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‘Gap Junctions and Cancer: Communicating for 50 Years’

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Abstract

Fifty years ago, tumour cells were found to lack electrical coupling, leading to the hypothesis that loss of direct intercellular communication is commonly associated with cancer onset and progression. Subsequent studies linked this phenomenon to gap junctions composed of connexin proteins. While many studies support the notion that connexins are tumour suppressors, recent evidence suggests that, in some tumour types, they may facilitate specific stages of tumour progression through both junctional and non-junctional signalling pathways. This Timeline article highlights the milestones connecting gap junctions to cancer, and underscores important unanswered questions, controversies and therapeutic opportunities in the field.

Introduction

In the 1960’s, electrical coupling and diffusion of small hydrophilic fluorescent tracers (< 1000 Daltons) between adjacent cells in animal tissues was described[1-3]. In a seminal *ex vivo* study published in 1966, Loewenstein and Kanno demonstrated that the electrical coupling found in healthy hepatocytes was lost in liver tumour cells[4] (FIG 1). Additional studies ensued in rat liver tumours[5], resected human thyroid cancer tissue[6], and in cultured mammalian cancer cells[7], supporting the hypothesis that loss of direct intercellular communication was a characteristic of cancer cells[7,8]. Concurrent electron microscopic

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approaches by McNutt and Weinstein demonstrated that the “nexus” of intercellular junctions normally observed was absent in human invasive cervical squamous cell carcinoma (SCC)[9]. These nexus sites were characterized as containing apposing hexagonal array structures with defined 2nm “gaps” between cells[10] (FIG 2). Subsequent permeability studies combined with structural and functional data led to the realization that these nexus sites were “gap junctions” consisting of clustered channels that enabled direct intercellular communication[11]. These gap junctions were later isolated and characterized by X-ray diffraction analysis[12], and the subsequent isolation of the structural protein subunits of gap junctions in 1974 led to their proposed naming as “connexins”[13]. This term later became mainstream following the cloning of the subunits towards the end of the 1980’s[14].

Meanwhile, evidence began to mount suggesting gap junctions may be causally important in tumorigenesis. Metabolic cooperation[15], a phenomenon whereby metabolites are shared with adjoining cells, was shown to be dependent on gap junctions[16] and this was frequently dysregulated amongst tumour cells and between tumour cells and their normal counterparts[17,18]. In other correlative but seminal studies, non-genotoxic chemicals often referred to as “tumour promoters” were shown to be effective inhibitors of gap junctional intercellular communication (GJIC) and metabolic cooperation[19,20]. For instance, the potent tumour promoter 12-O-tetra-decanoylphorbol-13-acetate (TPA) caused a rapid and significant decrease in the number of gap junctions in mouse interfollicular skin cells[21]. Not only tumour promoters, but also cancer-causing viruses, like the avian sarcoma virus, were shown to rapidly reduce GJIC[22]. Consequently, as a putative hallmark of cancer[23], loss of GJIC was proposed as a screening tool to identify reagents with tumour promoting activity[19,20].

In the years that followed, determining how gap junction channels were biosynthesized, assembled, and regulated would prove to be much more complex than initially imagined. In this Timeline article, we summarize key landmarks linking gap junctions to cancer focusing on the challenging observations that connexins display cancer type- and cancer stage-dependent functions.

The connexin family

The need to identify the gap junction genes became apparent in 1981, when the introduction of total mRNA from GJIC-competent cells was shown to be sufficient to restore GJIC in communication-deficient cells[24]. By 1986, several independent groups had isolated cDNAs of liver gap junction proteins[25-27] and the following year the gene encoding a cardiac gap junction protein of 43 kDa was cloned and named connexin43 (Cx43)[28]. Studies in *Xenopus* oocytes confirmed that cDNAs encoding connexins were necessary and sufficient for GJIC[29]. While the field continues to use the “connexin” prefix (Cx) followed by the predicted molecular mass of the human connexin protein in kilodaltons[14] as a nomenclature, the corresponding genes were named with a “*GJ*” (gap junction) prefix followed by a letter designating the family subclass and a number indicating the cloning order within that class. For example, the gene name of human Cx43 was assigned as gap junction alpha 1 (*GJA1*).

The ensuing decade of connexin gene cloning swiftly led to the realization that the connexin gene family was surprisingly large, consisting of 21 members in humans[30,31]. Connexin antibodies, proteolysis studies, and hydrophobicity analysis of the polypeptide sequences[32] revealed that all connexins consist of four-transmembrane domains and two extracellular loops that are remarkably similar amongst connexins (FIG. 2). The intracellular loop and C-terminal tail exhibit more divergence, with the C-terminal tail being the major determinant of connexin size, ranging from 23-62 kDa. It was quickly realized that connexins were expressed in every human organ, in a tissue-specific manner, and that cells almost always expressed multiple connexins[31], which can assemble into heteromeric hemichannels[33] that combine to form unique channels with specific permeability properties[34](FIG. 2). This diversity in channel permeability between different connexins posed a significant challenge to the field and sorting out the transjunctional selectivity remains a daunting task.

Expression and localization

Following their cloning, characterizing connexin expression, diversity and spatial localization in tumours became possible. These studies revealed a range of outcomes that reflects the complexity of tumour types and stages of disease[35]. Most tumours exhibited no discernible connexin expression whilst other tumours expressed connexins in the cytoplasm or at cell-cell junctions. Elevated mRNA and protein levels of connexins that were noted in some tumours were often correlated with connexin mislocalization (e.g., Cx26 (encoded by *GJB2*) in pancreatic[36] and colon[37] cancer, and Cx43 and Cx32 (encoded by *GJB1*) in prostate cancer[38]). Similarly recent studies tracking connexin levels as a cancer prognostic indicator produced a diverse set of outcomes. Over a dozen studies in the last decade correlate high connexin expression with a significantly better prognosis (e.g., Cx43 in prostate[39], pancreatic[40], breast[41], head and neck SCC[42], non-small-cell lung[43], and colorectal[44] cancers; and Cx26 in colorectal[45] and intestinal type-gastric[46] cancers). In contrast, more than a dozen studies correlate high connexin expression with a poor prognosis (e.g., Cx43 in oral SCC[47], esophageal SCC[48] and non-muscle invasive urothelial bladder cancer[49]; and Cx26 in breast cancer[50,51], lung SCC[52], esophageal SCC[53], colorectal cancer[37] and papillary and follicular thyroid cancer[54]). The complexity of these mixed findings may be partly explained by the connexin family member being assessed not only in the tumour but also in the host tissue. For example, in the same breast cancer series, elevated Cx43 and Cx30 (encoded by *GJB6*) were associated with improved and worse breast cancer outcomes, respectively[41]. In the case of Cx43, more studies favour its role as a tumour suppressor and a good prognostic indicator. Strikingly, the opposite is true for Cx26, as many more studies report its detection in tumours as a poor prognostic indicator. Thus, assessing connexin levels in human tumours is currently not a useful diagnostic as more direct functional analysis of connexins in tumorigenesis will be necessary.

Connexins as tumour suppressors

In the early 1990's, expression of specific connexins in cancer cell lines was found to be sufficient to restore GJIC and, in some cases, partially "normalize" their phenotype. Cx43 was shown to suppress growth of transformed mouse embryo cells *in vitro*[55], and to reduce rat glioma growth *in vitro*[56] and *in vivo*[57], whereas Cx32 was only shown to reduce human

hepatoma cell growth when cells were injected into mice[58]. Numerous studies followed and substantial evidence has now accumulated implicating connexins in cell proliferation[59], apoptosis[60], chemoresistance[61], migration[62] and invasion[63], although not all studies point towards a tumour suppressor role. Overexpression models also revealed that connexins regulate other features such as epithelial to mesenchymal transition (EMT), tumour cell differentiation and angiogenesis[64,65]. To date, there are many studies on how connexins regulate molecular pathways linked to cancer biology, which are reviewed extensively elsewhere[59-63,66].

Mouse models

The mid-90's saw the emergence of several connexin knockout (KO) mouse models (TABLE 1). Cx32 KO mice were shown to have a significant increase in both spontaneous liver tumours[67] and chemically-induced liver[68,69] and lung tumours[70]. These findings fit well with the loss or mislocalization of Cx32 that had been previously observed in liver tumours in rats[71] and humans[72], and the reversal of the neoplastic phenotype upon re-expression of Cx32 in rat liver cells[73]. An increase in susceptibility to chemical hepatocarcinogenesis was also observed in a transgenic mutant mouse model expressing a dominant-negative mutant (V139M) of Cx32 which causes a loss of channel function in the liver[74]. X-ray radiation also dramatically increased liver tumorigenesis in Cx32 KO versus wild type mice, as well as significantly augmented tumour formation in the lung, adrenal gland, lymph nodes and small intestine with an associated activation of the ERK pathway[75]. In line with this, a Cx32/p27 (encoded by cyclin-dependent kinase inhibitor 1b (*Cdkn1b*) double knockout (DKO) mouse model, displayed an increase in tumour formation in the intestine, adrenal gland and pituitary over that of the Cx32 KO, but had reduced liver tumours, pointing toward tissue and pathway specific interactions and crosstalk effects[76]. To complicate data interpretation further, there may also be sex-specific effects. For example, one study observed a significant increase in spontaneous liver tumours only in male Cx32 KO mice, while in female KO mice the incidence of pituitary adenoma was lower than that of control mice[67].

While early studies suggested that connexins co-expressed within the same organ would serve similar tumour suppressive roles, this turned out not to be the case. Unlike Cx32, conditional knockout of the other major liver connexin, Cx26, was not associated with a significant increase in chemically-induced liver tumour incidence[77]. Yet, in a mammary gland-specific Cx26 KO mouse model, 7,12-dimethylbenz[a]anthracene (DMBA)-treated KO mice developed significantly more primary and multifocal mammary tumours, compared to controls[78], again pointing to tissue-specific effects.

Given the availability of genetically-modified mice, questions arose as to whether the most widely expressed connexin in mammals, Cx43, would have tumour suppressive properties *in vivo*. To this end, heterozygous $GJA1^{+/-}$ mice were found to be significantly more susceptible to urethane-induced[79], DMBA-induced[80], and nicotine-derived nitrosamine ketone (NNK)-induced[81] lung tumours. Paradoxically, a correlation between increased Cx43 mRNA expression specifically in the NNK-induced tumour lesions and increased tumour aggressiveness was noted, suggesting the tumour-suppressive effect is lost at late stage lung

tumorigenesis[81]. In a DMBA-induced breast cancer model, a transgenic mouse harbouring a G60S Cx43 mutant that reduces overall GJIC crossed with an *Erbb2* overexpressing mouse exhibited significantly increased mammary gland dysplasia and tumour metastasis to the lungs[82].

Overall, genetically-modified connexin mouse models have supported the notion that connexins are tumour suppressors. These same mouse models have also served to elucidate more complex features of tumorigenesis such as the role of connexins in the surrounding tumour microenvironment that might affect tumour growth either independently or via direct communication with tumour cells. In this respect, a recent study showed that Cx40 KO mice exhibited reduced angiogenesis and tumour growth of subcutaneously implanted human melanoma or mouse lung tumour cells compared to wild type or KO mice specifically re-expressing Cx40 in endothelial cells[83]. Moreover, injecting wild type mice with peptides targeting Cx40 also reduced tumour growth[83]. This suggests that endothelial Cx40 conveys a benefit to the tumour by facilitating endothelial growth and tumour angiogenesis. Similarly, endogenous Cx43 in astrocytes appears to enhance glioma invasion in the brain through the exchange of proinvasive molecules (see below)[84]. It is clear that additional genetically-modified mouse models will be needed to gain further insights into the role of connexins in tumorigenesis.

Connexin gene mutations—Targeted gene sequencing has identified some somatic *GJA1* gene mutations in advanced stage colorectal tumours[85]. However, the advent of whole exome sequencing of human tumours should answer the question of whether driver mutations in connexin encoding genes can promote tumorigenesis and metastasis. Our search of the IntOGen platform[86], which systematically analyses many sequencing projects, did not find any mutated connexin genes as a driver in any tumour type (http://www.intogen.org/). However, from the mutational frequencies one can infer that the putative modulatory effect of connexins on cancers is likely to be connexin isoform and cancer type specific. For example, for the gene encoding Cx43, *GJA1,* mutations affecting the protein sequence are more frequent in some tumours, such as stomach adenocarcinomas (2.48%, 161 samples) and cutaneous melanoma (2.44%, 369 samples), compared to all tumour types (0.5%, 6792 samples). In contrast, the *GJA10* gene encoding Cx62 is more frequently mutated in tumours such as small cell lung carcinomas (4.34%, 69 samples) and lung SCC (3.45%, 174 samples) compared to 0.6% mutations in all tumours (6792 samples).

Since germline and somatic mutations in nearly half the connexin gene family have been linked to a wide range of developmental abnormalities, syndromes, and diseases[87], it should soon be possible to data mine these patients' records for any links to cancer or disease progression. To that end, *GJB2* mutations (encoding Cx26) (OMIM: #121011) causing the rare syndrome of keratitis-ichthyosis-deafness (OMIM: #148210) appear to be associated with an increased propensity to develop skin cancer[88]. Considering *GJB2* mutations that cause the loss of Cx26-based GJIC are the most common cause of congenital sensorineural deafness (OMIM: #220290) worldwide (the most common mutation, 35delG, has an estimated carrier frequency of 1 in 51 in the overall European population[89]), the field awaits population-wide epidemiology studies to assess cancer incidence and progression in this unique population cohort. Similarly, persons with X-linked Charcot-Marie-Tooth (CMTX)

neuropathy (OMIM: #302800), due to mutations in the *GJB1* gene encoding Cx32[90], will be another large group of patients to assess in detail, considering the strong links between this connexin and liver tumours in mice (Table 1) and in particular the observed tumour susceptibility of transgenic mice expressing the dominant negative CMTX mutation V139M[74].

Connexin regulation in cancer

Expression

Although connexins have a rather simple gene structure (in most cases the entire connexin protein is encoded by a single exon), their regulation from transcription to function is under tight control and subject to a wide range of regulatory mechanisms. At the gene level, at least some members of the connexin family are subject to extensive epigenetic control[91,92]. For example, promoter hypermethylation of *GJC1* encoding Cx45 was shown to reduce Cx45 expression in colon cancer cell lines and in colorectal tumours[93]. At the post-transcriptional level, several microRNAs (miRNAs) have been shown to downregulate Cx43 expression[91,92], e.g., the miR-221/222 cluster and miR-125b in glioma[94,95] and miR-20a in prostate cancer[96].

A less well understood aspect of gene regulation is the role of pseudogenes, ancestral copies of genes that have lost the ability to code for proteins. Some pseudogenes are transcribed and can have coding-independent functions related to tumorigenesis, as illustrated with the *PTENP1* pseudogene which competes with *PTEN* for miRNA binding and as a consequence, loss of *PTENP1* mRNA levels in tumours can lead to enhanced microRNA-mediated downregulation of PTEN protein expression[97].This same study also identified two miR-1 binding sites in the *GJA1* pseudogene *GJA1P*[97]. Thus, *GJA1P* may affect Cx43 expression indirectly since miR-1 is well known to inhibit Cx43 expression[92,98]. Further investigation of the *GJA1P* pseudogene is clearly warranted as reports have suggested that it is transcribed and even translated, and acts as a tumour suppressor in breast cancer cells[99,100].

Translational regulation of connexins is also tightly controlled (reviewed in[92]) and several connexins have been suggested to possess an internal ribosome entry site (IRES) in their 5′UTR allowing maintenance of translation where cap-dependent translation may be compromised, such as in differentiated or density-inhibited cells. Notably, the antiproliferative effect of somatostatin receptor type 2 was linked to IRES-dependent induction of connexin expression causing restoration of density-inhibition in pancreatic cancer cells[101]. Another recent discovery that highlights both the complex regulation and function of connexins is the finding that truncated isoforms of Cx43 are translated in some cell types. Smyth and Shaw[102] demonstrated internal translation of various N-terminally truncated isoforms of Cx43, with the major 20-kDa isoform acting as a chaperone protein critical for trafficking of full length Cx43 to the cell membrane. Interestingly, specific loss of the 20-kDa isoform (but not full length Cx43) in human breast cancer samples has been reported suggesting that these isoforms can be independently regulated[103]. This isoform was also described to reside in the nucleus of glioma cells where its functional role is unknown[104]. Internal translation of truncated Cx43 isoforms has been shown by several

groups to be strongly influenced by signalling pathways activated in cancer including MAPK-interacting serine/threonine protein kinase 1 (MNK1; also known as MKNK1) and MNK2, Akt and mTOR[102,105,106], and is also activated by hypoxia[106], a condition linked to mTOR activation, tumour progression and drug resistance. The notion of truncated Cx43 isoforms needs to be considered in the context of non-junctional Cx43 functions (discussed below), erroneous membrane trafficking of Cx43, and accumulation of Cx43 (and its truncated isoforms) in the cytoplasm or nucleus, all aspects frequently observed in tumours[44,47,81,107-109].

Phosphorylation—Connexin activity is modified by many post-translational modifications including SUMOylation, S-nitrosylation, palmitoylation, phosphorylation and ubiquitination[110]. In the context of cancer, by far the best-studied connexin modification has been phosphorylation. It was already suggested in 1983 that cell-cell communication was regulated by protein kinase activity[111] and in 1986, phosphorylation of Cx32 was demonstrated[112]. Subsequently, loss of GJIC was shown to occur following expression of specific oncogenes, including SRC[113] and HRAS[114], which was thought to be mediated by phosphorylation. In 1990, specific phosphorylation of Cx43 was demonstrated in Rous sarcoma virus transformed cells[115] and upon v-SRC expression[116]. Other studies followed verifying Cx43 as a phosphoprotein[117-119]. Notably, TPA was shown to rapidly inhibit Cx43-mediated GJIC[120-122], which is thought to occur through protein kinase C (PKC)- and ERK-mediated phosphorylation events[123,124]. Cx43 is now known to be controlled by a complex network of regulatory mechanisms whereby numerous kinases and phosphatases systematically target at least 16 different phospho-sites of Cx43[125]. Overall, phosphorylation of Cx43 regulates its trafficking, gap junction assembly and endocytosis[126], gap junction plaque (cluster of connexin channels) size and channel-gating[127], and degradation and protein-protein interactions[128], ultimately either enhancing or reducing GJIC (as reviewed in[125,129,130]). Several other connexins are phosphoproteins but much less is known about the role of kinases in regulating these other family members[130].

Reassessing connexins in cancer

Context-dependent effects

Despite extensive evidence supporting connexins as tumour suppressors, many exceptions to this concept have arisen in the last couple of decades. Thus, there has been an evolution towards the understanding that in some tumours or at later tumour stages, increased connexin expression may engender tumours with more aggressive tendencies or features[66,131] (FIG 3). Key evidence for this concept emerged in 2000, where mouse melanoma cells transfected with cDNA coding for Cx26 were shown to display increased metastatic potential when injected subcutaneously into mice[132]. The authors suggested this was due to enhanced intravasation and extravasation, as Cx26 facilitated heterologous GJIC between melanoma cells and endothelial cells *ex vivo*. Several other studies now suggest that increased connexin expression within the tumour (and even in the tumour stroma[133]) at late stage disease facilitates metastatic features such as migration and invasion[134-137] (reviewed in [63]), endothelial adhesion[138,139], intravasation and extravasation[132,139-143], and targeting to the metastatic site [144]. In addition, a recent study clearly demonstrates how

Cx43 can increase growth of brain metastases at a very late stage, after extravasation and remodelling of existing vascular networks[145]. Some of these features may be isoform specific effects. For example, Cx43 expression reversed EMT and prevented resistance to cisplatin chemotherapy in the A549 lung adenocarcinoma cell line[146], whereas Cx26 expression induced EMT via the PI3K/AKT signalling pathway and conferred resistance to the epidermal growth factor receptor (EGFR) inhibitor gefitinib in HCC827 and PC9 lung adenocarcinoma cells[147]. Similarly, tissue-specific effects also need to be considered; whilst Cx26 promotes EMT in lung cancer cells[147], it was shown to reverse EMT-like features in breast cancer cells[64]. Moreover, individual connexins may also display dual effects, such as acting as a tumour suppressor in primary tumour initiation only to have the opposite effect of facilitating cancer progression in later stage disease. Studies in the early 1980's showed a clear association between increased GJIC and resistance to radiotherapy, specifically in three-dimensional (3D) culture conditions[148]. Supporting this notion a recent study showed that, although restoration of Cx30 expression reduced growth of glioblastoma cell lines, it indeed conferred resistance to γ-radiation[149]. Nevertheless, in patient cohorts treated with radiation therapy, expression of Cx30 was associated with increased mortality[149]. Taken as a whole, stratification of tumour subtype, stage, and heterogeneity with connexin isoform profiles should be carried out to delineate connexin function in cancer.

Non-junctional functions

Increasing evidence suggests connexins also have functions unrelated to GJIC that are important in cancer progression. This idea arose from the observation that only Cx26, and not Cx32 or Cx43, was shown to repress tumorigenic features in HeLa cervical cancer cells, even though all three connexins enhanced GJIC[150]. A more recent study[151] has provided insight into a potential isoform-specific GJIC-dependent molecular mechanism behind these observations, whereby, Cx26, but not Cx32 and Cx43, maintains functional GJIC during the G2/M phase which favours intercellular redistribution of cyclic AMP (cAMP) delaying cell cycle progression. However, it is now clear that specific GJIC-independent mechanisms are operating (see reviews[59,152]) of which two different functional explanations have gained popularity: firstly, connexin hemichannels communicating with the extracellular environment and secondly, the role of connexin-interacting proteins.

Hemichannels—Evidence has emerged that unpaired gap junction channels otherwise known as "connexin hemichannels" act as direct channels between the cell cytosol and the extracellular milieu[153]. ATP release and modulation of Ca^{2+} concentrations regulate cell proliferation in a variety of cell types[154-156], and inappropriate hemichannel opening may underlie some hyperproliferative disorders such as hidrotic ectodermal dysplasia[157]. Hemichannel functions have also been linked to vascular disruption and haemorrhage within tumours[158], and recently Cx43 hemichannels of osteocytes are involved in suppression of breast cancer cell growth and bone metastasis[159]. However, the link between hemichannels and cancer is difficult to conclusively establish as most reagents that block gap junction channels also block connexin hemichannels making assignment of functional consequences specifically to hemichannel functions problematic[160]. Furthermore, studies may also be confounded by the action of pannexin channels that also allow communication between the cellular cytosol and extracellular milieu (BOX 1).

Connexin-interacting proteins—Connexin hemichannels alone are not sufficient to explain studies where connexins retained within intracellular compartments regulate cellular characteristics associated with cancer. Indeed, the ectopic expression of a carboxy-terminal tail fragment of Cx43 alone was as efficient as full length Cx43 in inhibiting the proliferation of the mouse neuroblast cell line Neuro2a[161]. This finding was supported by similar observations in human osteosarcoma U2OS cells and immortalized monkey COS-7 cells[162]. Cell growth was also reduced in non-tumorigenic cardiomyocytes, where the C-terminus of Cx43 was reported to be localized to the nucleus[163]. The mechanistic explanation for these observations are likely rooted in the Cx43 interactome. While all connexins likely interact with at least a few proteins, the Cx43 interactome is extensive (FIG 4 and Supplementary FIG 1 and Supplementary Table 1). Notably, Cx43 is known to bind directly with many key cancer-regulatory proteins including caveolin 1[164], nephroblastoma overexpressed (NOV, also known as CCN3)[165,166], discs large homolog 1 (DLG1)[167], SRC[168] and BCL-2-associated X protein (BAX)[169]. Some of the functional consequences are being elucidated in more detail. For example, Cx43 can regulate cellular migration via an interaction with calcium/calmodulin-dependent serine protein kinase (CASK; also known as LIN2)[170], but it is also clear that this and other Cx43 interactions occur through complex protein networks, as reviewed elsewhere[171]. Interactions between Cx43 and other proteins, such as zona occludens 1 (ZO1)[172], is also of importance for Cx43 channel assembly and regulation specifically during cancer progression. Apart from direct and indirect Cx43 interactions, Cx43 may change the expression levels of other proteins important in cancer, such as Cx43-mediated downregulation of p27 via E3 ubiquitin ligase complexes containing S-phase kinase-associated protein 2 (SKP2)[173].

Reports in recent years have noted that connexins may take up residence in unexpected intracellular compartments. Notably, Cx43 was detected in the inner membrane of the mitochondria of cardiomyocytes[174] possibly regulating cytochrome C-mediated apoptosis[175], raising the idea that mitochondrial Cx43 may also play a role in cancer. In pancreatic tumour cells, Cx43 was shown to induce apoptosis through an interaction with the mitochondrial anti-apoptotic protein BAX[169]. Moreover, in a human glioblastoma cell line Cx43 enhanced the efficacy of several chemotherapy agents via down-regulation of anti-apoptotic BCL-2 in a GJIC-independent manner[176]. However, this is contrasted by recent studies where the BAX/BCL-2 pathway was also shown to be regulated by Cx43 in human glioma cell lines, but in this case, Cx43 appeared to increase resistance to temozolomide chemotherapy, via both GJIC-dependent and independent pathways[177]. Cx43 downregulation was also shown to affect the cytoprotective properties of tumour cells by causing enhanced sensitivity and mitochondria-mediated apoptosis in response to low dose γ-radiation [178]. Mitochondrial translocation of Cx30 also appears to provide γ-radiation-resistance in human glioblastoma cells[149]. Going forward, additional studies are needed to determine if targeting the mitochondria-connexin relationship would have any putative therapeutic potential.

Novel connexin functions

The complexity of cancer progression is underscored by the continual identification of new pathways, concepts and features that regulate tumour onset and progression, many of which exhibit some level of cross-talk with connexins.

Cancer Stem Cells—As the concept and importance of cancer stem cells (CSCs) emerged, so did the hypothesis that these cells, like tumour cells were uncoupled or had a distinct connexin expression profile. Indeed, over a decade ago, loss of GJIC in CSCs was suggested as a potential hallmark of cancer (FIG 3)[179]. Some studies have supported this premise; notably, nestin+/CD133+ glioma CSCs were shown to have low levels of Cx43 expression and reduced GJIC[180]. Moreover, Cx43 expression in these CSCs inhibited growth, tumour-sphere self-renewal and invasion *in vitro*, as well as tumorigenicity in mouse xenografts[180]. In contrast, Cx32 was localized to the cytoplasm and was suggested to enhance the CSC self-renewal in Huh7 hepatoma cells[181] raising the question as to whether connexin isoform specificity is critical. Intriguingly, glioblastoma CSCs were shown to express Cx46 whereas non-CSCs expressed Cx43[182] suggesting specific channel permeability properties regulate self-renewal versus differentiation. This result also has implications for the therapeutic concept of the “bystander effect”, where spread of either death or survival signals between neighbouring cells may occur via functional GJIC as discussed in detail below. If connexins truly have different functions in CSCs versus non-CSCs, a significant reappraisal of their role in cancer is needed. Towards this end, the use of more accurate or representative CSC systems would be required, such as the emerging use of patient-derived xenograft (PDX) models[183]. Related to this, breast cancer PDX mice were recently used to study features of circulating tumour cells (CTCs), in which they identified a signature of four genes that included downregulated *GJA1* and was associated with both CTCs and lung metastasis[184]. Indeed this four-gene profile predicted a reduction in distant metastasis-free survival in early breast cancer patients[184]. Taking into account the rising importance of CSCs and CTCs in understanding metastasis, tumour cell response to treatment and disease recurrence, these models may assist the gap junction field in understanding some of the apparent contradicting results documented over the past 50 years.

miRNA transfer—Gap junction-mediated transfer of miRNAs is an emerging field that might help resolve some aspects of the connexin-carcinogenesis link. Lim *et al*[185] investigated tumour dormancy in bone marrow metastasis and suggested that gap junction-mediated transfer of C-X-C motif chemokine ligand 12 (CXCL12; also known as SDF1)-specific miRNAs between bone marrow stroma and breast cancer cells maintained cancer cell quiescence. Conceptually, co-culture experiments had already demonstrated gap junction-mediated miRNA transfer between miR-67-overexpressing and miR-67-negative glioma cells, a process blocked by the GJIC-inhibitor carbenoxolone[186]. More recently, miRNA transfer from glioma cells to astrocytes was shown to enhance the glioma pro-invasive potential[133]. In contrast, gap junction-mediated miRNA transfer between miR-124-3p transfected and non-transfected glioma cells had anti-proliferative effects, demonstrating a miRNA-mediated “bystander effect”[187]. Subsequently, several miRNAs associated with survival and chemotherapy-resistance were suggested to pass through gap junctions formed between astrocytes and lung tumour cells *in vitro*[188]. The discovery that

miRNAs transfer through gap junctions thus requires substantial reassessment of which intercellular signals are important in regulating carcinogenesis. There is a strong possibility that different tissues and cells express miRNAs with both positive and negative effects on growth (BOX 2), and it is likely that miRNA permeability will differ based on the connexin isoforms expressed[189]. Lim *et al.*[185] also noted that miRNA transfer occurred not only via gap junctions but by delivery from exosomes (secreted double-membrane structures that can carry proteins, lipids, miRNAs and mRNAs, which likely regulate cancer progression). Cx43 has now been shown to exist as hexameric channels in the membrane of exosomes and can facilitate the release of exosomal content into target cells[190], an exciting finding that needs to be further addressed.

Gap junctions in immune cells—Gap junctions are known to regulate (and to be regulated by) inflammatory responses such as cytokine release and, surprisingly, connexins are now known to be widely expressed in immune cells as reviewed in [191]. This line of investigation gained further interest following the 2005 report of cross-presentation of possible antigens via gap junction-mediated direct transfer of small peptides[192], a feature that may be lost as tumours shut down GJIC. In cancer, Cx43-derived gap junctions appear to participate in melanoma antigen transfer and cross-presentation between human dendritic cells (DCs), potentially facilitating a more effective DC-mediated T cell activation[193]. Another study suggested activation of autophagy in hypoxic melanoma cells selectively causes degradation of gap-junctional Cx43, potentially impairing natural killer cell-mediated tumour cell killing[194]. In addition to peptides, gap junction-mediated transfer of miRNAs from macrophages to hepatocellular carcinoma cell lines has been reported to regulate gene expression and inhibit tumour cell proliferation[195]. The physiological role of GJIC-mediated miRNA and peptide transfer in immune cells remains poorly defined, but, considering the recent promising advances in cancer immunotherapy, the idea of potentiating the anti-tumour immune response through modulation of GJIC deserves further attention. Towards this end, induction of Cx43 expression caused by infection of melanoma cells with bacteria allowed transfer of pre-processed tumour antigens from melanoma cells to DCs, improving DC-based tumour vaccination by increasing T cell activation, and anti-tumour immunity[196].

Multi-cellular interconnections—Tumours develop and grow in a complex microenvironment that contains both diseased and normal cells. Several recent studies suggest that GJIC between tumour cells and normal cells may be detrimental to the host, for example by facilitating metastasis and host colonization[132,142] or by enhancing the local brain invasion as seen in gliomas[84]. Another recent study suggests gap junction-mediated diffusion of pro-survival short RNAs between mouse astrocytes and human tumour cells provide increased resistance to chemotherapy[188]. Connexins have now been linked to yet another feature related to multi-cellular interconnections; Osswald *et al*[197] reported that astrocytoma cells were connected by microtubes that facilitate invasion and resistance to radiotherapy. These microtubes were shown to contain Cx43 that could facilitate the spread of toxic levels of calcium following radiation therapy. Consistent with a long-standing concept in the gap junction field, these authors suggest that intercellular microtubes allow individual tumour cells to promote cell survival by diluting out lethal levels of calcium or other toxic metabolites[197] (BOX 2). Whether networks of microtubes exist in other cancer

types, and their prognostic and therapeutic value, are important future questions to answer. Related to this, a recently described carcinoma-astrocyte interaction network was shown to promote brain metastasis of breast and lung cancers[145]. In this study, Chen *et al.* showed double-stranded DNA could induce the production of the second messenger 2′3′-cyclic GMP-AMP (cGAMP) in tumour cells, which upon traversing to astrocytes via Cx43 gap junctions activated the stimulator of interferon genes (STING) pathway. Subsequent STING-mediated production of interferon α (IFNα) and tumour necrosis factor (TNF) in astrocytes in turn act as paracrine signals stimulating signal transducer and activator of transcription 1 (STAT1) and nuclear factor-κB (NF-κB) survival pathways in the neighbouring cancer cells[145]. Thus, the elegant work of Osswald *et al.* and Chen *et al.* joins several emerging examples highlighting the profound impact of the tumour network and the stromal context, where connexins facilitate interactions between cancer cells or between cancer cells and the host providing support for aggressive late-stage tumours.

Therapeutic Potential

Since the 1966 hypothesis of Loewenstein suggesting intercellular communication can control cell growth[4], anti-cancer therapy targeting GJIC has been extensively explored. Twenty years later, in 1986, pioneering work directly demonstrated that inhibition of transformed cell growth was dependent on the degree of communication with normal cells, and that such heterologous communication and growth repression could be stimulated by cAMP-dependent phosphorylation of gap junctions and blocked by retinol and retinoic acid[198]. Seminal work by Yamasaki and Katoh[199,200] two years later demonstrated the therapeutic potential of targeting GJIC using two independent approaches. Firstly, they provided further evidence that adding chemicals such as dibutyryl cAMP, fluocinolone acetonide or dexamethasone, could be used to re-establish communication between transformed cells and normal neighbouring cells, leading to inhibition of cell transformation and potentially also reverting transformed cells to a normal phenotype[199]. Secondly, they took advantage of the lack of gap junction communication between transformed and non-transformed cells by injecting Lucifer Yellow (LY) dye so it would spread only between transformed cells[200]. Subsequent blue light irradiation (activating LY) specifically killed the tumour cells that had received LY via GJIC[200].

Bystander effect

The work of Yamasaki and Katoh[199,200] was followed by a period in the 1990's where studies suggested that GJIC and connexins could underlie the bystander effect observed during suicide gene therapy approaches, a concept stemming from the demonstration of GJIC-mediated metabolic cooperation[15,16]. Using the herpes virus thymidine kinase (HSV-TK) gene to render cancer cells sensitive to the drug ganciclovir, it was noted that HSV-TK free neighbouring cells also died. *In vitro* studies indicated a metabolite by-product of ganciclovir passed to uninfected cells via gap junctions causing cell death[201,202], and the extent of this bystander cytotoxicity was shown to correlate with GJIC activity[203]. Expression of connexins and the subsequent levels of GJIC correlated well with the bystander effect *in vitro*[204,205]. *In vivo* studies also suggested gap junctions promoted the bystander effect and enhanced ganciclovir therapy[206]. A number of articles substantiated the

role of GJIC in the bystander effect[207-211], including very recent studies in breast cancer[212], although some studies reported little or no effect[213]. Conversely, it was proposed[214] that HSV-TK transduced cells could be significantly protected from cell death by neighbouring cells via GJIC, possibly through the dilution of ganciclovir (or activated ganciclovir) or by the sharing of survival signals (the so-called “Good Samaritan effect”). Thus, whether actively enhancing GJIC would increase or decrease drug killing of tumour cells is not well defined[214]. The “kiss of death” or “kiss of life” conundrum (BOX 2) may be linked to the concentration of the effector, the connexin isoforms[215] and whether connexin-independent mechanisms are involved[213,216].

Regulating connexin function—A number of natural compounds have been shown to upregulate GJIC and potentially modulate cancer growth or enhance cytotoxic therapy; examples include retinoids and carotenoids[61,198,199,217-220], various flavonoid anti-oxidants such as genistein, quercetin, green tea catechins and caffeic acid phenethyl ester[221-227] and other non-flavonoid chemicals such as sulforaphane and red wine resveratrol[228,229]. However, these upregulating approaches are not connexin or GJIC specific. In a more promising and direct approach, a phase 2 trial has begun where a peptide mimetic is being used to increase Cx43-based GJIC (but reduce hemichannel activity) as a potential treatment for chronic wounds[230]. In cancer, it would be critical to use peptide mimetics that either enhance GJIC in early stage disease where it may suppress tumour growth or mimetics that block connexin functions in late stage disease where the connexin appears to give the tumour a survival advantage. However, using peptides to target connexins has not yet been endorsed for clinical trials in cancer treatment.

Since the early discovery of the reversible chemical inhibitor of GJIC 18-alpha-glycyrrhetinic acid[231], technological advances have brought a number of additional modalities (epigenetic modulators, antibodies, peptides, antisense RNA, miRNAs, CRISPR/Cas9) to inhibit GJIC and connexin functions. The long-standing hypothesis that connexins are tumour suppressors would argue against GJIC inhibition in a cancer treatment setting, particularly when treating primary tumours that have not yet metastasized. However, this position must be readdressed given the growing evidence that connexins facilitate metastatic disease in some specific cases. Indeed, the GJIC blocker oleamide was recently found to have anti-metastatic properties in MDA-MB-231 breast cancer cells *in vitro* and *in vivo* following intravenous delivery in mice[143]. From a therapeutic point of view, very promising and potent effects were recently reported in the aforementioned work of Chen and colleagues[145]. Blocking heterologous breast and lung carcinoma-astrocyte gap junctions either using gap junction inhibitors (tonabersat or meclofenamate) that pass the blood brain barrier or by Cx43 knockdown, clearly prevented metastasis progression in mouse models. Moreover, combining this treatment with traditional chemotherapeutics (carboplatin) was highly effective at blocking metastasis[145].

In addition to pharmacological approaches to regulate GJIC, Cx43 blocking antibodies have also been shown to reduce tumour growth either alone[232] or in combination with standard cancer therapy[233]. In other novel cases, Cx43 antibodies have been used as a guidance system to deliver diagnostic markers or therapeutic compounds such as cisplatin to Cx43-positive tumour cells[234,235].

Another strategy that has potential promise in cancer therapy is the use of short connexin mimetic peptides to modulate connexin function or gap junction permeability, although these have mostly been applied to treating inflammatory diseases[236]. For instance, a peptide (αCT1) mimicking the C-terminal of Cx43, blocking ZO1 interacting with Cx43, was shown to cause specific inhibition of Cx43 hemichannel function (but maintaining GJIC), which prevented temozolomide resistance in human glioblastoma cell lines[237]. In another study the same peptide augmented the effect of the oestrogen receptor modulator tamoxifen and the ERBB2 inhibitor lapatinib in breast cancer cell lines, although the authors suggested the key function of the peptide in this setting was to enhance GJIC[238]. The exact mode of action of this peptide appears complex and perhaps even context-dependent.

Nevertheless, these exciting new therapeutic developments provide hope for the development of novel anti-cancer drugs targeting connexins for the treatment of specific tumours. Although connexins are attractive therapeutic targets due to their exposure on the cell surface and the ability of drugs to block channel activity, there are significant challenges as connexins are also critical for healthy tissue function. Targeting (GJIC-independent) cancer associated connexin-protein interactions may be one approach towards reducing possible side effects associated with loss of essential GJIC-functions (for example electrical coupling in heart or brain). Using this strategy, a cell-penetrating peptide that mimicked and blocked the Cx43 binding site of SRC induced differentiation of glioma stem cells providing a proof-of-principal that this approach has merit[239].

Concluding Remarks

As personalised medicine continues to advance and focus on patient-specific global gene-expression profiles, the analysis of how gap junctions contribute to the physiology and pathology of patient-specific tumours will be highly informative. The connexin-isoforms expressed, the tumour type or sub-type, and the stage of disease significantly influence the role of connexins in tumours. Within this context, both channel-dependent and -independent functions are likely to operate, in a complex interplay between the tumour cells and the surrounding microenvironment. The breadth of studies linking connexins to cancer is daunting and an overall generic message has not emerged that can be applied to all tumour types. In fact, it is perhaps ironic that some of the apparent successful connexin-based anti-cancer treatment modalities reported in the last few years are based on blocking connexin and gap junction activity in advanced disease; a concept inconsistent with the mainstream beliefs of the field over the last 50 years. However, we cannot dismiss the importance of connexins in protecting against tumour onset and early disease progression as prevention is arguably more important than treatment. The success of translational efforts will clearly rely on the continual elucidation of the complex biological regulation and function of connexins. We predict that any useful treatments that emerge will take tumour type, stage and properties into account. It also seems clear that targeting connexins alone will likely not be enough, and combinatory treatments will be necessary. Future efforts to move this field forward will require multifaceted approaches to elucidate fundamental aspects such as the role of connexins in cancer stem cells and non-junctional functions, and to fully appreciate their role system-wide such as in the immune system or in tumour stroma. Our current

understanding of half a century worth of research on gap junctions and cancer can potentially be utilised to develop effective therapeutics of benefit to cancer patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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BOX 1

Pannexins: Communication in another language

Pannexins (a family of three members; pannexin 1 (PANX1), PANX2, PANX3) were identified in 2000 based on sequence homology to the invertebrate gap junction proteins, the innexins, and were quickly proposed to be a new family of ubiquitously expressed vertebrate gap junction proteins[240,241]. Even though they have no sequence homology to connexins, they exhibit a comparable topology with four hydrophobic transmembrane domains, one cytoplasmic domain and two extracellular loops. Early studies suggested they may form intercellular channels[241]. However, it is now clear that their main function is to make cell surface single-membrane channels that release autocrine and paracrine signals to the extracellular matrix[242], analogous to the proposed functions of connexin hemichannels (FIG 2).

Similar to what was described for Cx43 over two decades earlier[56], the overexpression of PANX1[243] or PANX2[244] in rat C6 glioma cells reduced monolayer cell growth and *in vivo* tumour growth in immunocompromised mice. In untransformed cells, PANX1 and PANX3 also reduced cell growth when overexpressed in rat epidermal keratinocytes[245]. Likewise, in chondrocytes PANX3 promoted a switch from proliferation to differentiation[246], and recently, PANX3 was shown to significantly inhibit osteoprogenitor cell growth through inhibition of the WNT pathway and via calcium-mediated regulation of cyclin-dependent kinase inhibitor 1A (*CDKN1A*, which encodes p21)[247].

As with connexins however, pannexins may also possess pro-tumorigenic features. PANX1 overexpression induced neural progenitor cell proliferation, possibly via ATP release[248] and knockdown of PANX1 in mouse melanoma cells induced cell re-differentiation and reduced tumour growth[249]. A recent elegant study identified PANX1-mediated ATP release as a mechanism of metastatic cell survival in the microvasculature, and channel inhibition significantly reduced breast cancer metastasis[250]. Another relevant finding is the link between PANX1, cell death and the release of “find-me” ATP and ADP signalling molecules that activate the immune system[251]. Finally, it is important to note that many of the pharmacological agents used today block both pannexin and connexin channels, and both channel types have been well documented as forming separate large-pore channels that engage in ATP release. Going forward, identification of isotype and disease stage specificity is needed to clearly discern the functional role of these two families of channel-forming proteins in cancer.

BOX 2

The complex nature of the intercellular signal: Kiss of Life or Kiss of Death?

An unresolved issue, partly due to its enormous complexity, is the full elucidation of the exact signalling molecules exchanged via gap junctional intercellular communication (GJIC) that modulate cancer malignancy. Gap junction-permeable signals known to be important include calcium, ATP, cyclic AMP (cAMP), cGMP, 2′3′-cyclic GMP-AMP (cGAMP), polyamines, nucleotides, glutathione, amino acids such as glutamate and other nutrients such as glucose. However, the Human Metabolome Database (HMDB, http://www.hmdb.ca/) currently lists 39674 metabolites smaller than 1500 daltons (thus potentially small enough to diffuse through gap junction channels). In addition, an increasing number of reports suggest miRNAs[185-189] and potentially even small peptides[192,193,196] can pass through gap junctions (FIG 2).

Depending on the specific signalling molecule exchanged through a gap junction channel, whether between tumour cells or between tumour cells and normal cells, it may provide either an advantage or a disadvantage to the target cell. This conundrum was first evident in the ganciclovir cancer therapy field and the associated “bystander effect”, whereby a possible dual-effect or “kiss of death” and “kiss of life” scenario was depicted[215]. The advantages of keeping versus sharing metabolites probably depend on the specific signal, its concentration, and perhaps the stage of the tumour. Altogether these variables would influence the balance of positive and negative growth of the tumour. In this sense, the specific channel permeability properties of various connexin isoforms may also dictate how these connexins affect tumour growth differentially.

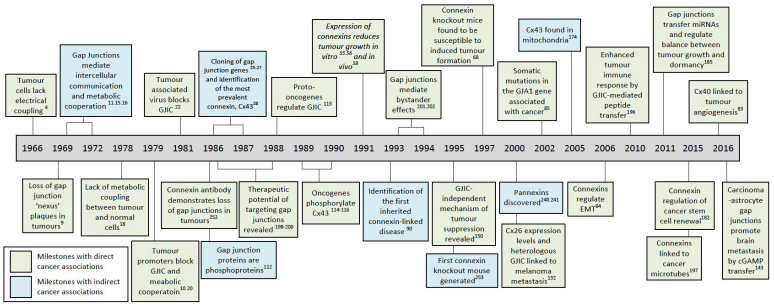

FIGURE 1. of key discoveries related to gap junctions and cancer

cGAMP, 2′3′-cyclic GMP-AMP; EMT, epithelial to mesenchymal transition; GJIC, gap junctional intercellular communication; miRNAs, microRNAs.

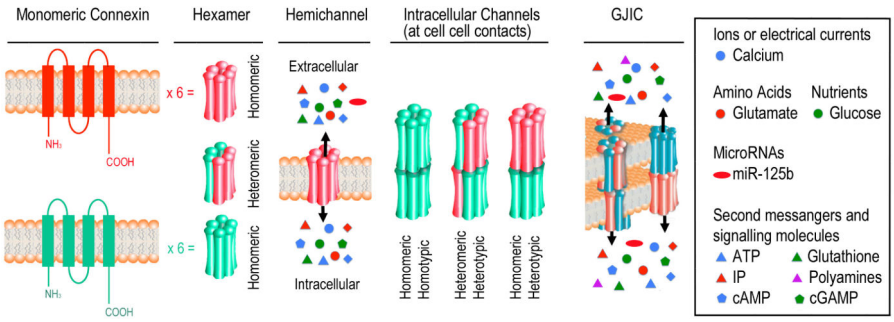

FIGURE 2. Assembly of connexins into gap junctions

Schematic depiction of typical connexins with the characteristic four-transmembrane topology consisting of four transmembrane domains, two extra cellular loop domains, a cytoplasmic amino terminal, one cytoplasmic loop and a highly variable cytoplasmic carboxy-terminal domain. Six connexins oligomerize into a connexon or hemichannel that docks in homotypic, heterotypic and combined heterotypic/heteromeric gap junction arrangements. The permeability properties depend on the connexin isoforms expressed, and since cells can coexpress and intermix different isoforms a huge number of possible combinations exist making functional evaluation highly complex. Exchange of possible types of cancer-associated signalling molecules between two cells or a cell and the extracellular environment is illustrated. For simplicity only a few examples for each class of signalling molecule are shown. cAMP, cyclic AMP; cGAMP, 2′3′-cyclic GMP-AMP; IP_3, inositol-1,4,5-trisphosphate; miR-125b, microRNA-125b.

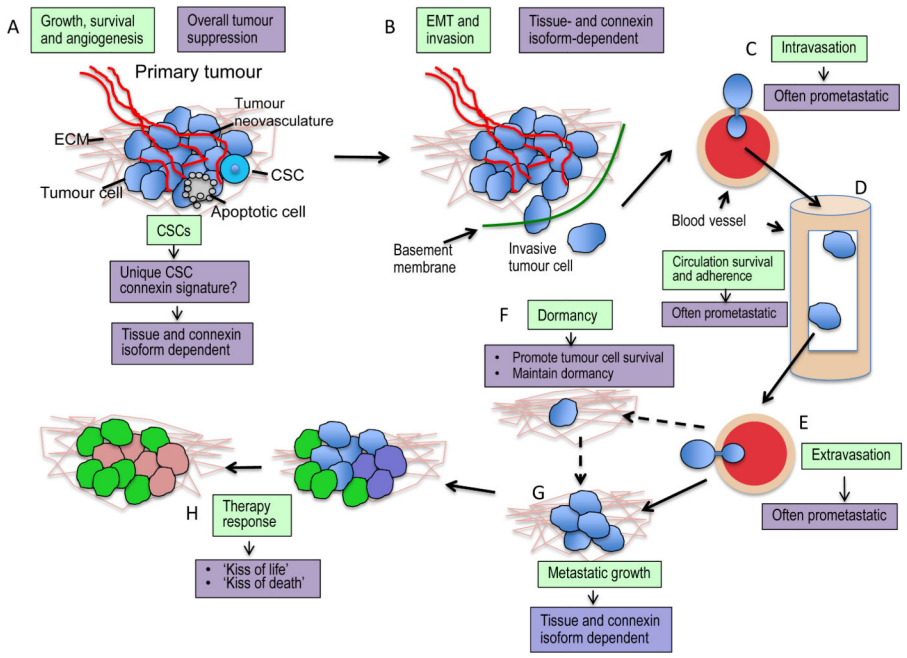

FIGURE 3. Connexin involvement during cancer progression

(a) At early stages, connexins appear to mainly act as tumour suppressors, whereby loss of connexin expression or gap junctional intercellular communication (GJIC) may promote growth, survival and possibly angiogenesis. Within this context, the role of connexins in cancer stem cells (CSCs) remains unclear, as isoform-specific effects of connexins may be opposing. (b) Likewise, different connexins play different roles during epithelial to mesenchymal transition (EMT) and invasion, although overall loss of connexins seems to promote this phenotype. (c) During later stages, when cancer cells metastasise, connexin expression generally seems to facilitate rather than block intravasation of tumour cells into blood vessels. (d) Increased connexin levels can also promote tumour cell survival and adherence within the circulation (e) Extravasation of tumour cells out of blood vessels is also supported by upregulation of connexin expression. (f) Once at the metastatic site, the roles of connexins are more unclear, with some evidence for connexins promoting tumour cell dormancy but also promoting survival within that context. (g) Some evidence suggests connexins reduce cell growth directly in metastases, but at the same time may stimulate local invasion and survival. (h) Therapy response, which includes chemoresistance, can be achieved via multicellular connections between cancer cells or between cancer cells and healthy cells and may be connexin isoform-specific. Moreover, this process will be highly influenced by the specific microenvironment and whether the overall exchange of signals promotes the “Kiss of Life” or “Kiss of Death” (BOX 2). Purple boxes indicate overall connexin effect and green boxes denote the specific stage in cancer progression where connexins function.

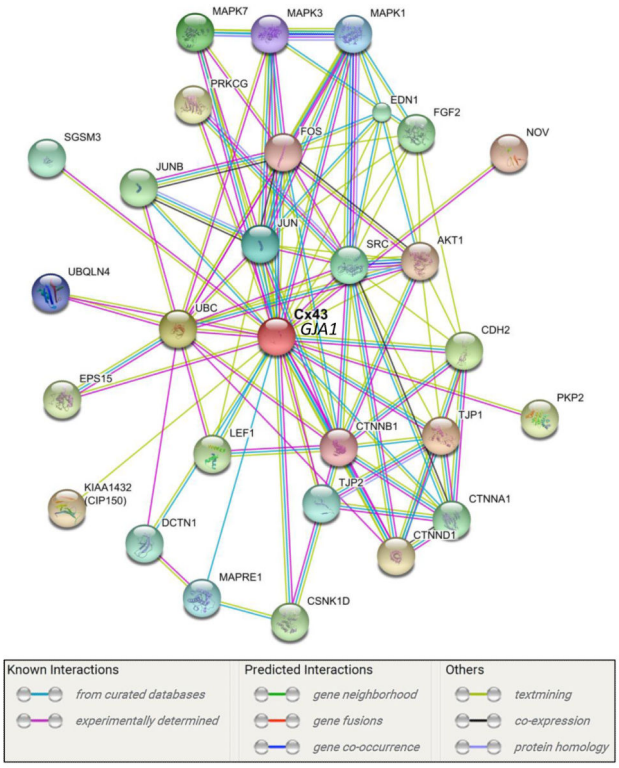

FIGURE 4. The Cx43 interactome

Example of STRING analysis of the most prevalent connexin Cx43 (encoded by *GJA1*) revealing a wide range of putative interactions, of which many are growth regulators or oncogenes such as SRC, AKT1, JUN, FOS and nephroblastoma overexpressed (NOV). The network was retrieved and constructed using the STRING database version 10.0 (http://string-db.org), using the most stringent confidence score prediction setting (>0.9), resulting in 28 interactions. Extended interaction-network (high confidence prediction, >0.7, 99 interactions) can be viewed in Supplementary Figure 1. Full names, score prediction and details of specific proteins are available in Supplementary Table 1.

TABLE 1

Connexin genetically-modified mouse models and cancer.

Connexin	Encoding Gene	Mouse Model	Carcinogen	Outcome	References
Cx26	*GJB2*	KO	DMBA	Increased breast tumour	[78]
Cx32	*GJB1*	KO	None	Increased spontaneous liver tumours in males	[67]
Cx32	*GJB1*	KO	DEN	Increased liver tumours	[69, 68]
Cx32	*GJB1*	KO	X-ray	Increased multiple tumour types	[75]
Cx32	*GJB1*	KO	DEN	Increased lung tumours	[70]
Cx32	*GJB1*	Cx32/p27 DKO	X-ray	Increased adrenal, pituitary, intestinal tumours	[76]
Cx32	*GJB1*	V139M	DEN	Increased liver tumours in males	[74]
Cx43	*GJA1*	G60S /ERBB2	DMBA	Increased breast metastasis	[78]
Cx43	*GJA1*	+/−	urethane	Increased lung tumours	[79]
Cx43	*GJA1*	+/−	DMBA	Increased lung tumours	[80]
Cx43	*GJA1*	+/−	NNK	Increased lung tumours	[81]

DEN, diethylnitrosamine; DMBA,7, 12-dimethylbenz[a]anthracene; DKO, double knockout; KO, knockout; NNK, nicotine-derived nitrosamine ketone; p27 encoded by cyclin-dependent kinase inhibitor 1b (*Cdkn1b*) .