli & White, 2019; Srinivas et al., 2019). The concept that leaky hemichannels are associated with skin disorders is also true for Cx30 (Essenfelder et al., 2004) and Cx31 (Chi et al., 2012). Leaky hemichannels can induce cell death by the loss of important metabolites (i.e., ATP) and the massive uptake of ions (i.e., Ca^{2+} and Na^{+}) (Retamal et al., 2015). On the other hand, under physiological conditions, Cxs are important in skin wound healing processes; thus, for example, downregulation of Cx43 promotes angiogenesis, epithelial-to-mesenchymal transition (EMT) processes, and cell proliferation (Mori et al., 2006). Contrary to Cx43, an increase in Cx26 expression has been associated with the upregulation of cell proliferation and EMT-associated mechanisms (Goliger & Paul, 1995). The association between Cx expression with cell proliferation, wound healing and EMT is very important also because the deregulation of these processes is one of the features of cancer. Indeed, since the initial observation that the absence of GJ-mediated intercellular communication (GJIC) is associated with increasing cell division in liver cancer cell lines (Loewenstein, 1979), Cxs were considered as tumor suppressors. Nowadays, the current concept is that Cxs could suppress or promote tumors, depending on the Cx isoform, cancer type, or cancer stage (Aasen et al., 2019; Wu & Wang, 2019).

3 | MELANOMA

Melanoma is the most aggressive and deadly skin cancer, which has shown a continuous increase in its incidence rate, more than any other type of solid cancer (Bray et al., 2018). It is responsible for about 75% of skin cancers deaths and predominantly affects fair-skinned populations living in countries exposed to high levels of UV radiation (Cosgarea et al., 2017; Ferlay et al., 2002). New Zealand and Australia have the highest incidence and mortality rates followed by North America and Northern Europe (Cosgarea et al., 2017). The survival rate for patients varies significantly depending on whether melanoma is detected in the early or late stages (Sandru et al., 2014). At early stages, the surgical resection represents an effective treatment, increasing the survival rate about 98%; however, if melanoma cells spread to lymph nodes, this falls to 64%, and in late stages, where melanoma colonizes distant

organs, the survival rate decreases dramatically to 23% (Global Cancer Observatory; https://gco.iarc.fr/). Melanoma tumorigenesis is associated with accumulative oncogenic DNA mutations and cellular damages induced by progressive exposure to the UV rays in melanocytes, which finally lead to the deregulation of growth regulatory genes, augmented secretion of autocrine growth factors, evasion of apoptotic signals, and immune response escape (Gray-Schofer et al., 2007; Shain & Bastian, 2016). Moreover, UV radiation impacts melanoma progression affecting the tumor microenvironment, causing a neutrophilic inflammatory response that promotes melanoma metastasis (Bald et al., 2014). On the other hand, the malignancy of melanocytes of the eye uvea (the iris, ciliary body, and choroid) leads to uveal melanoma, which despite sharing the same cell origin is biologically different from cutaneous melanoma (van der Kooij et al., 2019).

It is well known that metastatic melanoma is highly refractory to conventional therapies such as chemotherapy and radiotherapy due to its resistance to cytotoxic agents (Soengas & Lowe, 2003). Several studies using next-generation sequencing (NGS) have shown that melanoma possesses the highest burden of genetic and epigenetic modifications as compared to other cancer types (Lawrence et al., 2013). These molecular changes lead to the generation of different cancer cell clones, increasing intra- and intertumoral heterogeneity, and thus limiting the efficacy of target therapeutic approaches (Andor et al., 2016; Grzywa et al., 2017; Rizos et al., 2014). On the other hand, this high tumor mutational rate leads to neoantigen burden and presentation, which are associated with better responses to immunotherapy, particularly to immune-checkpoint blockade (ICB) (Cristescu et al., 2018; Van Allen et al., 2015). However, while ICB has resulted in durable clinical response in melanoma patients (Wolchok et al., 2017), these approaches are still limited to a subset of patients, because those can cause non-negligible side effects, including immunotoxicity, and a significant percentage of patients develops resistance to these therapies (Restifo et al., 2016. Therefore, the study of new therapeutic targets can increase the possibilities to find strategies with few side effects and/or that enhance the effect of the current treatments.

Melanoma cells can form GJCs with themselves and with fibroblasts, but do not with keratinocytes (Hsu et al., 2000), supporting the idea that Cx deregulation has a huge impact on the homeostatic control that keratinocytes exert over melanocytes. Moreover, melanoma cells establish GJIC with immune cells, such as natural killer (NK) cells, T cells, and dendritic cells (DCs), indicating that the immune system also controls melanoma progression by Cx-mediated mechanisms (Saccheri et al., 2010; Tittarelli et al., 2014; Tittarelli et al., 2015; Gleisner et al., 2017; Hofmann et al., 2019; Tittarelli et al., 2020; Navarrete et al., 2020). Several proteins that allow cell-to-cell communication have been described as key factors in tumor cell transformation. Thus, for example, cadherins are proteins that maintain cell anchorage, and, through this mechanism, help to control cell differentiation and EMT (Corso et al., 2020; Sommariva & Gagliano, 2020). In melanoma, the loss or gain of E-cadherin or N-cadherin has a great impact on GJIC (Li et al., 2002). In the next sections, we will discuss what is currently known about the role of the most relevant Cxs in melanoma pathology.

3.1 | Role of Cx26 in melanoma

Numerous reports indicate an important role for Cx26 in promoting melanoma metastasis. A study using murine melanoma B16-BL6 and B16-F10 cell lines described that these two cell types constitutively express Cx26, with BL6 having a higher expression (~5-fold) as compared to F10 cells (Ito et al., 2000). This result could suggest that these cells may form hemichannels and GJCs; however, BL6 and F10 show GJC-mediated dye transfer, neither with themselves nor in co-culture with fibroblasts or endothelial cells. These results were congruent with the lack of GJ structures observed by immunofluorescence (Ito et al., 2000). Interestingly, when BL6 cells were co-cultured with a vein tissue segment, they showed positive dye transfer, suggesting that they can establish GJIC under certain conditions. On the contrary, F10 cells were unable to form functional GJCs in the same conditions (Ito et al., 2000). Interestingly, whereas both melanoma cell lines showed the same metastatic capacity when injected through a mouse tail vein, only BL6 cells showed spontaneous metastasis to lung tissue in a metastasis footpath model (Ito et al., 2000 and Ito et al., 2004). Moreover, F10 melanoma cells transfected with Cx26 developed functional GJs in vitro and displayed a spontaneous metastatic phenotype similar to BL6 cells (Ito et al., 2000). Accordingly with these results, when BL6 cells were transfected with a Cx26-negative dominant form, their dye transfer capability in vitro was noticeably lower and their capacity to develop metastasis in vivo was abrogated, suggesting that Cx26 and its GJC formation play key roles in spontaneous metastasis capacity, and positions this Cx as an attractive target for melanoma therapy (Ito et al., 2000). To confirm this idea, GJC´s inhibitors as oleamide derivatives and Camellia oil fractions abrogate the in vivo spontaneous metastasis of BL6 cells by suppressing Cx26 GJ functional structures (Ito et al., 2004; Miura et al., 2007; Ohba et al., 2007). Moreover, another study revealed that Cx26 is necessary to induce B16 melanoma cell colony formation in vitro and melanoma brain colonization in chicken embryo models (Stoletov et al., 2013). In addition, the use of the GJ pharmacological blocker carbenoxolone (CBX) inhibited the capacity of B16 cells to colonize the brain by interfering with GJIC between the tumor and vessel cells (Stoletov et al., 2013).

A comparative study between melanotic and amelanotic canine oral melanomas showed that Cx26 mRNA expression levels were similar in these two melanoma types; however, the amelanotic cells possess lower Cx26 protein levels, correlating with a more aggressive phenotype compared with its melanotic counterpart (Teixeira et al., 2014). The authors of this work suggested an anti-tumorigenic role of Cx26. Interestingly, they also described that Cx26 immunostaining was observed not only at the plasma membrane but also in granular cytoplasmic marks, which was defined as an aberrant localization (Teixeira et al., 2014). Accordingly with this study, in a triple-negative breast cancer cell line, Cx26 localized in the cell nucleus

interacting with Nanog, a transcription factor associated with cancer stem cells (CSC) (Thiagarajan et al., 2018). Therefore, the final effect of Cx26 on cancer cell phenotype not only depends on its levels of mRNA or protein but also on its localization and its protein–protein interaction pattern.

In human melanoma, Cx26 expression levels are still controversial. Thus, some studies have found an upregulation of Cx26 in melanoma surrounding tissue (endothelial and keratinocyte cells) and undetected expression in melanoma cells, suggesting that Cx26 may not have an active role in melanocyte malignant transformation (Haass et al., 2006; Saito-Katsuragi et al., 2007; Sargen et al., 2013). However, association analysis revealed that Cx26 expression is related to melanoma advanced stages and the ulcerative phenotype, leading to poor patient prognosis (Haass et al., 2010). In addition, Cx26 could facilitate the intra- and extravasation of melanoma cells by GJIC with endothelial cells, favoring the establishment of new niches into distant organs, such as lung or brain tissues (Nojima et al., 2008). Following the same line of evidence, the Oncomine database analysis revealed a positive correlation between Cx26 in primary tumors with poor patient prognosis (Stoletov et al., 2013), supporting the idea that Cx26 could favor intra- and extravasation in endothelial cells (Haass et al., 2006; Ito et al., 2000; Nojima et al., 2008; Saito-Katsuragi et al., 2007). In conclusion, Cx26 expression in melanoma tissue (melanoma, endothelial, and fibroblast cells) promotes a metastatic cell phenotype and enhances the establishment of new tumor niches through cell-to-cell communication with the surrounding tissue.

3.2 | Role of Cx43 in melanoma

Cx43 is the most studied Cx type in human research, probably because it is the most ubiquitously expressed. There is a large number of studies showing that Cx43 has both pro- and anti-tumor roles (Crespin et al., 2010; Grek et al., 2016; Deen et al., 2019; Uzu et al., 2018), the current consensus is that the final effect of Cx43 in a given cancer will depend on different variables, such as cancer type, disease stages and the tumor microenvironment (Crespin et al., 2010; Gleisner et al., 2017; Uzu et al., 2018; Varela-Vázquez et al., 2020).

The role of Cx43 channels in melanoma progression is quite controversial and still is under deep investigation. Thus, on the one hand, recent in silico analysis revealed downregulation of Cx43 during melanoma progression (Kiszner et al., 2019). Accordingly, in primary cultures of human and mouse melanocytes, Cx43 was absent or its levels were lower enough not to be detected (Alaga et al., 2017). Interestingly, gene expression comparison between two human melanoma cell lines, UACC903 and modified induced Chr 6 UACC903 (+6), showed that the decrease in Cx43 enhanced the anchorage-independent growth of the malignant cells (Su et al., 2000). On the other hand, an immunohistochemical tissue microarray analysis over 272 pigmented lesions, including common nevus, atypical nevus, and melanoma, found an elevated expression of Cx43 in melanoma tissue along with all tumor stages (Rezze et al., 2011). Similarly, Cx43 was detected in melanoma cells that invaded lymph nodes and in metastatic niches in distant organs with a marked intracellular pattern; thus, Cx43 could have a pro-tumoral role in a GJIC-independent manner (Alaga et al., 2017). In addition, this apparent discrepancy could be due to differences between the antibodies used to detect Cx43, post-translational modifications, activated/inactivated intracellular pathways, or others. Therefore, the need to unify criteria to generate comparable results and thus determine the true role of Cx43 in melanoma becomes evident.

Regardless of whether the expression of Cx43 increases or decreases in human melanoma, it is clear that when Cx43 is present in melanoma cells, there is a decrease in some parameters associated with the metastatic process. Thus, for example, murine B16 melanoma cells exposed to hyper-adhesive substrates develop increased levels of Cx43-GJIC, which is associated with inhibition of cancer cell motility, suggesting that Cx43 could modulate the migration capacity involved in melanoma carcinogenesis (Daniel-Wójcik et al., 2008). Similarly, BL6 cells transfected with Cx43 showed a reduction in tumorigenic properties as anchorage-independent growth, proliferation, and primary tumor development, in heterocellular co-cultures with keratinocytes and independently of GJIC (Ableser et al., 2014). Additional reports, using melanoma cells derived from patients, confirm that Cx43 expression downregulates cell growth and metastatic potential in vitro and in vivo (Tittarelli, Guerrero, et al., 2015). Moreover, melanoma cells overexpressing Cx43 display higher basal and TNF-α-induced apoptosis. Indeed, lung metastasis of melanoma cells expressing Cx43 developed more GJ plaques and showed more active caspase 3 staining than its Cx43-low cell counterpart (Tittarelli, Guerrero, et al., 2015). Further, the effects of Cx43 as tumor suppressor protein in melanoma cells can be reverted by specific Cx43 downregulation by miR-106a in melanoma cells (Wang et al., 2019). Yet, as mentioned before, the role of Cx43 in melanoma is not so simple; thus, when premalignant and malignant cells derived from the same melanoma patient were transfected with wild type or a mutant Cx43 (which cannot form functional GJ structures), an increase in both growth rate and invasiveness was observed in malignant but not in premalignant cells (Zucker et al., 2013). Therefore, both channel-dependent and channel-independent roles of Cx43 could depend on the melanoma tumor progression state.

Accordingly to the idea that Cx43 could have relevant roles in more advanced melanoma states, immunohistochemistry analysis from human choroidal melanoma and benign nevi has shown that Cx43 was strongly expressed in choroidal melanoma with marked expression in cells surrounding the blood vessels, suggesting Cx43-mediated endothelial–melanoma cell communication (Mou et al., 2011). Therefore, Cx43 expression may improve tumor cell interaction with its surrounding cells, increasing vascular attachment and favoring the formation of metastatic niches (Braeuer et al., 2011). Correspondingly with this idea, astrocytes have been proposed as facilitators of the settle down of metastatic cancer cells by mechanisms involving Cx43-GJIC. In human brain metastasis, astrocytes are surrounded by melanoma tumor tissue (Schackert

et al., 1990). Importantly, co-cultures of astrocytes and melanoma cells show cell-to-cell interactions by direct contact of multiple podia and functional GJIC structures (Lin et al., 2010). When these co-cultures were confronted with chemotherapy treatment, astrocytes act as protectors of the melanoma cells through a mechanism involving cytoplasmic Ca^{2+} exchange by Cx43-mediated GJIC, decreasing the melanoma cytotoxicity induced by chemotherapeutic drugs (Lin et al., 2010).

Melanoma cells can also communicate with other immune cells (in addition to astrocytes) by Cx43 channels. Indeed, it has been shown that Cx43 is the major Cx type expressed by immune system cells such as monocytes, DCs, NK cells, B cells, and T cells (Gleisner et al., 2017). Thus, Cx43 GJs have been implicated in melanoma antigen cross-presentation (Mendoza-Naranjo et al., 2007), DC maturation, and melanoma-associated antigen (MAA)-specific T-cell activation by DCs (Matsue et al., 2016; Mendoza-Naranjo et al., 2011). Human melanoma cells are capable to form Cx43 GJs with autologous endothelial cells when co-cultured in vitro, allowing the transfer of MAA from melanoma to endothelial cells and its cross-recognition and elimination by autologous MAA-specific cytotoxic T lymphocytes (CTL) (Benlalam et al., 2009). Interestingly, Cx43-GJs can be detected among melanoma and endothelial cells in metastatic biopsies from melanoma patients (Benlalam et al., 2013), suggesting that CTL-mediated elimination of endothelial cells may contribute to control melanoma progression. Moreover, overexpression of Cx43 in human melanoma cell lines under normoxic conditions increases its susceptibility to NK cell-mediated melanoma cell killing via stabilization of the immunological synapses (Tittarelli, Janji, et al., 2015). However, under hypoxic conditions, these cells were less susceptible to NK cell-mediated lysis due to selective degradation of Cx43-GJ by autophagy, and the subsequent destabilization of the immune synapse (Tittarelli, Janji, et al., 2015). Furthermore, the upregulation of Cx43 on murine and human melanoma cells by Salmonella infection has been associated with enhancing anti-melanoma immune responses and tumor control. Specifically, melanoma cells share processed tumor antigens with DCs through GIJC, which finally results in an efficient DC-mediated melanoma-specific T-cell activation and T-cell-mediated tumor cell elimination (Saccheri et al., 2010).

As mentioned before, some intra- and extracellular signaling can modify the role of Cx43 in melanoma, and therefore explicate the variable results obtained until now. Among them, endothelins (ETs) are a family of proteins that induce melanocyte migration and differentiation under physiological and malignant conditions (Cichorek et al., 2013). In melanoma cells, ETs decrease Cx43-mediated GJIC through its phosphorylation in a time-dependent manner (Bagnato et al., 2004; Rosanò et al., 2004). Contrarily, Cx43 can be induced by proteinase-activated receptor 1 (PAR-1), a molecule highly associated with the progression of several cancers including breast, colon, prostate, and melanoma (Villares et al., 2011). When in the highly metastatic human melanoma cells A375M and C8161, the expression of PAR-1 was silenced by specific shRNA, downregulation of Cx43 was also observed (Villares et al., 2009). Interestingly, PAR-1-silenced melanoma cells shown a decrease in both surface attachment capacity and GJ-mediated dye transfer; accordingly, the silencing of PAR-1 induced significantly lower melanoma tumor growth and reduced experimental lung metastasis in nude mice (Villares et al., 2008 and Villares et al., 2009). In addition, p54nrb is an RNA-binding nuclear protein that has Cx43 as one of its targets (Dong et al., 1993), inhibiting its translation in embryonic stages and melanoma. Interestingly, the levels of p54 are higher in advanced stages of melanoma (Schiffner et al., 2011). The silencing of p54 on melanoma cell lines upregulates Cx43 expression, which in turn is associated with lower cell proliferation, migration, apoptotic rate, and higher cell attachment to the matrix (Schiffner et al., 2011).

Altogether, these evidences suggest that Cx43 is a targeted candidate for melanoma treatment. Indeed, it has been shown that resveratrol, an anti-inflammatory molecule isolated from grapes, is capable of increasing Cx43 expression in human K1735 and murine B16F10 melanoma cells. The combination of resveratrol with cisplatin causes a potentiated apoptotic effect in vitro, and reduced tumor cell growth to a higher extent than cisplatin therapy alone in vivo (Cheng et al., 2015). Similar results were obtained using regular 5-FU anticancer therapy in combination with eicosapentaenoic acids (EPA) that upregulate Cx43, enhancing the drug cytotoxicity through the activation of mitogen-activated protein kinase (MAPK) signaling pathways (Yang et al., 2019). In addition, the bystander effect is the basis of gene suicide therapy success, where dying cells share toxic substrates via GJCs to neighbor cells, promoting massive cell death (Nardin et al., 2019). Accordingly, the treatment of B16 melanoma cells with tanshinone (Tan IIA) or dioscin (chemical substances extracted from roots), induces the upregulation of Cx43 and the consequent formation of functional Cx43-GJ-like structures that allow the bystander effect of gene suicide therapy and ionizing radiation therapies (Ohshima et al., 2012; Xiao et al., 2013 and Xiao et al., 2017). Moreover, Cx43 upregulation by dioscin treatments inhibits melanoma progression not only by suppressing melanoma cell malignancy but also by inducing the polarization of macrophages to M1 phenotype (Kou et al., 2017).

3.3 | Potential role of Cx46 in melanoma

Cx46 is transcribed from the GJA3 gene, which is located at chromosome 13 (13q12.11). This protein has a predicted MW of about 46 kDa; however, it can be modified by phosphorylations (Jiang et al., 1993). Thus, for example in mouse seminiferous tubule-enriched samples, Cx46 immunoreactive bands of 51 and 68 kDa were observed, and both bands were diminished after the addition of alkali phosphatase to the samples (Pelletier et al., 2015). However, it seems that other post-translational modifications such as carbonylation and S-nitrosylation do not change its MW (Retamal et al., 2009 and Retamal et al., 2020). In humans, the expression of Cx46 only has been reported in the eye lens (Berthoud & Ngezahayo, 2017). The lens is an avascular organ, and Cx46 GJCs help to mobilize ions and metabolites from the lens periphery to the fiber cells at the lens center (Beyer & Berthoud, 2014; Mathias et al., 2010). On the other

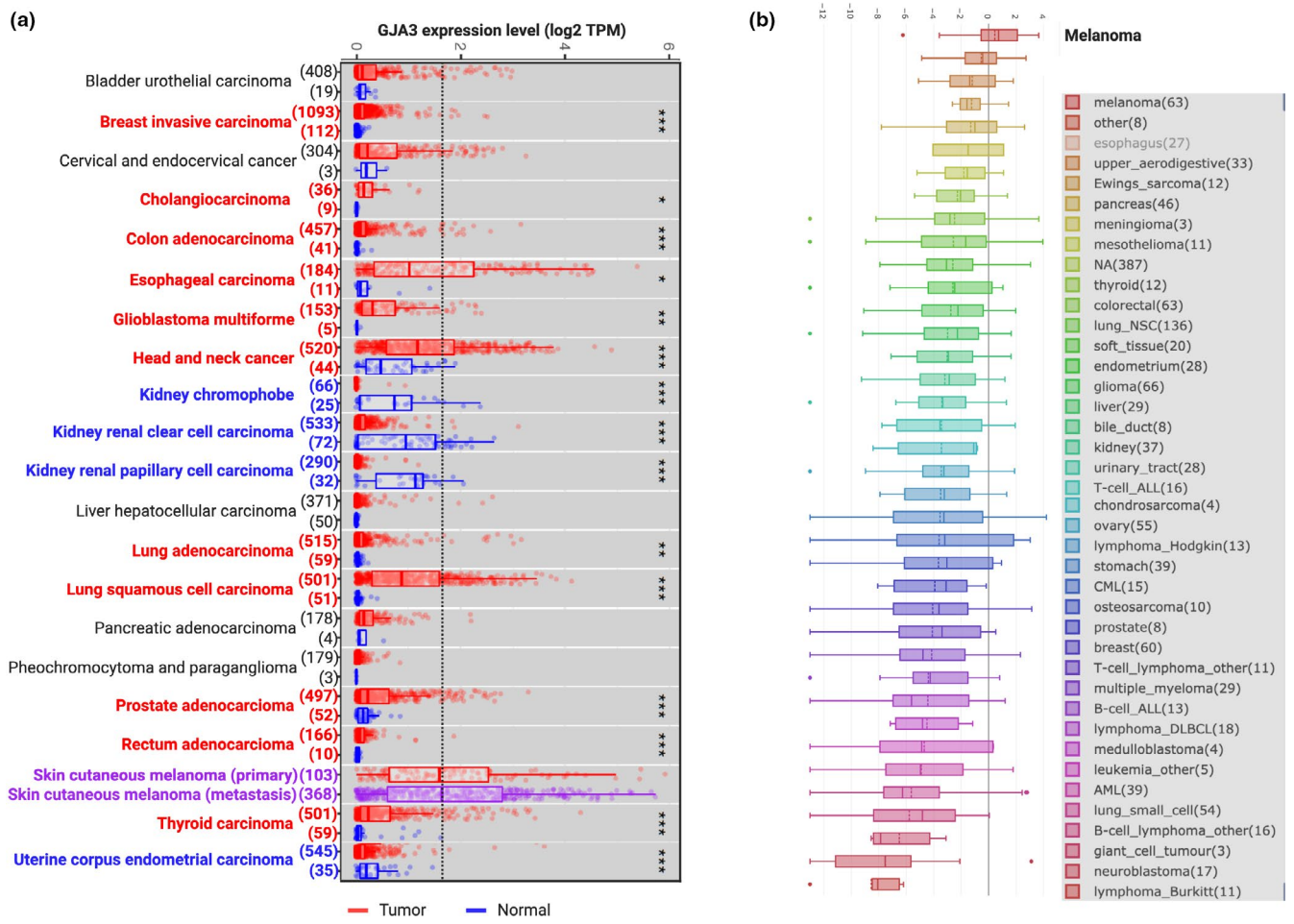

FIGURE 2 GJA3 expression in normal, cancer tissue, and cancer cell lines. (a) GJA3 differential expression levels between tumor and adjacent normal tissues (RNA-Seq analysis from the TCGA database using TIMER2.0). (b) GJA3 gene expression in a dataset of cancer lineages from cell lines. The number next to the lineage name indicates how many cell lines are in the lineage. The highest average distribution is on the left and is colored red. The dashed line within a box is the mean. Courtesy of The Cancer Cell Line Encyclopedia (Ghandi et al., 2019). Image available at the following URL: https://portals.broadinstitute.org/ccle/page?gene=GJA3

hand, Cx46 hemichannels help to the glutathione transport from lens fiber cells, which in turn help to protect them against oxidative stress (Shi et al., 2018). The malfunctioning of Cx46 is very well correlated with cataract formation in both animal models and humans (Gong et al., 1997; Minogue et al., 2005; Pal et al., 2000). Thus, several reports are showing that GJA3 gene mutations are very well correlated with cataract formation (Berthoud & Ngezahayo, 2017). Accordingly, mice lacking Cx46 developed nuclear cataracts associated with increasing intracellular Ca^{+2} concentration of about 1 μM in fiber cells (Gao et al., 2004) and crystalline aggregation and proteolysis due to activation of calcium-dependent proteases m-calpain and Lp82 (Gong et al., 1997).

In 2010, for the first time, Cx46 was correlated with the progression of human cancer (Banerjee et al., 2010). In this work, it was demonstrated that Cx46 protein levels are elevated in breast-infiltrating ductal carcinoma. Interestingly, when a human-derived breast cancer cell line (MCF-7) is exposed to shRNAs against Cx46, it becomes highly susceptible to hypoxia, suggested that Cx46 is a "protective factor" against low oxygen levels (Banerjee et al., 2010). Accordingly to a pro-tumorigenic role of Cx46, MCF-7 cells formed larger tumors in a xenograft mice model compared with MCF-7 cells treated with an anti-Cx46 shRNA (Banerjee et al., 2010). A similar effect was observed in a xenograft model using the Y79 retinoblastoma cell line (Burr et al., 2011). Supporting a malignant role of Cx46 in cancer cells, Acuña and co-workers (2020) demonstrated that Cx46-expressing MCF-7 cells released more extracellular vesicles (EV, likely exosomes) than its Cx46-negative MCF-7 counterpart, and these EV presented Cx46 in their membranes (Acuña et al., 2020). Interestingly, the presence of Cx46 in the EV enhanced the exchange of "information" between EV and target cells (Acuña et al., 2020). Despite all these evidences, it has not been found yet a correlation between the patient`s prognosis and Cx46 mRNA levels in breast cancer (Teleki et al., 2014). On the other hand, in human-derived glioblastoma cell lines, Cx46 controls CSC self-renewal by a GJIC-dependent mechanism (Hitomi et al., 2015; Mulkearns-Hubert et al., 2019). Interestingly, Cx43 and Cx46 seem to have opposite effects on CSC properties in glioblastoma (Hitomi et al., 2015). In summary, Cx46 seems to increase cancer aggressiveness through the

promotion of CSC-like properties and/or a protective role against hypoxia. Undoubtedly, future works correlating cancer patient prognosis should take into account both Cx46 protein and mRNA levels.

Under normal conditions, Cx46 is not expressed in any human skin cell type, and as far as we know, few reports are showing its expression in melanoma cells (Kiszner et al., 2019). Accordingly, using gene expression data of the Cancer Genome Atlas Program (TCGA) available in web analysis platform TIMER2.0 (http://timer.cistrome.org/; Li et al., 2020), we found that Cx46 mRNA levels are significantly upregulated in several tumors, including melanoma (Figure 2a), strongly suggest a potential role of Cx46 in melanoma biology. Then, we analyzed whether human melanoma cell lines express Cx46. To do that, we used the Cancer Cell Line Encyclopedia (ECCL) that possesses a database of mRNA expression from numerous cancer cell lines. We found that melanoma cell lines were the ones with the highest expression of Cx46 mRNA (Figure 2b), suggesting that melanoma cell lines can be used for the study of the role of Cx46 on melanoma biology. An analysis of TCGA data showed that, although GJA3 expression does not correlate with skin cutaneous melanoma patient's survival (Figure 3a), patients with uveal melanoma with higher GJA3 tumor expression have a significantly worse prognosis than those with lower GJA3 tumor expression (hazard ratio = 2.063, $p = .000055$; Figure 3b). The same analysis was performed on breast cancer and glioblastoma, where mRNA levels and patient survival did not correlate, although the experimental data strongly suggest that Cx46 protein expression is well correlated with an enhancement of cancer aggressiveness and CSC phenotype, respectively (Acuña et al., 2020; Banerjee et al., 2010; Hitomi et al., 2015; Mulkearns-Hubert et al., 2019). It is well accepted that not always mRNA levels correlate with their protein levels (Liu et al., 2016) and this discrepancy seems to be higher in cancer than in normal cells (Kosti et al., 2016). Another interesting question is whether Cx46 is important in all the stages of melanoma. Using TCGA data, we found that the Cx46 mRNA levels are high at stages I, II, and III and become low at stage IV (Figure 4), suggesting that Cx46 could have an important role in melanoma growth and early stages of metastasis. Therefore, we encourage the community to analyze the expression of Cx46 (as protein) in melanoma tumors and then correlate these results with patient survival.

3.3.1 | How Cx46 could modulate melanoma cell aggressiveness?

Channel-independent actions

In the last years, it has been noted that not only Cx expression levels but also the Cx channel-dependent and channel-independent functions must be considered in order to decipher their role in cancer. Thus, Cxs exert their channel-independent way of action mainly through the interaction with other proteins, and the vast majority of these protein interactions are mediated by the Cx C-terminal (hereafter named as free CT) (Giepmans et al., 2001; Hervé et al., 2004; Jiang & Gu, 2005; Kanemitsu et al., 1997; Moorby & Patel, 2001; Sorgen et al., 2018; Van Campenhout et al., 2020; Zhou & Jiang, 2014). Thus for example, in triple-negative breast cancer cells, Cx26 interaction with Nanog promotes CSC renewal (Thiagarajan et al., 2018). Similarly, Cx32 accumulation in the cytoplasm enhances CSC renewal of HuH7 hepatoma cells (Kawasaki et al., 2011), probably by a mechanism involving protein–protein interaction. Recently, it has been demonstrated that Cx-CT can be transcribed separately from the rest of the Cx (Salat-Canela et al., 2014; Ul-Hussain et al., 2012) through an mRNA IRES-entry site (Salat-Canela et al., 2014). This peptide has its own protein–protein interactions (Joshi-Mukherjee et al., 2007; Leithe et al., 2018). In this context, the Cx43-free CT increases glioma cell migratory capacity via its interaction with the actin cytoskeleton (Crespin et al., 2010). On the other hand, Cx43-free CT expression in U2OS osteosarcoma cells and HeLa cells decreased their cell division rate (Dang et al., 2003; Zhang et al., 2003). Similarly, a peptide derived from the CT of Cx43 (TAT-$Cx43_{266\text{-}283}$) reduces CSC properties via inhibition of c-Src in a patient-derived glioma model (Gangoso et al., 2014; Jaraíz-Rodríguez et al., 2017). In the case of Cx46, it has been observed that in the folliculostellate cell's nucleus, Cx46 interacts with Nopp-140, which is a transcription

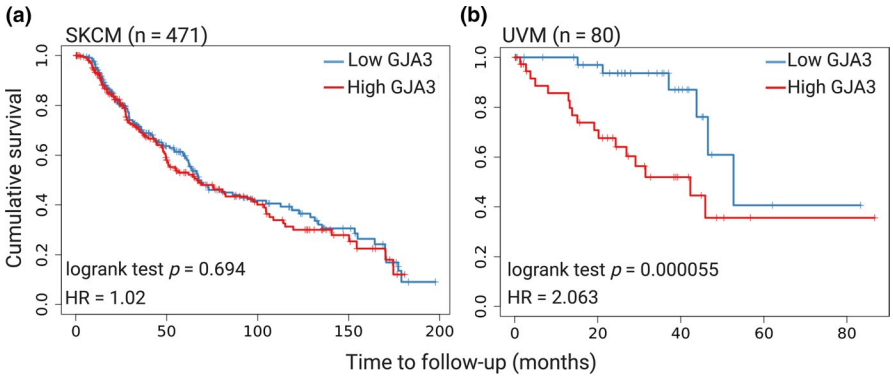

FIGURE 3 Correlation between GJA3 expression and melanoma patient survival. Kaplan–Meier survival plot of skin cutaneous melanoma (SKCM) and uveal melanoma (UVM) patients generated using TIMER2.0 resource. The Cancer Genome Atlas (TCGA) data of 471 SKCM and 80 UVM melanoma patients were assigned into low or high groups according to the expression level of GJA3 reported as RNA-Seq values.

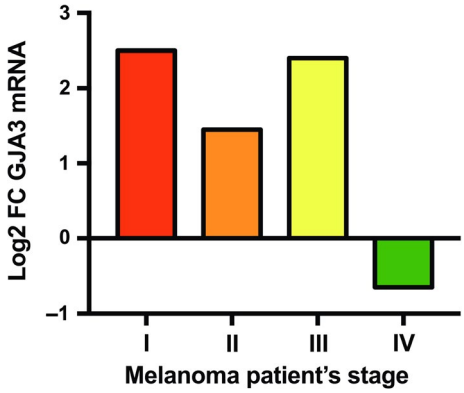

FIGURE 4 Differential gene expression of Cx46 mRNA over stages of melanoma. RNA-Seq analysis was performed using DESeq2 and cutoff $p \leq .05$ over FPKM data of melanoma stages and normal tissue samples obtained from The Cancer Genome Atlas (TCGA) repository.

factor involved in rRNA processing (Vitale et al., 2017). Thus, these data suggest that a channel-independent role of Cx46 in cancer cells cannot be ruled out.

Channel-dependent actions

In general, as mentioned before, it is well accepted that cancer cells do not form functional GJCs (Leithe et al., 2006). Actually when GJC communication is re-established by re-expression of Cxs, a reduction in cell division and decrease in cancer cell characteristics are observed (Eghbali et al., 1991; Tittarelli, Guerrero, et al., 2015; Yamasaki, 1990). Thus, for example, Cx43 re-expression and GJC formation inhibit CSC properties in lung cancer cells (Ruch, 2019). However, the case of Cx46 seems to be different, because as mentioned before, its expression is strongly associated with the establishment of CSC (Hitomi et al., 2015). Thus, Cx46 expression increases CSC´s proliferation, self-renewal, and tumor propagation in human glioblastoma (Hitomi et al., 2015). Importantly, these effects were inhibited by a Cx46-GJC blocker (Clofazimine), suggesting that Cx46-GJCs could be also involved in CSC. The CSC are important melanoma initiators and are responsible for chemotherapy resistance (Zhang et al., 2020). Therefore, it could be interesting to investigate whether in melanoma Cx46 could co-localize with CSC markers, such as aldehyde dehydrogenase (Zhang et al., 2020).

As mentioned, Cxs can also form functional hemichannels. On the contrary to the role of GJCs in cancer, the role of hemichannels has been poorly studied. Hemichannels are permeable to ions and signaling molecules. Thus, Cx26 and Cx43 hemichannels are permeable to Ca^{2+} (Sánchez et al., 2010; Schalper et al., 2010), which in the case of Cx26 can activate PI3k (Fig ueroa et al., 2013), a very important player in cancer progression, including in melanoma (Chamcheu et al., 2019). Another possibility is allowing the release of ATP to the extracellular milieu (Stout et al., 2002), which can activate P2X and P2Y receptors, both being able to increase the $[Ca^{2+}]_i$ (Burnstock, 1990). According to this model, it has been observed that Cx43 hemichannels—via ATP release—regulate H9c2 cell proliferation by an increase in $[Ca^{2+}]_i$ (Song et al., 2010). Additionally, it has been proposed that through the release of ATP, hemichannels can activate Akt (Chi et al., 2014) and PI3k (Fig ueroa et al., 2013) in NRK-E52 and HeLa cells, respectively. In melanoma, high expression of P2X7 has been reported, and its activation by extracellular ATP contributes to cancer cell survival (Gilbert et al., 2019). In addition, it is worth to mention that pannexins (Panxs), which are a family of three transmembrane proteins (Panx1, Panx2, and Panx3), share similar topology to Cxs, but only can form non-junctional hemichannels (Penuela et al., 2013). Panx1 is highly expressed in human melanoma tumors, and recently, it has been shown that blocking Panx1 channels by using probenecid reduced both ATP release and tumorigenic properties in melanoma cells (Freeman et al., 2019). Collectively, these evidences strongly suggest a pro-tumor role of ATP release by hemichannels (Cx- and Panx-formed) in melanoma aggressiveness. Although there is no information about the role of Cx46 as hemichannels in cancer, at least in HeLa cells, Cx46 can form functional hemichannels (Retamal et al., 2020). In summary, if expressed in melanoma cancer cells in vivo, Cx46 could be associated with a higher EV release and generation of CSC, through both channel-dependent and channel-independent ways.

Probably Cx26 and Cx43 are the most studied Cx types, and their biophysical and biochemical characteristics and control mechanism are well established. However, Cx46 has been much less studied; for instance, little is known about the effect of phosphorylations and other post-translational modifications on hemichannels and GJCs formed by this Cx type. Because of the growing amount of evidence that points to Cx46 as a key element in cancer aggressiveness, we encourage the scientific community to study more in detail this Cx type.

4 | CONCLUSIONS AND FUTURES DIRECTIONS

Melanoma is one of the deadliest cancer types, so the study of the molecular mechanisms that govern its aggressiveness is essential for the design of efficient treatments that improve patient survival. Cxs are proteins involved in cellular communication, which are crucial for the correct skin function. Among them, the deregulation of Cx26 and Cx43 has been associated with melanoma growth and metastasis, although its molecular mechanisms of action are still not well understood. Recently, Cx46 has been associated with breast and brain cancer malignancy through enhancing tumor growth, CSC-maintenance, and releasing of EVs. Nowadays, the role of Cx46 in melanoma has not been studied; however, we proposed that it could be relevant, based on the analysis of data available in public repositories, such as ECCL and the TCGA. Therefore, this work was designed to provoke the scientific community to explore the possibility that Cx46 could be a fundamental protein in the progression of melanoma, and thus to generate new therapeutic and diagnosis Cx46-related molecules, that in the future could increase the life expectancy of patients who suffer this type of cancer.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

VPO and MAR designed, wrote, and edited the paper; AT edited the paper.

ORCID

Mauricio A. Retamal https://orcid.org/0000-0003-2562-6686

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