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Review

Connexin-dependent intercellular stress signaling in tissue homeostasis and tumor development*

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Cellular stress responses determine tissue development, homeostasis and pathogenesis. Paracrine signaling, exchange of mechanical stimuli and intercellular transfer of small metabolites *via* connexin-built gap junctional channels are involved in the cellular stress detection and propagation of stress stimuli in multicellular networks. Cellular stress responses are also regulated through the activity of unpaired connexons (hemichannels) and *via* the intracellular interference of connexins with the cell cycle and pro-apoptotic machinery. Therefore, connexins are considered as multidirectional transmitters of the "outside-in" and "inside-out" stress signaling that are crucial for tissue homeostasis, regeneration and pathology. In particular, the disturbance of connexin function during the multi-stage process of tumor development leads to abnormal reactions of tumor cells to stress stimuli. In this review, we outline the current knowledge on the multidirectional role of connexins in the detection of stress signals. We also discuss the role of connexin-mediated intercellular transmittance of stress signals in tumour promotion, progression and metastatic cascade.

Highlights:

1. Connexins and gap junctions protect cells from the microenvironmental stress and are involved in propagation and intracellular processing of stress signals.

2. The quality and quantity of stress stimuli, which may lead to cell adaptation or death by apoptosis, is determined by intrinsic properties of connexins and the cell phenotype.

3. Connexin deficiency increases the resistance of tumor cells to the "outside-in" stress signaling.

4. The connexin-mediated "inside-out" stress signaling participates in tumor cell invasion during the metastatic cascade.

Key words: carcinogenesis; connexin; gap junctions; cellular stress; tumor

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Abbreviations: AKT, protein kinase B; AML cells, acute myeloid leukemia cells; ATRA, all-trans retinoic acid; Bax, bcl-2-like protein 4; Bcl2, B-cell lymphoma 2; cAMP, cyclic adenosine monophosphate; Cxs, connexins; EMT, epithelial-mesenchymal transition; ERK1/2, extracellular signal-regulated kinases 1/2; GJIC, gap junctional intercellular coupling; GSK-3β, glycogen synthase kinase 3 beta; HEK, human embryonic kidney cells; IP3, inositol trisphosphate; MAP, mitogen-activated protein kinase; MDR, multi-drug resistance; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; ROS, reactive oxygen species; STAT3, signal transducer and activator of transcription 3; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; TNF, tumor necrosis factor; TPhT, triphenylotin; ZO-1/2, tight junction protein 1/2

INTRODUCTION

Cells that reside in multicellular systems are exposed to miscellaneous stress signals. The way, a cell responds to exogenous stress stimuli is determined by their quality, amplitude and duration, as well as by a cellular long-term phenotype and momentary physiologic status. The initial response of cells to a stress stimulus aims at preserving their integrity through activation of survival pathways. However, when the noxious stimulus is unresolved, death signaling pathways that eventually eliminate damaged cells are activated. Cellular responses to stress stimuli (i.e., growth arrest, differentiation apoptosis, necrosis or autophagic cell death) depend on the "secular" ability of cells to manage stressful conditions and on their permanent phenotype (Fulda *et al.,* 2010; Samali *et al.,* 2010). Integrated intercellular communication networks propagate stress stimuli between cells and synchronize cellular stress reactions in tissues. They consist of membrane receptors of soluble factors, ion channels, juxtacrine receptors and gap junctional channels.

Gap junctions are semicrystalline clusters of intercellular channels, which consist of connexin family proteins (Sohl & Willecke, 2004). Human connexins (Cxs) represent a relatively conservative family of at least 20 integral membrane proteins ranging in molecular mass between 25 (Cx25) and 57 (Cx57) kDa. Connexins include four transmembrane α-helical domains, two intracellular termini, two extracellular and one intracellular loop. They spontaneously form hexameric hemichannels, the so-called connexons. When docking to its counterpart contributed by an adjacent cell, connexons form aqueous intercellular channels which mediate intercellular exchange of small (<1.5 kDa) metabolites and second messengers in all the vertebrate tissues (Fig. 1; Nakagawa *et al.,* 2010). These channels provide a route for metabolic and electrical synchronization of multicellular compartments in the process of gap junctional intercellular coupling (GJIC). Electrical coupling is crucial for synchronous excitability of tissues. In turn, metabolic coupling synchronizes cellular functions in non-excitable tissues through gap junction-mediated intercellular propagation of metabolites (Nielsen *et al.,* 2012). The gap junction-mediated "outside-in" and "inside-out" signaling participates in the formation of local multicellular networks and governs tissue development and homeostasis in all Metazoans (Nelson & Bissell, 2006). However, undocked connexons can also serve as membrane channels which couple the cells with their extracellular milieu (Saez *et al.,* 2005). Finally, single connexin molecules and their fragments act as effectors of intracellular signaling pathways that regulate cell proliferation, differentiation and apoptosis (Mroue *et al.,* 2011).

Accumulating reports show that gap junctions confer physiologic and pathogenic stress stimuli between cells and/or mediate intercellular exchange of information on tissue constraints (Dbouk *et al.,* 2009). Connexins also interfere with multiple pathways which regulate cell reactions to intracellular stress in the GJIC-independent manner. Cellular stress reactions are involved in developmental malformations and in chronic diseases, such as tumorogenesis (Lopez-Otin *et al.,* 2013). In this review, we outlined the involvement of connexins in the "outside-in" and "inside-out" stress signaling between cells and their microenvironment. We also discussed the involvement of connexins in the regulation of tumor cells reactivity to stress, in particular to (i) tissue constraints and to (ii) systemic defense systems. Finally, we outlined the role of connexins in the "inside-out" stress signaling that facilitates cancer cell invasion and metastasis.

THE ROLE OF CONNEXINS IN INTERCELLULAR STRESS SIGNALING

According to the canonical view on the functions of connexins, these proteins participate in cell adaptation to microenvironmental dynamics and to tissue constraints *via* constituting the routes for intercellular metabolic cooperation. For example, the gap junctional channels mediate the intercellular transfer of signaling molecules, nutrients and oxygen (Berthoud & Beyer, 2009). GJIC can locally limit cellular stress resulting from shortages of food and oxygen supply. Furthermore, gap junctions and unpaired connexons participate in the intercellular dissipation of metabolic products (e.g., CO_2 and urea), reactive oxygen species (ROS) and antioxidants. Reactive products of oxygen are amongst the most potent and omnipresent threats which cells face. Import of the ROS scavengers through gap junctions can help cells to recover from the pro-oxidant:antioxidant imbalance. GJIC intensity is determined by the abundance of connexins in cell-to-cell interfaces and selectivity of channels' conductance. The composition of connexons and phosphorylation status of connexin molecules affect the quality and quantity of the transmitted molecules in a cell context-specific manner (Fig. 1; Maeda & Tsukihara, 2011; Ek-Vitorin & Burt, 2013; Su & Lau, 2014). Together with GJIC-independent functions of undocked connexons and connexins localized in cytoplasm, GJIC-mediated metabolic coupling evokes tissue-specific protective responses at the cellular and tissue level. Connexin-modulated stress signaling regulates local tissue integrity and architecture (cell density and positioning, extracellular matrix properties, quality and quantity of physical cell-cell interactions) through the activation/inhibition of cell proliferation/differentiation and apoptosis. Below, we outline consequences of intrinsic sensitivity of connexin molecules to stress stimuli and of connexin-mediated inter- and intracellular stress signaling for the physiology of single cells and multicellular networks.

Cytoprotective connexin responses to stress stimuli

The efficiency of GJIC depends on the availability of gap junctional channels at cell-to-cell interfaces. Therefore, connexins need to be efficiently transported from the places of their synthesis to plasma membranes to fulfill these canonical (GJIC-dependent) functions. Connexins are usually synthesized in the perinuclear zone. Upon translation, they are incorporated into the membranes of endoplasmic reticulum. Connexins localized in the cytoplasm regulate numerous intracellular signaling pathways in a GJIC-independent manner (Dbouk *et al.*, 2009). Concomitantly, they spontaneously oligomerize into hexameric hemichannels (connexons; Laird, 2006; VanSlyke *et al.*, 2009), undergo post-translational modifications (i.e. glicosylation and acetylation) in the Golgi apparatus and are trafficked towards plasmalemmae, where they can either reside as unpaired connexons or form intercellular channels (Fig. 1). Vesicular transport of connexins is predominantly governed by microtubules, whereas the "gap junction proteome", i.e. proteins associated with gap junctions (such as β-catenin, ZO-1 and ZO-2, vinculin, myosins, small G proteins, kinases etc.), regulates the structure and functional status of the gap junctional plaques (Laird, 2006; Mroue *et al.*, 2011). Abundance of gap junctional channels in cellular interfaces is determined by the transport of newly-synthesized connexons, the stability of gap junctional plaques and the rate of their degradation. Stress signals affect GJIC *via* the effect on the expression and oligomerization of connexins, their intracellular trafficking, docking of connexons and recruitment of the gap junction proteome. Actually, numerous studies demonstrated that cells can activate or attenuate connexin turn-over in response to external stress (VanSlyke & Musil, 2005).

A relatively high rate of connexin turn-over enables cells to adapt GJIC efficiency to the dynamics of microenvironmental stress conditions (Leithe, 2016; Wong *et al.*, 2017). Additionally, the gating of gap junctional channels is often evoked by extreme deviations in the concentrations of ions, nutrients, oxygen, metabolic products, temperature, pH, and osmolarity of interstitial fluids (Chovatiya & Medzhitov, 2014). When a cell dies due to the exposure to permanent starvation, the inhibition of protein glycosylation, disturbance of Ca^{2+} homeostasis and/or oxygen deprivation, the intrinsic mechanisms which close gap junctions are activated (for review see: Oshima, 2014). Activity of Cx40, Cx43 and Cx45 channels in cardiac tissue constitute an example of this function. These connexins built gap junctions that enable rapid transfer of Ca^{++} waves between cardiac cells. Due to the accidental cell necrosis, intracellular Ca^{++} concentrations typically reach pathologic (milimolar) concentrations. Gap junctional channels are closed in such conditions, thus preventing Ca^{++} influx from dying to intact cells (Orellana *et al.*, 2012). A similar mechanism has been described in endothelial and epithelial cells, neurons and astrocytes, fibroblasts and muscle cells (for review see: Decrock *et al.*, 2011). Gap junctional permeability is also sensitive to pathologic changes in intracellular pH, Mg^{2+}, Cl^- and Na^+ levels (for review see: Oshima, 2014), to the reactive oxygen species (ROS), plant toxins and to heavy metal ions. For instance, H_2O_2-induced inhibition of gap-junction intercellular communication (GJIC) in liver epithelial cells (Kim *et al.*, 2016). The inhibitory effect of oxidative stress on GJIC was reported in neurons (Quintanilla *et al.*, 2012), lung (Johnson & Koval, 2009), lenses (Berthoud & Beyer, 2009) and cardiac cells (Pogoda *et al.*, 2016; Sovari, 2016). Moreover, administration of phorbol esters from croton oil attenuates GJIC in numerous cell types (for review see: Lampe, 1994). Cell-protective responses of gap junctions are also elicited by the challenges that are not stress signals themselves but can disrupt homeostasis (e.g. infections and allergens; Chovatiya & Medzhitov, 2014). Consequently, the attenuation of GJIC restricts the intercellular flux of harmful compounds, whereas the quality and quantity of intercellular transfer of cytoprotective and stress molecules is determined by the selectivity of channels' conductance.

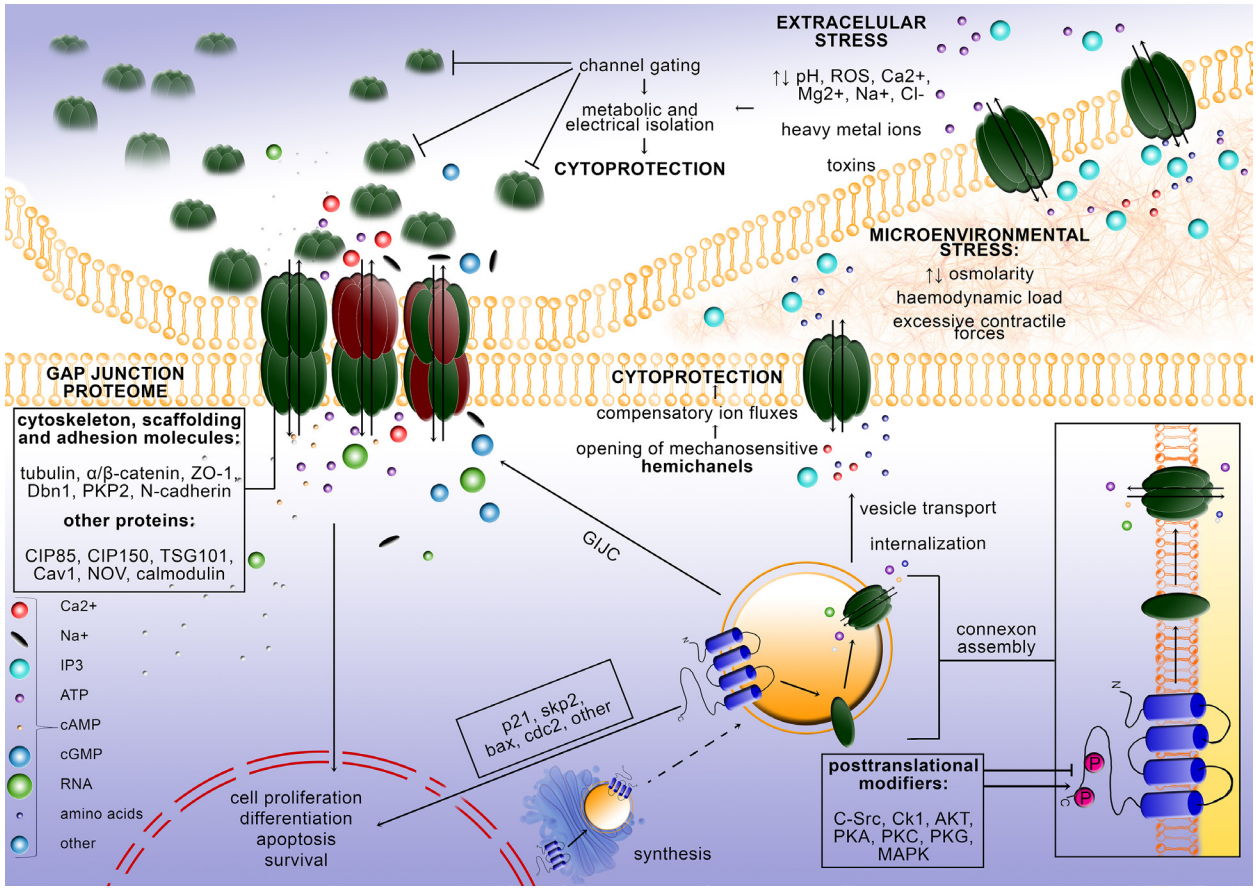

Figure 1. Gap-junction-dependent and gap-junction-independent involvement of connexins in stress signaling.
Oligomerized connexins interact with intercellular stress signaling pathways that control cell proliferation, differentiation and viability in a manner independent of gap junction-mediated intercellular coupling. When incorporated into plasmalemmae, connexons can act as paracrine conduits of intercellular stress signaling. Upon docking of two opposing connexons, an aqueous channel that enables intercellular propagation of stress stimuli is established. Compatibility of connexons and interactions of connexins with other structural and signaling molecules (i.e. the gap junction proteome) determines the channel permeability to stress signals. Abbreviations: C, C-terminus; Cdc2, cyclin-dependent kinase 1; IP_3, inositol trisphosphate; MAPK, mitogen-activated protein kinase; PKA, protein kinase A; PKC, protein kinase C; PKG, protein kinase G; skp2, S-phase kinase-associated protein 2; ZO-1, tight junction protein ZO-1.

Furthermore, unopposed connexons can fulfill cytoprotective function by facilitating cellular adaptation to hypoxia/reoxygenation, metabolic starvation and excessive mechanoosmotic stress. Connexin hemichannels mediate the rapid exchange of ions, second messengers and metabolites between the cell interior and interstitial space. They predominantly exist in a closed state under normal physiological conditions. However, it was reported that stress conditions cause the opening of hemichannels (Saez *et al.*, 2010; Retamal *et al.*, 2015; Pogoda *et al.*, 2016). Increased microenvironmental osmolarity, haemodynamic load and excessive contractile forces can open mechanosensitive hemichannels and trigger compensatory ion fluxes between the cells and their milieu (De Vuyst *et al.*, 2006; Evans *et al.*, 2006; Saez *et al.*, 2010; Plotkin & Stains, 2015). In this way, the cells regulate their osmolarity, adapt cytoskeletal architecture to the vectors of excessive mechanical load and prevent the activation of pathologic cell death signalling. Collectively, sensitivity of connexin trafficking and gap junction permeability to stress signals determines the involvement of connexins in tissue and cell homeostasis. Channel gating mechanisms protect the cells from extrinsic stress via regulation of the balance between intercellular cooperation and metabolic isolation (Sohl & Willecke, 2004; Maeda & Tsukihara, 2011). They participate in cell protection from toxic microenvironment, for instance from the "by-stander" propagation of cell death signals (Little, 2006). These mechanisms situate connexons and gap junctions in the category of effective and relatively universal sensors of cellular stress that isolate the cells from stressful conditions.

Connexin-mediated protective cell responses

Cellular responses to stress signals depend on their amplitude and the cell phenotype. They range from cell adaptation to necrotic or apoptotic cell death. Although channel perm-selectivity determines the quality and amplitude of stress signals received by cells, the connexin-dependent regulatory system extends beyond the rapid channel opening and closure events associated with channel gating. As already mentioned, GJIC-dependent intercellular dissipation of toxic substances, such as ROS, helps to sustain equilibrium between pro-oxidant species and antioxidant defense mechanisms such as ROS-metabolizing enzymes. This mechanism is present in lenses (Berthoud & Beyer, 2009) and in myocardium, where sarcolemmal and mitochondrial Cx43 contribute to activation of a major cytoprotective PI3K in PI3K-Akt-GSK-3β signaling in cardiomyocytes (Ishikawa *et al.*, 2012). Finally, connexins, connexons and gap junctions

participate in cellular perception of stress signals and in intracellular signalling responsible for cellular adaptation to stress conditions.

An illustrative example of the role of connexins in adaptative cell responses is the involvement of GJIC in regulation of cell proliferation. GJIC cooperates with chemical (paracrine) and nanomechanical stimuli during the transition of normal cells from the "activated" to "dormant" phenotype (Schalper *et al.,* 2012). The so-called contact-inhibition of cell proliferation (Abercrombie, 1970; Castor, 1970) is commonly observed in confluent monolayers of normal cells. It is accompanied by (*i*) a dramatic decrease of cell motility along with the increasing cell density and by (*ii*) establishment of a stationary post-confluent state which is insensitive to nutrient renewal (for review see: Heckman, 2009; Puliafito *et al.,* 2012). A picture of the mechanism that regulates these processes is still incomplete. GJIC-dependent intercellular transmission of inhibitory signals, such as cAMP and Ca^{2+} may account for contact inhibition of growth in crowded cell populations. For instance, it is required for endothelial quiescence in stabilized vessels. Actually, down-regulation of Cx43 can be sufficient to release endothelial cells from contact-inhibition of growth (Choudhary *et al.,* 2015). Also toxic aryl hydrocarbon receptor (AhR) ligands (such as 2,3,7,8-tetrachlorodibenzo-p-dioxin; TCDD) decrease the amount of gap junction plaques, down-regulate GJIC in the AhR-dependent manner and disrupt contact-inhibition growth in liver cells (Andrysik *et al.,* 2013). Down-regulation of Cx43/GJIC may also be an inherent part of disruption of anchorage-dependence of thyroid cells (Jensen *et al.,* 2011).

Regulation of the contact phenomena extends beyond the events associated with GJIC-dependent functions of connexins. Contact inhibition of cell proliferation may be regulated by GJIC-independent interactions of gap and adherens junctions. The recruitment of E-cadherin to cell-cell contacts and the subsequent maturation of the adherens junctions in epithelial cells (Heckman, 2009; Tinkle *et al.,* 2008) usually inhibits cell proliferation, while their disruption can induce cell proliferation. E- and N-cadherin and β-catenin are present within the gap junction proteome (Sirnes *et al.,* 2015), where they can interact with protein kinases, ion channels and small G proteins (for review see: Laird, 2006). Apparently, cadherin- and connexin-dependent sub-membranous protein complexes constitute a system (Meens *et al.,* 2013) that integrates regulation of "social" cell behavior in tissues with the intracellular cell cycle machinery.

Moreover, numerous studies demonstrated interactions of single connexin entities with STAT3, ERK1/2 and src-dependent pathways (for review see: Vinken *et al.,* 2011; Vinken *et al.,* 2012). The interactions of connexins with intracellular signaling are often attributed to C-terminal of connexins and to their phosphorylation. This is well manifested in cardiomyocytes, where phosphorylation of serine262 in Cx43 inhibits DNA synthesis independently of GJIC (Doble *et al.,* 2004). Cx43 interferes with the function of cyclins, $p27^{kip}$ and S phase kinase-associated protein 2 (skp2), which regulates p27 ubiquitination in a GJIC-independent manner (Zhang *et al.,* 2003b; Zhang *et al.,* 2003c). Notably, these interactions inhibit logarithmic cell growth rather than participate in retardation of cell growth in confluent cultures.

Collectively, connexins take part in imposing "social" tissue constraints through suppressive "by-stander effects" mediated by GJIC. Even though the signals from neighbor cells in crowded populations do not necessarily affect cell welfare, contact-inhibition of cell proliferation can be interpreted as a pre-stress adaptation response. Multiple interceptions of connexins, connexons and gap junctions with sub-membrane assemblies of cytoskeletal and signaling molecules (i.e. gap junction proteome (Mroue *et al.,* 2011)) and with the cell cycle machinery prevent excessive cellular crowding in tissues. They apparently create conditions for the initiation of cell differentiation and permanent reprogramming, thus participating in the maintenance of tissue integrity and functionality (Iyyathurai *et al.,* 2016).

Consequences of connexin-dependent stress signaling at the tissue level

Whereas most insults can be overcome by the cells' natural defenses, sustained perturbations of tissue homeostasis and/or tissue rearrangements during morphogenesis or regeneration may result in the execution of pro-apoptotic programs. Whether the extrinsic and intrinsic connexin-mediated stress stimuli are interpreted by a cell as a death signal, depends on the cellular context, i.e. a phenotype of the stress-generating and stress-receiving cells. Furthermore, the amplitude, permanence and quality of stress signal, as well as the abundance, functional status and "perm-selectivity" of gap junctional channels, determines the quality and quantity of apoptotic cell responses to extrinsic stress signals.

There is ample evidence for the involvement of GJIC-mediated transfer of Ca^{2+}, cAMP, IP_3, and reactive oxygen/nitrogen species in the programmed cell death. "By-stander" effects, i.e. gap junction-mediated intracellular propagation of stress signals induces the apoptosis in virtually all tissues (Krysko *et al.,* 2005). The involvement of GJIC in the intercellular propagation of pro-apoptotic stimuli is illustrated by the spread of apoptotic cell death in ischemia (Contreras *et al.,* 2004; Jeyaraman *et al.,* 2012) and in the morphogenic processes (Krutovskikh *et al.,* 2002). These signals can trigger the release of Ca^{2+} from endoplasmic reticulum. Intracellular Ca^{2+} contributes to the regulation of apoptotic cascades and mitochondrial permeability, thereby amplifying the intracellular pro-apoptotic signaling (Orrenius *et al.,* 2003). Corresponding involvement of GJIC in apoptotic cell responses was observed in liver, where hepatic gap junctions play a crucial role in local propagation of antiviral immune response signaling (Knabb *et al.,* 2007). By contrast, GJIC inhibition in hepatocytes down-regulated the activity of caspase-3, a major contributor in the pro-apoptotic cascades (Naiki-Ito *et al.,* 2010). Gap junctions and hemichannels built of Cx43 are also involved in H_2O_2-mediated cell death in epithelial cells and osteocytes (Ramachandran *et al.,* 2007; Hutnik *et al.,* 2008; Kar *et al.,* 2013). Cx43 modulates H_2O_2- and H/R-induced cell death in astrocytes and these distinct effects of Cx43 correlate with differential regulation of Cx43 phosphorylation and spatial distribution.

Furthermore, the intracellular fraction of connexins participates in GJIC-independent intracellular amplification of pro-apoptotic signals (Kardami *et al.,* 2007). For instance, mitochondrial connexons may confer cell fate/death signals (Baines, 2010). Along with a panoply of mitochondrial proteins/complexes involving Bcl2, Bax and K(ATP) channels, Cx43 has been implicated in mitochondrium-related cell death. These functions can also be executed by single connexin molecules and hemichannels localized in the nucleus (Rodriguez-Sinovas *et al.,* 2007). Finally, due to the multiple interceptions between signaling pathways that regulate cell proliferation/growth arrest, the interference of connexins with the function

of MAP kinases, p27 and other cell cycle effectors can be translated into apoptotic cell responses (Vinken *et al.,* 2012).

Collectively, connexin-mediated stress signaling activates cell adaptation mechanisms, which prevent abnormal tissue hyperplasia and are important for elimination of excessive or irreversibly damaged cells. These mechanisms add to the role of connexin-mediated stress signaling in tissue homeostasis, development and regeneration. Notably, "outside-in" signalling pathways can activate signaling loops that close gap junctional channels in the apoptotic cells. This mechanism represents an adaptation system that protects tissue homeostasis through metabolic isolation of apoptotic cells. Multidirectional functions of connexins in the regulation of cell proliferation and apoptosis place them in the category of multifunctional, signaling micro-domains involved in "outside-in" stress signaling that preserves tissue integrity and homeostasis (Dbouk *et al.,* 2009).

CONNEXIN-MEDIATED CELLULAR STRESS IN TUMOR DEVELOPMENT

Tissue homeostasis, development and regeneration depend on the cooperation between connexin-dependent intra- and intercellular pathways and paracrine/mechanical signaling (Nelson & Bissell, 2006). Accordingly, abnormal propagation and amplification of intercellular stress signaling, which disturbs cell reactivity to extrinsic stress stimuli, is the physiological outcome of connexin dysfunctions. At the tissue level, the disturbances in generation, detection and propagation of stress signals lead to numerous abnormalities, such as developmental malformations, inflammatory diseases and tumor (for review see: Wong *et al.,* 2016; Wong *et al.,* 2017). For instance, increased proliferation of keratinocytes in psoriasis is attributed to increased Cx26 levels (Aasen, 2015). Notably, subtle changes of connexin expression, trafficking and turnover participate in dysfunctional social behavior of the cells, leading to cell transformation. Cx32-knockout mice exhibited resistance to liver cell death induced by D-galactosamine and carbon tetrachloride (Asamoto *et al.,* 2004), and the increased predisposition to liver cancer (Hokaiwado *et al.,* 2007). It is conceivable that injured hepatocytes may escape apoptosis upon Cx32 removal, which poses a risk factor in carcinogenesis (Naiki-Ito *et al.,* 2010). Indeed, associations between low expression of connexins in tumors and a poor prognosis have been reported for numerous tumors, including prostate, colorectal and breast cancer (Benko *et al.*, 2011; Teleki *et al.*, 2014; Sirnes *et al.,* 2015; Grek *et al.*, 2016). The relevance of connexin (dys)function for carcinogenesis was also supported by reports (i) on the interference of chemical carcinogens with the function of connexins in normal cells, (ii) on the postulated role of connexin deficiency in anchorage-independent and contact-resistant tumor cell proliferation, (iii) on the interrelations between connexin (dys)functions and the resistance of tumor cells to apoptosis and (iv) on the role of connexin-mediated "inside-out" signaling in tumor invasion and metastasis. Below, we propose how the combination of these effects can contribute to cancer promotion and progression (Fig. 2).

Connexin deficiency and cell transformation

Occasional transmission of stress signals through gap junctions can lead to "physiologic" apoptosis that eliminates excessive or damaged cells from tissues. Therefore, the resistance of tumor cells to these signals may result from connexin dysfunction. The role of intracellular transfer of metabolites in cancer promotion was experimentally analysed for the first time in the late 1960s, i.e. long before connexins had been discovered (Loewenstein & Kanno, 1966; Loewenstein & Kanno, 1967). Early demonstrations of the fact that cancer cells are less communicated than their normal counterparts were underlined by a plethora of reports on connexin dysfunction in cancer cells (Laird, 2006; Leithe *et al.,* 2006). Chemical carcinogenes, such as phorbol esters, exert an inhibitory effect on the expression and/or function of connexins in numerous cell types, incl. hepatocytes and keratinocytes (Ren *et al.,* 1998; Langlois *et al.,* 2010). Accordingly, it is commonly assumed that connexins, gap junctions and GJIC may stabilize a "normal" cell phenotype, whereas connexin dysfunctions participate in the resistance of tumor cells to stressful microenvironment of primary tumors.

Phorbol esters impair GJIC through the protein kinase C (PKC)-dependent phosphorylation of connexins (Chipman *et al.,* 2003). A similar activity of lindane (hexachlorocyklohexan) in liver and myometrial cells was attributed to the oxidation of glutathione (Loch-Caruso *et al.,* 2004; Caruso *et al.,* 2005). We have demonstrated that triphenylotin (TPhT) closes Cx43 channels in human embryonic kidney (HEK) cells through ROS-dependent activation of PKC (Sroka *et al.,* 2008). This finding confirms that Cx43 functions as a sensor of oxidative stress. Metabolic isolation of tumor cells is the primary outcome of connexin deficiency and dysfunction. Thus, the cells are released from the control regime of the tissue because GJIC impairment prevents intercellular propagation and intracellular amplification of stress stimuli. On the other hand, the dysfunction of connexins can also attenuate intercellular fluxes of harmful compounds from dysfunctional cells to their intact neighbors, leading to accumulation of toxic metabolites (such as ROS) in metabolically isolated cells (Tsujino *et al.,* 2007; Vinken *et al.,* 2012). Deficient/abnormal connexin expression, disturbance of connexin trafficking (Leithe, 2016), deregulated gating and selective permeability of gap junction channels may thus contribute to the accumulation of mutations in tumor cells' genome (incl. connexin-coding genes). Such mutations are commonly observed in the genes encoding the effectors of GSK3β, src/PKC and cAMP-dependent cascades. Because these pathways participate in the"oncogenic transformation" and concomitantly regulate connexin expression (reviewed by: Lee *et al.,* 1991; Chipman *et al.,* 2003), "vicious circles" that account for heritable Cx43, Cx32 and Cx26 dysfunction in tumor cells may be constituted. Due to the possible involvement of connexin dysfunction in disturbed genetic stability of tumor cells, connexins are often claimed to represent class II suppressors.

Connexin deficiency and stress-resistance of tumor cells

Tumor cell populations *in vitro* and *in vivo* are characterized by extreme phenotypic heterogeneity, which concerns gene expression, proteome, secretome, composition and architecture of surface complex, adhesive properties, morphology, motility, contractility etc. Apparently, this heterogeneity illustrates the complexity of microevolution routes undertaken by tumor cell lineages in the dynamic microenvironment of primary tumor. In contrast, anchorage-independent growth and relative insensitivity to cellular crowding are characteristic for the vast majority of tumor cells. Even though there are several reports on cytoprotective effects of Cx32, Cx43 and

Table 1. Effect of connexin manipulations on the susceptibility of tumor cells to pro-apoptotic and cytostatic signals

Connexin	Tumor	Parameter		Mechanism	References
Cx43↑	prostate cancer	apoptosis↑	proliferation↓	n.d.	(Lu *et al.,* 2015; Li *et al.,* 2016)
Cx43↑	glioma	apoptosis↓	proliferation↑	GJIC-independent	(Gielen *et al.,* 2013)
Cx43↑	glioma	apoptosis↑	n.d.	GJIC-independent	(Huang *et al.,* 2001)
Cx43↑	hepatoma	apoptosis↑	proliferation↓	GJIC-dependent	(Liu *et al.,* 2009)
Cx43↑	mammary carcinoma	apoptosis↑	proliferation↑	GJIC-dep/indep	(Shishido & Nguyen, 2016)
Cx43↑	breast cancer	apoptosis↑	n.d.	GJIC-dependent	(Chang *et al.,* 2013)
Cx43↑	medulloblastoma	apoptosis↑	n.d.	n.d.	(Sun *et al.,* 2012a)
Cx43↑	mesothelioma	apoptosis↑	proliferation↓	GJIC-independent	(Sato *et al.,* 2009)
Cx43↑	nasopharyngeal tumor	apoptosis↑	proliferation↓	GJIC-independent	(Hattori *et al.,* 2007)
Cx43↑	melanoma	apoptosis↑	proliferation↓	GJIC-dependent	(Tittarelli *et al.,* 2015)
Cx43↑	pancreatic cancer	apoptosis↑	n.d.	GJIC-independent	(Sun *et al.,* 2012b)
Cx43↓	prostate cancer	apoptosis↓	proliferation↓	n.d.	(Li *et al.,* 2012)
Cx43↓	bladder cancer	apoptosis↑	proliferation↓	n.d.	(Ai *et al.,* 2017)
Cx43↓	glioma	apoptosis↓	proliferation↑	n.d.	(Jin *et al.,* 2013)
Cx43↓	Giant-cell tumor of the bone	apoptosis↓	n.d.	GJIC-dependent	(Balla *et al.,* 2015)
Cx32↑	hepatoma	apoptosis↑	proliferation↓	n.d.	(Wu *et al.,* 2016; Liu *et al.,* 2009)
Cx32↑	lung adenocarcinoma cells	apoptosis↑	n.d.	n.d.	(Sato *et al.,* 2007a)
Cx32↑	renal carcinoma	apoptosis↑	proliferation↓	n.d.	(Sato *et al.,* 2007b)
Cx32↑	renal cancer	apoptosis↑	proliferation↓	GJIC-dependent	(Fujimoto *et al.,* 2004)
Cx26↑	prostate cancer	apoptosis↑	proliferation↓	n.d.	(Tanaka & Grossman, 2004)
Cx26↑	bladder cancer	apoptosis↑	proliferation↓	n.d.	(Tanaka & Grossman, 2001)
Cx37↑	gastric tumor	apoptosis↑	n.d.	n.d.	(Jing *et al.,* 2014)
Cx37↑	insulinoma	apoptosis=	proliferation↓	n.d.	(Burt *et al.,* 2008)
Cx46↓	breast cancer	apoptosis↑	proliferation↓	n.d.	(Banerjee *et al.,* 2010)
Cx25↓	leukemia	apoptosis↑	proliferation↓	GJIC-dependent	(Sinyuk *et al.,* 2015)
Cx30↑	glioma	apoptosis↓	proliferation↓	n.d.	(Artesi *et al.,* 2015)

Cx46 on cancer cells (Banerjee *et al.,* 2010), their dysfunction generally attenuates the sensitivity of tumor cells to growth-retarding and pro-apototic signals (Table 1). Accordingly, misperception of the presence of neighboring cells is considered as a fundamental milestone in the development of tumors. Numerous studies showed that the ectopic Cx26, Cx32 and Cx43 expression in connexin-deficient glioma (Goldberg *et al.,* 2000), melanoma (Su *et al.,* 2000), breast (Hirschi *et al.,* 1996; Momiyama *et al.,* 2003) and prostate cancer cells (Mehta *et al.,* 1999) leads to a partial reversion of their transformed (anchorage-independent) phenotype and to restoration of the contact-inhibited growth.

The up-regulation of connexins has also been shown to increase the sensitivity of tumor cells to the pro-apoptotic stimuli (for review see: Kandouz & Batist, 2010). For instance, Cx43 up-regulation induced by all-trans retinoic acid (ATRA) increased the sensitivity of prostate cancer cells to docetaxel (Nehme *et al.*, 2001). Ectopic Cx43 expression was shown to sensitize HeLa cells to apigenin (Czyz *et al.,* 2005). Cx43 also increases the sensitivity of prostate cancer cells to ganciclovir after adenoviral delivery of the herpes virus thymidine kinase suicide gene and to combined ganciclovir/tumor necrosis factor (TNF) therapy (Hattori & Maitani, 2005; Wang *et al.,* 2007). Similarly, Cx43 up-regulation increased the sensitivity of human glioblastoma cells and AML cells to etoposide, paclitaxel and doxorubicin (Li *et al.,* 2006; Foss *et al.,* 2010). Chemosensitivity of tumor cells may also be increased by the ectopic expression of Cx26 and Cx32 (Foss *et al.,* 2010). Collectively, disturbed intercellular propagation of growth inhibitory and pro-apoptotic signals, which results from connexin deficiency/dysfunction in tumor cells, supports their clonal expansion and unrestricted growth. In turn, restoration of connexin functions augments the sensitivity of tumor cells to pro-apoptotic signals (Table 1).

In addition to the GJIC-dependent connexin functions, the GJIC-independent signaling pathways regulate cytostatic and pro-apoptotic cell responses to extrinsic stress (Carette *et al.,* 2014). Accordingly, connexin deficiency may attenuate the activity of these pathways in tumor cells, thus augmenting their resistance to stress signaling. For instance, GJIC-independent interference of C-terminal fragments of Cx43 with skp2/p21-dependent cascade is dysfunctional in tumor cells. Down-regulation of cyclin D1 was shown upon Cx43 transfection in E9 mouse lung carcinoma and osteosarcoma cells. This effect correlated with up-regulation of p27kip-1 (Koffler *et al.,* 2000; Zhang *et al.,* 2003c; Vinken *et al.,* 2011). On the other hand, GJIC-independent involvement of Cx26 in the regulation gene expression was seen in breast tumor cells (Qin *et al.,* 2003). Cytoplasmic, mitochondrial and nuclear connexins also regulate pro-apoptotic cascades in the GJIC-independent manner. They contribute to

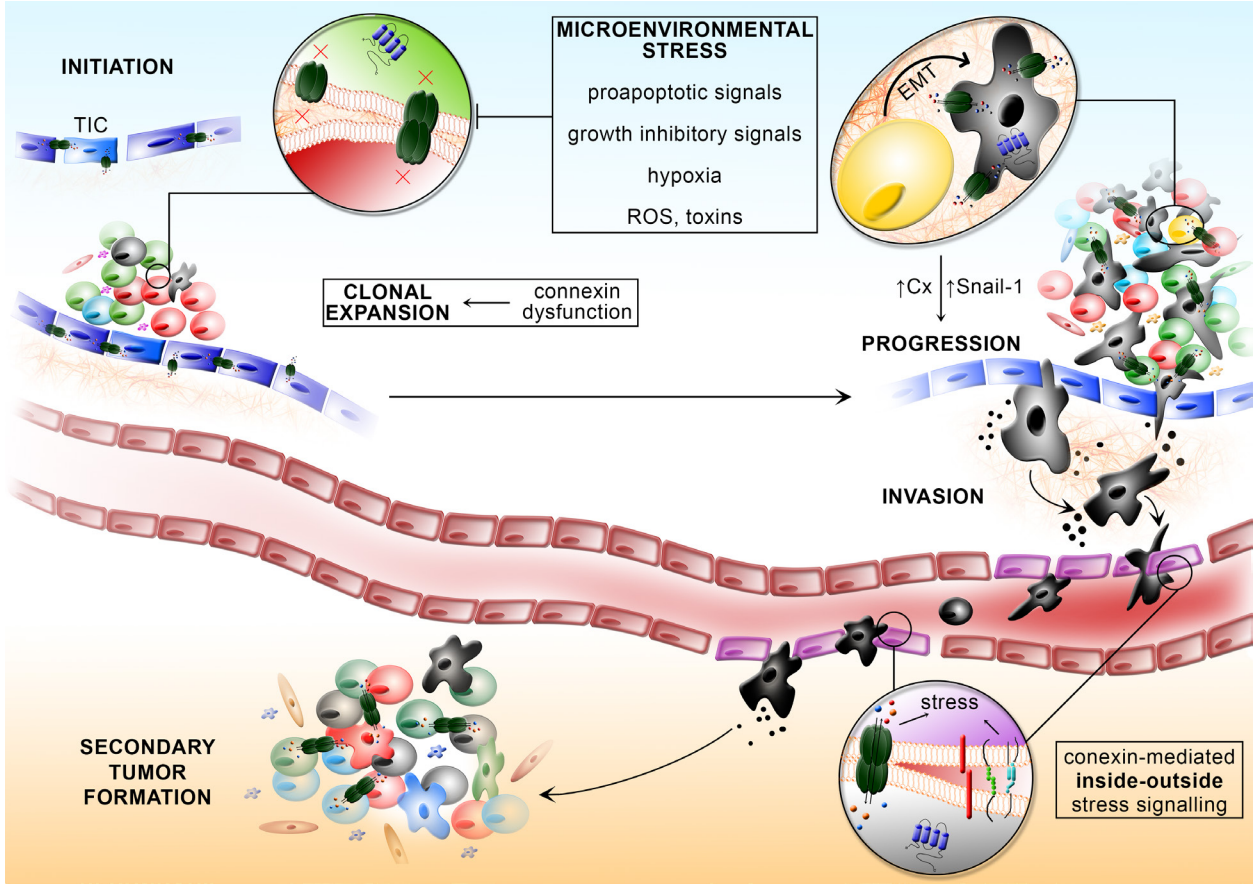

Figure 2. Connexin-dependent stress signaling in cancer promotion and progression.
Connexin deficiency/dysfunction promotes aberrant cancer cell proliferation and differentiation during tumor initiation and promotion. It drives the phenotypic diversity of the cells constituting the primary tumor cell mass and the expansion of invasive cancer cell sub-populations. They are characterized by high motile activity, nanomechanical elasticity, and the expression of connexins (Cx). During tumor progression, connexins expressed by invasive (post-EMT) cells participate in the inside-out stress signaling which facilitates tumor cell diapedesis and metastatic cascade.

bcl2, bax- and caspase-dependent signaling pathways and to the release of ROS from mitochondria (Krysko *et al.,* 2005; Rodriguez-Sinovas *et al.,* 2007). Thus, deficiency of Cx43, Cx32 and Cx26 in tumor cells can disturb the apoptosis-related gene expression in a GJIC-independent manner, whereas their re-expression is associated with the re-activation of pro-apoptotic signaling in tumor cells (Huang *et al.,* 2001) (Table 1).

Collectively, the accumulating data show that connexin dysfunctions augment the resistance of connexin-deficient tumor cells to steric tissue constraints and to systemic defense mechanisms (for review see: Vinken *et al.,* 2011; Aasen, 2015). Resistance of tumor cells to contact-phenomena facilitates the expansion of stress- and drug-resistant sub-clones within the primary tumors. Disturbed propagation/amplification of pro-apoptotic signals in connexin-deficient tumor cells cooperates with hyperactive multi-drug resistance (MDR) and autophagy systems, further augmenting tumor cells' resistance to adverse micro environmental factors, i.e. to toxic metabolites, ROS species or chemotherapeutics. The connexin dysfunction in tumor cells can also help them to overcome the pressure from the immune system (Oviedo-Orta & Evans, 2002). Consequently, tumor cells are more resistant to extreme deviations of the parameters that determine tissue and cellular homeostasis. Together with reprogrammed energy metabolism (i.e. Warburg effect), the dysfunction of connexins facilitates the microevolution adaptation of the cells to the dynamic conditions of the developing tumor. Furthermore, it can increase their predilection to undertake erroneous differentiation programs, which result in the formation of invasive sub-populations (Loewenstein, 1979; Yamasaki *et al.,* 1999; Leithe *et al.,* 2006; Naus & Laird, 2010; Hanahan & Weinberg, 2011; Czyz *et al.,* 2012) (Fig. 2).

Connexins, intercellular stress signaling and tumor progression

Clonal evolution of invasive tumor cells governs tumor malignacy, i.e. its predilection for colonisation of distant organs. Tumor cell invasiveness is determined by their susceptibility to permissive microenvironmental signals, chemotactic motility and nanomechanical elasticity (Wysoczynski *et al.,* 2007; Kumar & Weaver, 2009; Boiko *et al.,* 2010; Bechyne *et al.,* 2011; Friedl & Alexander, 2011; Langley & Fidler, 2011; Visvader, 2011; Fredebohm *et al.,* 2012; Shibata & Shen, 2013). Although connexins inhibit tumorigenesis at its early stages, relatively high expression of connexins often correlates with the invasive potential of tumor cells (Leithe *et al.,* 2006; Mol *et al.,* 2007; Czyz, 2008; Czyz *et al.,* 2012). Data on the involvement of connexins, connexons and gap junctional channels in the "metastatic cascade" of tumors started to accumulate in 1990s (Brauner *et al.,* 1990; Brauner & Hülser, 1990). Since then, interrelations between high Cx26, Cx32 and Cx43 levels and an invasive cell pheno-

type were described in the populations of glioma, lung, prostate, breast cancer and melanoma cells (for review see: Defamie *et al.,* 2014). Nowadays, it is clear that the erroneous up-regulation of connexins is a part of heritable switches that promote invasive behavior of tumour cells during tumor progression. These findings prompted the discussion on the stage-dependent connexin function during tumor promotion and progression, incl. the formation of metastases (Czyz, 2008; Dbouk *et al.,* 2009; Czyz *et al.,* 2012; Defamie *et al.,* 2014). However, they also stimulated discussions on the significance of microenvironmental stress for the formation and expansion of Cx26-, Cx32- and Cx43-positive cell sub-populations within the connexin-negative primary tumors.

Numerous reports show the mechanistic links between the invasive cell behavior and connexin expression in normal and cancer cells (Czyz *et al.,* 2005; Li *et al.,* 2007; Omori *et al.,* 2007; El Saghir *et al.,* 2011; Czyz *et al.,* 2012; Sin *et al.,* 2012; Zucker *et al.,* 2013). Conceivably, these links are related to the GJIC-dependent and GJIC-independent involvement of connexins and connexons in cell adhesion, mechanosensitivity and directed motility (Elias *et al.,* 2007; Cotrina *et al.,* 2008; Cronier *et al.,* 2009). The increased expression of Cx26 and Cx43 has been correlated with the increased motility of glioma, glioblastoma, melanoma cells as well as prostate, gastric and breast cancer cells. It also enhances their nanomechanical elasticity, susceptibility to chemotactic and haptotactic cues, and secretion of cytokines and metalloproteinases (Tate *et al.,* 2006; Bechyne *et al.,* 2011; Garcia-Rodriguez *et al.,* 2011; Lamiche *et al.,* 2011; Szpak *et al.,* 2011). This correlation results from interactions of connexins and connexons with a myriad of submembrane protein assemblies within the gap junction proteome (for review see: Dbouk *et al.,* 2009; Mroue *et al.,* 2011). Noteworthy, cells that constitute malignant tumors are predominantly characterized by over-active multi-drug resistance (MDR) systems, dysfunctional pro-apoptotic pathways and reprogrammed energy metabolism. Thus, they are less susceptible to connexin-dependent generation, propagation and amplification of stress signals. Consequently, phenotypic shifts that prompt the "re-expression" of connexins in originally connexin-deficient cells can enhance their malignant behavior without a considerable effect on their welfare.

For instance, Cx43 increases the invasive potential of prostate cancer cells through direct involvement in epithelial-mesenchymal transition (EMT). EMT is a sequence of phenotypic shifts that augments the invasive potential of cancer cells. These shifts include the acquisition of rear-front cell polarity, plasticity and motility by originally benign epithelioid tumor cells. EMT is induced by numerous extrinsic stimuli and regulated by a plethora of transcriptional regulators, including Snail-1 (Berx *et al.,* 2007; Thiery *et al.,* 2009; Savagner, 2010). We have shown that Cx43 constitutes a positive feedback loop with Snail-1/Smad2-dependent signaling, which induces EMT in prostate cancer cells (Ryszawy *et al.,* 2014). Its activation leads to the concomitant EMT and Cx43 up-regulation in prostate cancer cells. Because EMT increases the ability of tumor cells to cross tissue barriers, it can help them to avoid/escape stressful conditions. Moreover, EMT was found to correlate with the resistance of tumor cells to the extrinsic stress (for review see: Koumenis, 2014). Further studies are necessary to verify whether Cx43/Snail-1/Cx43 loop may regulate the activity of MDR systems. However, it is clear that connexins can participate in the microevolution of stress-resistant tumor cell lineages and in the functional stabilization of the invasive front of tumor (Brabletz, 2012).

The relationships between Cx43 and the invasive phenotype of tumor cells may also underlie the mechanisms of tumor cell homing in a "comfortable" metastatic niche. Connexins participate in the penetration of natural barriers by invasive tumor cells, thus facilitating their homing in metastatic niches. Connexins and gap junctions apparently confer "inside-out" stress signaling from tumor cells to the stroma that favors tumor cell extravasation. Cx43 is involved in the diapedesis of melanoma, glioblastoma, breast, lung and gastric cancer cells (El Sabban & Pauli, 1991; El Sabban & Pauli, 1994; Ito *et al.,* 2000; Zhang *et al.,* 2003a; Pollmann *et al.,* 2005; Naoi *et al.,* 2007; Elzarrad *et al.,* 2008; Tang *et al.,* 2013; Piwowarczyk *et al.,* 2015; Ryszawy *et al.,* 2014). Cx43 function during this process may be attributed to the disturbance of endothelial calcium homeostasis by GJIC-mediated calcium fluxes from tumor to endothelial cells (Lewalle *et al.,* 1998). We have recently demonstrated that prostate cancer cells can also activate the "inside-out" stress signaling axis with endothelial cells in Cx43-dependent, GJIC-independent manner (Piwowarczyk *et al.,* 2015; Piwowarczyk *et al.,* 2017). Other studies showed increased apoptosis of endothelial cells in the proximity of tumor cells, however the involvement of connexin in this process still requires experimental verification. Collectively, multifaceted functions of connexins provide the background for their complex involvement in generation, propagation and detection of stress signals during tumor invasion. They may be responsible for high levels of Cx43, Cx32 and Cx26 in secondary tumors *in vivo* (Kanczuga-Koda *et al.,* 2006; Kanczuga-Koda *et al.,* 2007; Chao *et al.,* 2011; Stoletov *et al.,* 2013) and for increased drug-resistance of progressed tumors.

SUMMARY AND OUTLOOK

Gap junctional channels provide a route for metabolic and electrical synchronization of multicellular compartments. As such, they are indispensable in limiting cellular stress resulting from nutritious starvation, hyper- and hypoxia. However, connexins can also serve as universal and sensitive transmitters and sensors of intercellular stress signals. Connexin-dependent stress signaling activates cell death programs that eliminate damaged cells from the organism. It also regulates cellular adaptation to tissue constraints, and preserves tissue functionality through promoting cellular specialization/apoptosis during developmental and regenerative processes. These multiple, context-specific functions situate connexins in the centre of the integrated system that generates, propagates and amplifies stress signals. Further studies on the role of connexins and gap junctions in regulating cell sensitivity to environmental stress signals, in cell adaptation to stressful conditions and in cell death signaling may help to better understand the mechanisms underlying embryogenesis and tissue homeostasis.

In the light of these data, pharmacological modulation of connexin-mediated stress responses presents a potentially attractive strategy in the therapy of numerous chronic diseases. For instance, there are numerous speculations concerning application of the connexin-based gene transfer technology in combination with conventional approaches in a tumor therapy. During cancer development, connexin-dependent stress signaling and cell responses to extrinsic stress signals participate in the selective pressure conferred by different environments,

which lead to different growth rates of the cell clones within neoplasm. In particular, connexins cooperate with stochastic genetic and epigenetic changes in determining the heterogeneity of primary tumor cells and in the microevolution of invasive cancer cell subsets. Due to the role of connexins in determining tumor cells' sensitivity to stress, the approaches based on the restoration of connexin expression in connexin-deficient tumor cells could help to increase the efficiency of chemotherapeutic approaches in tumor treatment. However, connexins-dependent stress signaling cooperates with dynamically established loops of intercellular communication during tumor progression and metastatic cascade (Marusyk & Polyak, 2013). Connexins and gap junctions also participate in "inside-out" stress signaling between tumor cells and microenvironment that increases their drug-resistance, and facilitates their expansion and metastasis. Further studies should elucidate the consequences of switching between connexin-positive and connexin-negative cell phenotypes for survival strategies of tumor cells in fluctuating environment. Owing to this pleiotropic involvement of connexins in tumor development, therapeutic regimens based on the modulation of connexin-mediated cellular stress responses should be considered with caution and tailored to individual patients.

Conflicts of interest

The authors declare no conflict of interest.

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