

Mini review

THE STAGE-SPECIFIC FUNCTION OF GAP JUNCTIONS DURING TUMOURIGENESIS

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Abstract: Tumour development is a process resulting from the disturbance of various cellular functions including cell proliferation, adhesion and motility. While the role of these cell parameters in tumour promotion and progression has been widely recognized, the mechanisms that influence gap junctional coupling during tumorigenesis remain elusive. Neoplastic cells usually display decreased levels of connexin expression and/or gap junctional coupling. Thus, impaired intercellular communication via gap junctions may facilitate the release of a potentially neoplastic cell from the controlling regime of the surrounding tissue, leading to tumour promotion. However, recent data indicates that metastatic tumour cell lines are often characterized by relatively high levels of connexin expression and gap junctional coupling. This review outlines current knowledge on the role of connexins in tumorigenesis and the possible mechanisms of the interference of gap junctional coupling with the processes of tumour invasion and metastasis.

Key words: Gap junctions, Connexin, Tumour, Neoplasia, Metastasis

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Abbreviations used: Cx – connexin, FACS – fluorescence-activated cell sorting

GAP JUNCTIONS – THEIR STRUCTURE AND BASIC FUNCTIONS IN TISSUE HOMEOSTASIS

Gap junctions are aqueous intercellular channels which directly link the cytoplasmic compartments of adjacent cells (Fig. 1). These channels are formed upon the docking of two connexons, hexameric hemichannels built of proteins belonging to the connexin family [1]. Gap junctions are present in almost all vertebrate tissues, where they permit the intercellular exchange of small (< 1 kDa) metabolites and second messengers, and thus synchronise cellular functions in tissues [2-4]. Representative examples of the synchronising function of gap junctional coupling in tissues include electrical and metabolic coupling. In the former, gap junctions take part in the intercellular spreading of membrane depolarisation by permitting the rapid cell-to-cell transfer of calcium ions between cardiac muscle cells [1], or by constituting electrical synapses in the nervous system [5]. Metabolic coupling is established when the stimulation of one cell is propagated to a cluster of cells through the spreading of active substances, such as monosaccharides and cyclic nucleotides [2, 6]. While electrical coupling predominantly synchronizes cell functions in excitable tissues, metabolic coupling affects the homeostasis of a spectrum of multicellular systems, including both excitable and non-excitable tissues.

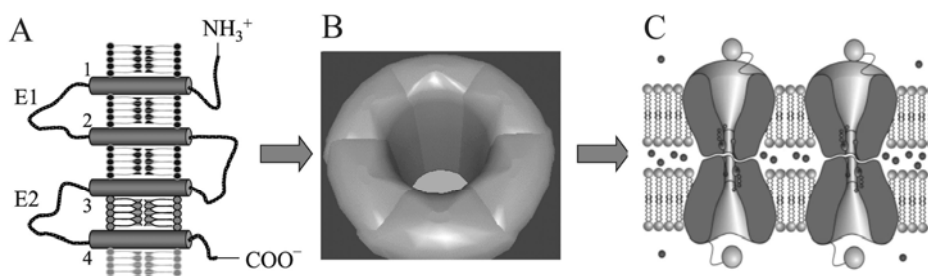


Fig. 1. The structure of gap junctions. Proteins of the connexin family consisting of intramembraneous (1-4), extracellular (E1, E2), and intracellular domains (A), compose hexameres (connexons; B), which give rise to intercellular channels built of connexons contributed by adjacent cells (C). R. Eckert and D. Huelser, private communication, modified.

The crucial role of gap junctions in the regulation of tissue homeostasis is illustrated by the functional impairment of many vertebrate tissues occurring when there is deficient gap junctional communication [7, 8]. For instance, disruption of the function of the gene encoding Cx32 is associated with the progressive degeneration of the peripheral nerves, resulting from the impairment of the diffusion of nutrients and signalling molecules between the perinuclear and periaxonal Schwann cell cytoplasm [for review see [9]. Furthermore, abnormalities have been observed in Cx32-deficient mouse livers: Cx32 disruption leads to a decrease in the discharge of glucose from glycogen stores [10].

However, the relative non-specificity of the channel allows the passage of a wide variety of small molecules, and a multitude of signalling systems affect the function of gap junctional channels [11, 12], thus making the mechanistic elucidation of the role of gap junctional coupling in tissue homeostasis difficult. Cx32 knockout mice appeared to be more susceptible to liver carcinogenesis than their wild-type counterparts [13], suggesting that the role of gap junctions during the development of tumours may be of special importance. In this paper, the state of research on the involvement of gap junctions in the processes of tumour arousal and development is outlined.

THE INVOLVEMENT OF GAP JUNCTIONS IN THE REGULATION OF TUMOUR CELL PROLIFERATION

Many reports indicate that natural or provoked deficiency in connexin gene function correlates with increased cellular susceptibility to transformation. *In vivo* observations indicating the influence of gap junctions on tumorigenesis are supported by *in vitro* data demonstrating the down-regulation of connexin expression and gap junction assembly in a wide range of neoplastic cell lines and primary tumours [14-17]. Furthermore, viral transfection was shown to reduce the levels of gap junctional coupling in cell populations, while a forced expression of genes encoding connexins inhibited the proliferation and affected the contact behaviour of transfected tumour cells [16]. Retarded tumorigenicity was described upon connexin transfection of hepatoma SKHep1, breast carcinoma, glioblastoma, PG lung carcinoma, cervical adenocarcinoma HeLa and prostate cancer LNCaP cells, which correlated with the inhibition of their growth *in vitro* and *in vivo*, the decrease of their saturation density, i.e. the density attained by cells in culture upon entering the plateau phase [18-20], and the inhibition of anchorage-independent growth [21], i.e. the partial reversion of the tumour phenotype represented by a decrease in the proliferation rate of connexin-transfected tumour cells in semi-solid media [16, 22, 23].

An inverse relationship between connexin expression and tumour cell proliferation has long been regarded as resulting from the synchronising effect of gap junctional coupling on cell physiology. Since the principal role of connexins is the formation of intercellular channels, the mechanisms by which connexins affect cellular proliferation were originally proposed to depend on the channel-forming property and gap junction-mediated exchange of putative growth inhibitory signals [24, 25]. On the other hand, more recent studies have provided evidence for a dissociation of gap junctional coupling intensity and the ability of connexins to suppress cell proliferation [26-28]. For example, a direct, coupling-independent interference of connexins with the signaling pathways involved in the regulation of cell proliferation was demonstrated. A down-regulation of cyclin D1 upon Cx43 transfection was revealed in human breast tumour cells [28] and in normal cell lines [29]. A similar correlation was observed for E9 mouse lung carcinoma and osteosarcoma cells [30].

Mechanistic studies demonstrated that the exogenous expression of Cx43 might exert its inhibitory effect on cell proliferation via the inhibition of the expression of S phase kinase-associated protein 2 (skp2), the protein that promotes the ubiquitination of cyclin-dependent kinase inhibitor p27kip. A down-regulation of cyclin D1 in osteosarcoma cells was shown upon Cx43 transfection, which correlated with up-regulation of p27kip [31]. The deletion mutation analyses revealed that the C-terminal domain of Cx43 that did not form gap junctions was still sufficient to inhibit skp2 expression, while coupling inhibitors did not affect the inhibitory effect of Cx43 on skp2 expression, indicating that coupling-independent mechanisms of Cx43 interfere with the cell cycle [30, 31]. It was also recently shown that the phosphorylation of Cx43 on S262 by protein kinase C cancels HEK-293 cell growth inhibition by Cx43 independently of the channel-forming ability [32]. This finding defines a novel, connexin-dependent and gap junctional coupling-independent mechanism for tumour promotion by PKCs. Taking all this into account, it would seem that intercellular diffusion of putative growth inhibitory factors via gap junctions may play an important role during early stages of tumour development in a way complementary to mechanisms including direct, coupling-independent interference of connexin molecules with signalling pathways regulating the cell cycle.

THE EFFECT OF GAP JUNCTIONS ON THE INVASIVE POTENTIAL OF TUMOUR CELLS

In a simplistic model of tumour development, the potentially neoplastic cell, when released from the controlling regime of the tissue, begins uncontrolled proliferation, thus giving rise to the primary tumour. Gap junctional coupling may take part in this process, as impairment of gap junctional communication may facilitate this process. On the other hand, some data shows that the function of gap junctions may be dependent on the stage of tumorigenesis, i.e. it can differ between neoplastic and metastatic cell populations.

Sequential obligatory steps must be completed after neoplasia (the initial step of tumorigenesis) for the occurrence of metastases. Upon detachment from neighbouring cells and penetration of natural barriers such as the basement membrane and endothelial continuum, neoplastic cells invade blood vessels and/or lymphatic vessels in the process known as intravasation, which enables them to access the circulatory system. After reaching the bloodstream, tumour cells enter the microcirculation and, finally exit the bloodstream by extravasation. They then form metastases via expansive growth in the parenchyma of the target tissue [33]. Thus, a deregulation of the mechanisms determining cell proliferation is followed by the acquisition of the ability of unrestricted migration by tumour cells, a prerequisite of invasion [34].

Although a correlation between the loss of gap junctions and the metastatic capability of cancerous cells was reported (see [35]), several studies have revealed relatively high levels of connexins and gap junctional coupling in

populations of invasive tumour cells. Kamibayashi *et al.* [36] demonstrated that even though the expression of Cx26 and Cx43 was reduced in the early stages of mouse skin carcinogenesis, both connexins were expressed on the plasma membranes of cells invading the lymph nodes. Similar observations were made for breast cancer [35], prostate cancer [37, 38] and glioma cell populations [39]. In melanoma cells residing in the basal layer, Cx26 levels remained unchanged, but were significantly up-regulated in the cells invading the dermis. Furthermore, gap junction formation was observed between melanoma cells and mammary adenocarcinoma cells and the endothelium, which could take part in the processes of tumour cell intra- and extravasation [40, 41]. Moreover, transfection of human cervical carcinoma (HeLa) cells with a gene encoding Cx43 significantly increased their *in vitro* invasive potential [42]. Thus, gap junctions and gap junctional coupling facilitates tumour cell invasion in versatile cellular models, but the specific mechanisms of the correlation between the expression and function of connexins and the metastatic potential of tumour cells remain to be determined.

INTERRELATIONS BETWEEN PROLIFERATION, CELL MOTILITY, AND GAP JUNCTIONAL COUPLING IN INVASIVE AND NON-INVASIVE TUMOUR CELL LINES

As mentioned, the invasive potential of tumour cells *in vivo* is predominantly based on their ability to penetrate the endothelial barrier, and to spread and proliferate in the adjacent tissue. In order to elucidate how gap junctional coupling facilitates tumour invasion, we have established an experimental approach enabling the quantitative analyses of connexin expression and the function of gap junctions in relation to other cellular traits involved in tumorigenesis, such as cell proliferation and motility [43, 44]. This method exploits pairs of spontaneously aroused tumour cell lines differing in their connexin expression levels and metastatic potential *in vivo* [37], as well as cell lines established through the stable transfection of connexin-deficient tumour cell lines with gene(s) encoding particular connexins [42, 45, 46]. Cell proliferation and apoptosis was quantified by cell counters and FACS [44], time-lapse video analysis of cell motility provided statistically significant quantitative data on the parameters of tumour cell motility [47, 48], and a calcein spreading assay [49] enabled the quantification of gap junctional coupling in homo- and heterologous cellular systems. Thus, quantitative analyses of tumour cell proliferation, motility and the connexin function of tumour cell lines of common origin differing in terms of connexin expression enabled the *in vitro* determination of the interrelations between the cell parameters involved in the process of tumorigenesis.

Using this experimental approach, we compared the properties of two sub-clones of HeLa differing in the levels of Cx43 expression and gap junctional coupling, namely wild-type HeLa cells and HeLa Cx43 transfectants. Growth curve and

time-lapse analyses revealed that both cell lines display similar proliferation rates and motility. Since the induction of gap junctional coupling correlates with the increased invasive potential of HeLa Cx43 cells, this data indicates that Cx43-mediated gap junctional coupling directly affects the invasive properties of tumour cells and facilitates the process of metastasis [42, 44]. Similar observations have been made for rat prostate cell lines. Highly metastatic MAT-LyLu cells display significantly higher expressions of Cx43 and coupling levels than non-invasive AT-2 cells, while no significant differences in motile activity on underlying aligned fibroblasts [37] or in growth rate [50] were observed. Thus, cellular systems based on pairs of tumour cell lines differing in the intensity of gap junctional coupling [42, 43] are a suitable tool for analyses of the influence of gap junctions on tumour development in relation to the tumour stage and other cellular traits relevant for tumorigenesis, i.e. cell motility and proliferation.

SUMMARY AND OUTLOOK

Evidence exists that the function of gap junctions affects the development of tumours in a manner dependent on the stage of tumorigenesis. Gap junctions seemingly act as tumour suppressors in the initial stages of this process, while the re-expression of connexins in migrating tumour cells may help these cells to invade surrounding tissue, and intra- and extravasate (Fig. 2). Interestingly, the

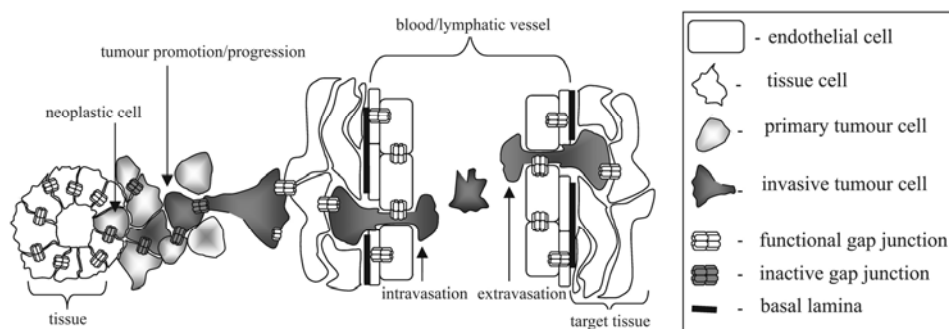


Fig. 2. The stage-specific function of gap junctions during tumour development. While the loss of connexin expression/function can initiate and promote tumorigenesis in its initial stages, the re-expression of connexins and/or re-constitution of their function seem to facilitate tumour progression and invasion.

corresponding interrelation has been described for the cells of the immune system. Gap junctional coupling between human monocytes and endothelial cells seems crucial for monocyte/macrophage transmigration in a blood-brain barrier model [51]. Moreover, the expression of three connexin isoforms, namely Cx37, Cx40 and Cx43, has been reported in circulating blood cells as well as in

vascular endothelial cells [52, 53]. The expression of connexins in blood and endothelial cells is modulated by pro-inflammatory agents such as LPS and TNF- α [51] and the switch from Cx37/Cx40 to Cx43 expression takes place in endothelial cells during inflammation [54]. Thus, gap junctional coupling between circulating tumour and endothelial cells may represent a functional link between inflammatory processes and the local progression of primary tumours.

While the correlation between the intensity of gap junctional coupling and the invasive potential of blood and tumour cells including their penetration through the endothelium seems well documented, the molecular mediators of these processes remain unidentified. Since tumour cells are often characterised by elevated cytoplasmic calcium levels, and gap junctions mediate the intercellular transfer of calcium ions, gap junctional coupling between tumour and endothelial cells may elevate calcium concentrations in the endothelium and induce the retraction of the endothelial layer, thus reducing its barrier function [55]. However, other systems involved in the transmigration of tumour cells can be activated by connexin expression, for example those dependent on metalloproteinases and cell cycle-related regulatory factors.

In summary, connexins should be recognised as potential factors determining the invasive and metastatic potential of tumour cells. They maintain the intercellular communication pathways that malignant cells need in order to overcome tissue barriers and invade the surrounding tissues.

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