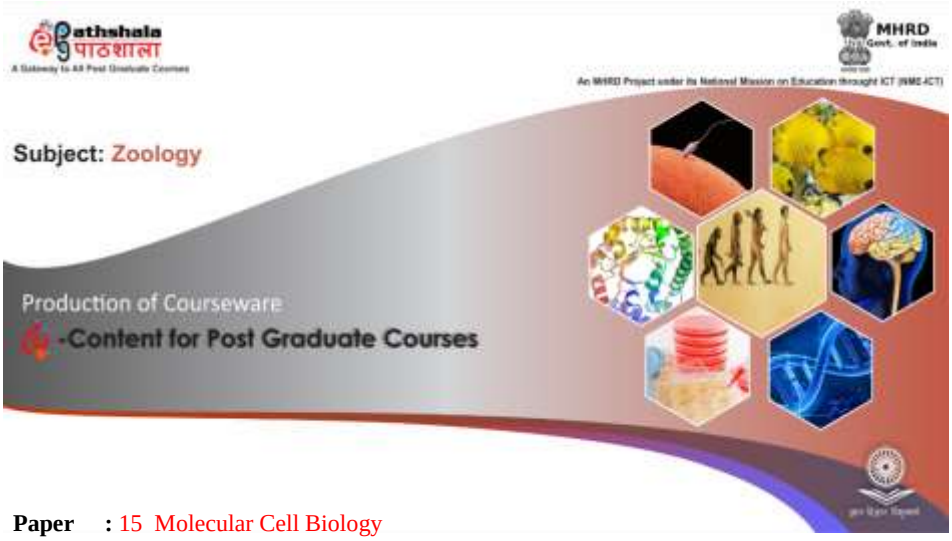

Paper : 15 Molecular Cell Biology
Module :16 Cell-Cell Adhesion and Communication: Gap Junctions

athshala पाठशाला MHRD

Development Team

Principal Investigator:	Prof. Neeta Sehgal Department of Zoology, University of Delhi
Co-Principal Investigator:	Prof. D.K. Singh Department of Zoology, University of Delhi
Paper Coordinator:	Prof. Kuldeep K. Sharma Department of Zoology, University of Jammu
Content Writer:	Dr. Jaspreet Kaur Maitreyi College, University of Delhi
Content Reviewer:	Prof. Rup Lal Department of Zoology, University of Delhi

Description of Module	
Subject Name	ZOOLOGY
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Module Name/Title	Cell-Cell Adhesion and Communication
Module ID	M16: Gap Junctions
Keywords	Cellular communication, connexin, hemichannel, junction plaque, connexinopathy

Contents

1. Learning Outcomes

After studying this module, you shall be able to

- appreciate the importance of cellular communication in multicellular organisms
- understand the assembly, functioning and regulation of gap junctions
- describe the role of gap junction channels in maintenance of homeostasis in body tissues and organs
- understand the critical role of connexins and the human diseases associated with them like cancer

2. Introduction

The evolution of multicellular form of life from the simpler, unicellular organisms was one of the most momentous events in the history of biology on the planet Earth. With an exceptional increase in the biological complexity, there was an emerging necessity of cellular communication in order to carry out all the physiological functions of the multicellular body in a coordinated and efficient manner. Thus, different means of such communication include distant interactions facilitated by neural or endocrine mechanisms or the short-range interactions, which include the cell-cell contact. In animal cells, one such communication system includes **cellular junctions.** They are of varying types, but they mediate the transport of chemical signals that mediate cellular communication, just like humans use their sensory organs for communicating with each other. This cellular communication is very important in the maintenance of homeostasis at the tissue or organ level, especially in case of multicellular organisms. The situation can be compared to the maintenance of the structure of a building, not by bricks alone, as the role of cement and nails are extremely important. Likewise, the integrity of a tissue or an organ cannot be maintained properly without the appropriate cell junctions and cell adhesion molecules.

This excellent mechanism of cellular communication works in a very systematic manner, wherein; a signal sent across neighboring cells can result in generation of a coordinated response or isolation of some cells from the rest of the cell population, in order to maintain tissue integrity. This kind of communication is important in different physiological processes

like cell growth, differentiation, morphogenesis, regulating cellular events during embryogenesis and synchronization of electrical and/or metabolic cellular responses (Mese& White, 2007).

3. Types of Cell Junctions

There are three types of cell junctions, each performing a critical role in homeostatic maintenance. These are tight junctions, anchoring junctions and communicating junctions. In this chapter, we would be discussing the communicating junctions, which are also called as **gap junctions** in animal cells, while they are termed as **plasmodesmata** in plant cells. As they are termed, they create physical link between the cytoplasm of the two adjoining cells and provide passage for the transport of small molecules or ions between the cells. In fact, they have been termed as "*the original cellular phone*" by Trosko & Goodman in 1994.

4. Gap Junctions in Animals

The discovery of intercellular channels of gap junctions came from two interesting observations, i.e., the existence of continuous core of conductivity despite the multicellular nature of Purkinje fibers of mammalian hearts (Weidman, 1952) and electrical transmission of the giant crayfish motor synapse (Furshpan & Potter, 1959).

The term 'gap' came from the histological and electron-micrograph studies (Revel & Karnovsky, 1967), wherein lanthanum, a silvery-white heavy metal was used to fill the extracellular space between two cells, thereby indicating the presence of a 'gap' between the plasma membranes of two neighboring cells (Fig. 1). In contrast, the tight junctions or '*zonulaoccludens*' are characterized and therefore named due to the absence of this extracellular gap.

After the observation of an extracellular space between cells, further studies indicated the presence of several intercellular channels that directly connect the cytoplasm of one cell to the other adjoining cell (Sosinsky & Nicholson, 2005). They are present in most cells of body tissues, except some cells like circulating lymphocytes, skeletal muscles and erythrocytes, which are terminally differentiated. However, the progenitors of these cells do express gap junctions (Sáez et al., 2003). The transport across these junctions occurs by passive diffusion

and is generally non-specific but they selectively transport small molecules with molecular size less than 1 KDa (Kumar & Gilula, 1996), for example, ions such as K^+ & Ca^{2+}, messenger molecules like cAMP, cGMP & inositol 1,4,5-triphosphate (IP_3), small metabolites like glucose, small interference RNAs and small informational molecules like morphogens (regulate pattern of tissue development), but not the macromolecules like proteins, polysaccharides and nucleic acids.

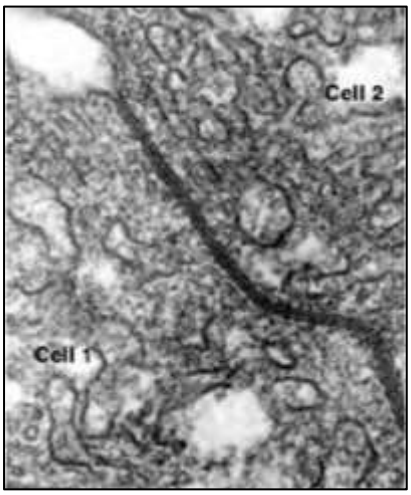


Fig. 1: Electron micrograph of gap junction, depicting the extracellular space or 'gap' between cell 1 and cell 2.
Source:https://www.slideshare.net/openmichigan/090808-epithelialtissue

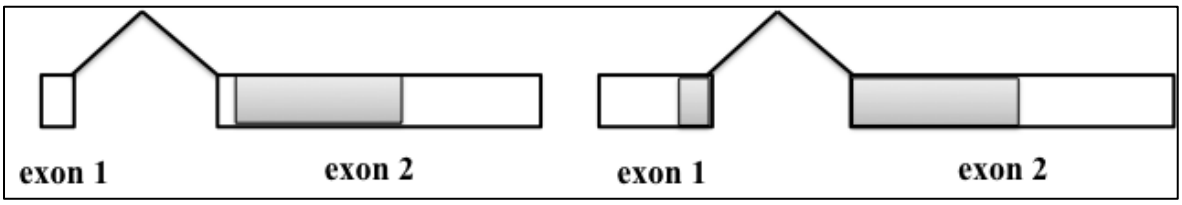
5. Structure of Gap junction

The three-dimensional (3D) structure of gap junction was first deduced by Unwin et al in 1980 in rat liver using electron crystallography. The intercellular hydrophilic channels forming the gap junctions in chordate animals are encoded by a family of genes called connexins (Cx). There are 21 members of connexin gene family in human genome and 20 in mice (Nielsen et al., 2012). In invertebrates, the intercellular channels are formed of proteins called innexins (Inxs). Interestingly, innexins do not show any sequence homology with connexins, but they form gap junctions with similar functional properties like that of connexins (Bukauskas & Verselis, 2004). Also, there is another group of proteins, called *pannexins* (Panxs), which are present in chordates and are homologous to innexins. The pannexin protein family consists of three members only: Panx1, Panx2 and Panx3, which oligomerize into aqueous pores between the intracellular and extracellular space. Although no

germ-line mutations in pannexins have been reported to be associated with human diseases but the degree of pannexin expression has been linked to the onset and/or progression of disease (Penuela et al., 2014, Bond & Naus, 2014).

5.1. Connexins

The connexins are membrane spanning integral proteins, named on the basis of the molecular mass of the connexin polypeptide (Table 1). These genes can be categorized in three groups- α, β and γ, based on their gene structure, homology and certain sequence motifs (Messe et al., 2007). The connexin genes have a simple genomic structure, comprising of a 5′ untranslated exon 1 (5′-UTR), followed by an intron of variable length and a 3′ untranslated exon 2 (3′-UTR), which consists of the complete coding sequence (CDS) for connexin protein (Söhl & Willecke, 2004). But, different isoforms of this gene structure can be seen due to alternative splicing in the 5′-UTR or the coding region can be interrupted by an intron (Fig. 2).



(a) (b)

Fig. 2. (a) The general genomic structure of connexin genes present in mouse, wherein the shaded box represents the CDS and the black lines separating the two exons is the intron (b) alternate splicing of connexin gene, as seen in mCx36, hCx36, mCx39, hCx40.1 and mCx57 (m: mouse and h: human).
Source: Author

Connexins are transmembrane proteins, six of which are assembled or oligomerized to form *connexon or connexin hemichannel*. The connexons or hemi channels of the adjacent cells dock together, forming an axial channel, spanning the two plasma membranes with a narrow (~2 nm) extracellular 'gap', for which the junction is named (Good enough& Paul, 2009). Similar type of connexins can assemble to form '*homomericconnexon*', or '*heteromericconnexon*', which is formed by more than one kind connexin protein (Fig. 3). Consequently, the resulting gap junctions formed with identical homomericconnexins is

termed as '*homotypic junction*' and '*heterotypic junction*' formed with two connexons of different homomeric or heteromeric composition (Sosinsky & Nicholson, 2005).

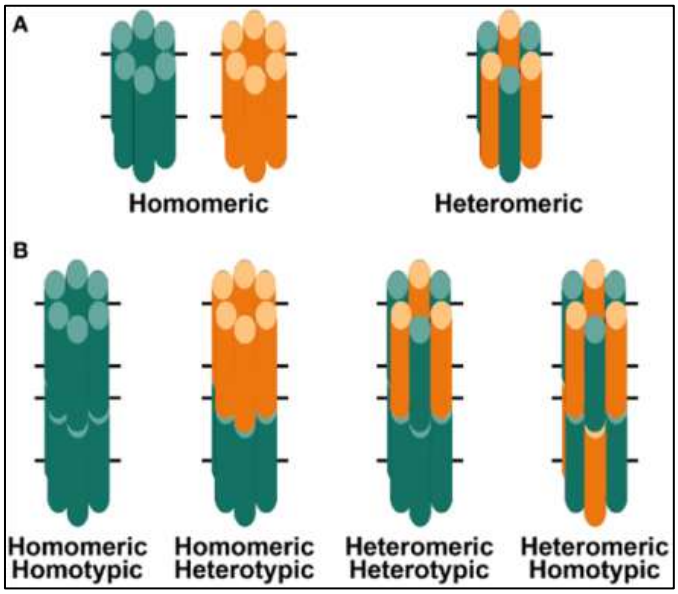


Fig. 3: Diagram depicting the connexin hemichannel or connexon, formed from (A) 6 identical connexin subunits (green/orange) or different connexin subunits (green & orange); (B) Docking of two hemichannels with each other to form a complete gap junction channel. Two hemichannels of identical composition form homotypic channels whereas two hemichannels of different composition form heterotypic channels.
Source: Beyer, et al., 2013, http://journal.frontiersin.org/article/10.3389/fphar.2013.00043/full

Table 1: Different Cx with their cellular localization (**Source:** Kumar & Gilula, 2001)

Molecular Mass Nomenclature	Predicted Molecular Mass (kDa)	Examples of Organs with Expression
Cx43	43	Heart
Cx38	37.8	Embryo
Cx46	46	Lens
Cx37	37.6	Lung
Cx40	40.4	Lung
Cx45	45.7	Heart
Cx33	32.9	Testis
Cx50	49.6	Lens
Cx32	32	Liver
Cx26	26.5	Liver
Cx31	31	Skin
Cx31.1	31.1	Skin
Cx30.3	30.3	Skin

The transmembrane domains, which comprise the wall or pore of the channels, include four membrane-spanning segments (M1, M2, M3&M4), which form alpha-helix bundles. The cytoplasmic domains include N-terminus, the cytoplasmic loop or the connecting loop between M2&M3 helices and the C-terminus. The extracellular domains include E1&E2 loops, which are involved in cell-cell recognition and docking, process (Fig. 4). One of the most striking features of the extracellular domains is the order of three cysteine residues in each extracellular loop, which is highly conserved in all the connexin gene family members, except Cx31. These cysteine residues form intra molecular disuphide bridges, which helps in stabilization during the docking of two opposing connexons (Hoh et al., 1991 & Dahl et al. 1992). Also, Gong et al in 2013 reported that minimum of four hydrogen bonds are required between each second extracellular loop of the opposing hemi channels to dock together.

There is high degree of conservation among the members ofconnexin gene family for transmembrane, extracellular loops and N-terminus of cytoplasmic loop. On the other hand, the cytoplasmic loop and the C-terminus show variation in terms of sequence and length. For example, C-terminus is shorter in Cx26 protein while it is longer in Cx50 protein.

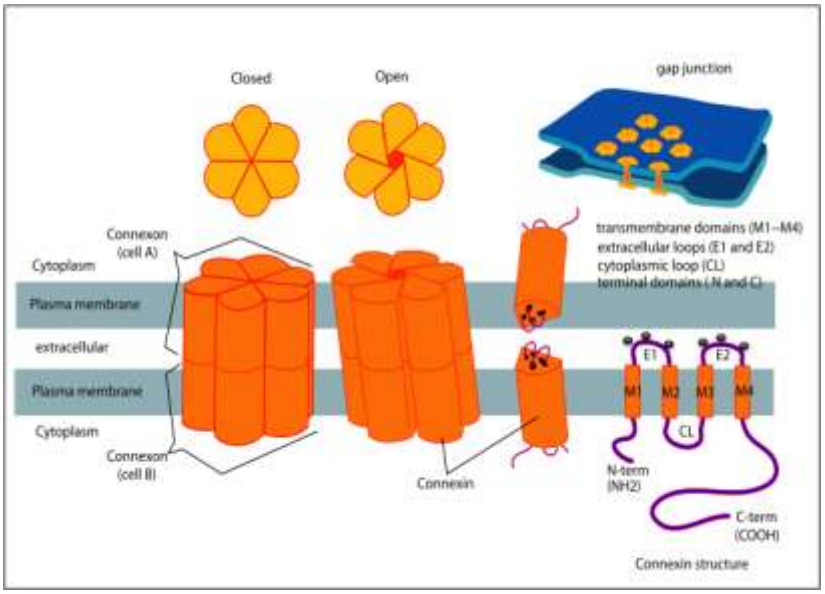


Fig. 4: Structure of gap junction (**Source:** https://en.wikipedia.org/wiki/Connexin. The three conserved cytseine residues in each extracellular loop are depicted as small black circles.)

5.2. Connexin-Protein Interactions

Most of the connexin proteins contain phosphorylation sites (except Cx26), hence they undergo phosphorylation as a post-translational modification and also interact with several kinases and phosphatases. Some of the kinases reported to interact with connexins include v and c-src kinase, protein kinase C, MAPK (mitogen-activated protein kinase), cdc2 kinase (also known as cyclin-dependent kinase), casein kinase 1 and protein kinase A. Connexin phosphorylation acts as a regulatory event for the assembly, gating as well as internalization and degradation of gap junction channels (discussed in next sections). Several other protein molecules also bind to connexin like ZO-1 (zonulaoccludens 1), MAGUK (membrane-associated guanylate kinase, ZO-2), which regulate the stability of gap junctions. Also, calmodulin is an important Ca^{2+} dependent binding partner of Cx43 and modulates hemichannel and gap junction function (Kelly, 2014).Further, β-catenin plays a role in cell signaling and regulation of gap junction intercellular channels (GJIC) (Fig. 5). Cx43 also binds with caveolin-1 at the cytoplasmic tail (Schubert et al., 2002), suggesting its role in the internalization of Cx43 for its degradation. Also, α & β-tubulin bind to the C-terminal tail of Cx43, suggesting a synergistic interaction of Cx43 and microtubules. The microtubules help in regulating the trafficking and transport of connexin during gap junction assembly and degradation, while connexins may aid as microtubule-anchoring locations, thereby regulating the cellular activity (Laird, 2006). Thus, it can be suggested that several proteins regulate the life cycle of connexins in the gap junction channels, starting from their biosynthesis, assembly, internalization and degradation.

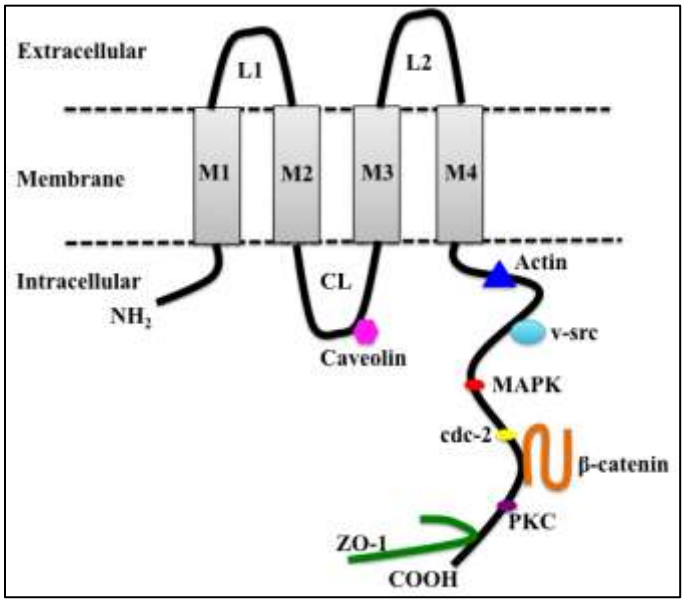


Fig. 5: Connexin-interacting partners. (**Source:**Author)

5.3. Biosynthesis of Connexin and Gap junction assembly

In eukaryotes, all the secretory proteins and plasma membrane proteins (with few exceptions) are synthesized in the endoplasmic membrane (ER) and follow the secretory pathway. The connexins also follow this pathway, wherein; the newly made proteins are incorporated into small (~50 nm in diameter) transport vesicles. These vesicles then fuse with either *cis*-Golgi or with each other and by *cisternal progression*, further move towards the *trans*-Golgi network (TGN). The oligomerization of connexins into a hemichannel or connexon occurs either in ER or TGN, for example, Cx32 assembles in endoplasmic reticulum, while Cx43asssembles in trans-Golgi network (Sáez et al., 2003). These connexons are then carried to the cell surface by vesicles transported through microtubules, which then fuse to the plasma membrane. At this stage, the fate of these hemichannels can be either to form 'nonjunctional' channels with unopposed areas of cell membrane or to form intercellular channels with an opposing hemichannel with a neighboring cell (Harris, 2001). The unopposed channels can also become functional under pathological conditions like ischaemia and they can release metabolites like ATP, glutamate and NAD^+ into the extracellular space (Evans et al., 2006; Messe et al., 2007).

Ca^{2+} dependent molecules called cadherins mediate the formation of gap junction channels. The gap junction channels aggregate to form a distinctive two-dimensional array of channels,

called as *plaque* (Fig. 6).These plaques can extend from less than 100 nanometers to several micrometers, consisting of less than 12 to more than 1000 individual channels (McNutt & Weinstein, 1970; Forge et al., 2003). Increased levels of cAMP induce the clustering of Cx43 gap junction channels to form the junction plaque and the plaque is increased either by polymerized actin or trafficking through intracellular membrane compartments. Plaque development takes place when the old channels are removed from the center of the plaque and the new channels are incorporated from the periphery of the existing gap junction channels.

6. Gap Junction Degradation

The half-life of a gap junction ranges from 1-5 hours both *in vivo* and *in vitro*; however, some lens fiber connexins are much stable, with their half-lives of $\geq$ 2-3 days (Segretain& Falk, 2004; Sáez et al, 2003). The removal of old gap junction channels from the middle of junction plaque involves their internalization into vesicular structures called '*annular junctions*' or connexosomes. Then these vesicles are either degraded by lysosomal or proteasomal degradation pathways. The multivesicular body, lysosomal and autophagosomal compartments have been often found in pathological condition of ischaemia. Phosphorylation of Cx43 by different kinases like protein kinase C (PKC), extracellular signal-regulated kinase (ERK), casein kinase 1 (CK1) has been found to stimulate gap junction removal from the plasma membrane by proteasomal degradation in lens epithelial cells (Segretain & Falk, 2004). Also mono-ubiquitination of Cx43 acts as an internalization signal, which targets it to proteasomal degradation (Leithe & Rivedal, 2004, Saffitz et al., 2000).

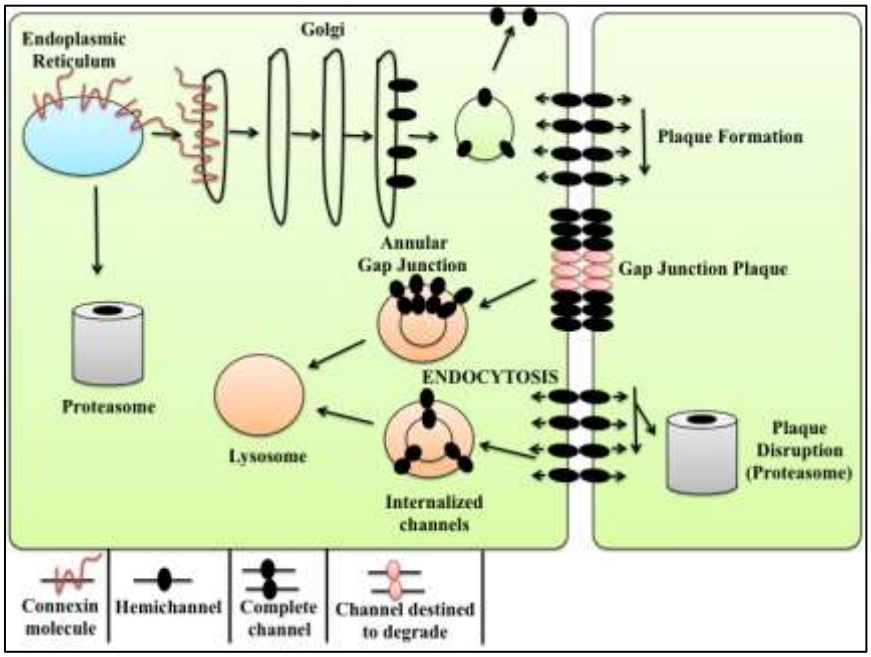


Fig. 6: Assembly and degradation of gap junction channels. (**Source:** Author)

7. Opening and Closing of gap junction

The activity of gap junction channel is affected by physiological processes like phosphorylation or response to cell trauma, like drop in pH, increase in Ca^{2+} or voltage differences between cells (Nicholson et al., 1998, Bennet et al., 2016). The gating mechanism of gap junctions can be divided into two categories:

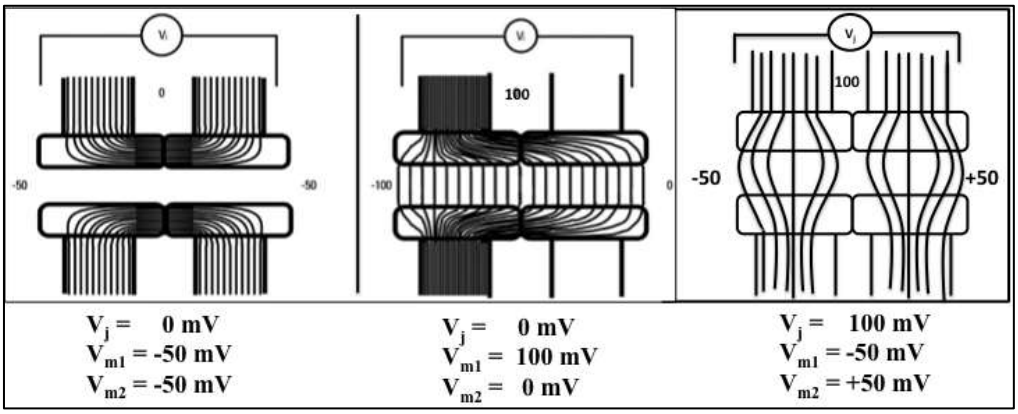
1. Voltage gating, which involves rapid (~2 msec) responses to electrical stimuli like changes in transjunctional voltage (V_j), i.e., the potential difference between cytoplasm of two adjacent cells, and

2. Chemical gating, which involves slower (~10-40 msec) responses to chemical stimuli, like intracellular Ca^{2+} and pH.

7.1. Voltage Gating

Like several ion channels exhibit the property of voltage gating, wherein, the conductance across the channel is sensitive to voltage, the activity of gap junction is also regulated by transjunctional (V_j) and transmembrane voltages (V_m). V_m is defined as the difference in voltage between the cell interior and the lumen of the channel relative to the outer

extracellular space. Some gap junctions are V_j sensitive only, while others are sensitive to both V_j and V_m (Revilla et al., 2000). When the transmembrane voltage, V_m is equal in two adjoining cells, for example, -50 mV, while V_j=0 mV, it creates a strong electric field (E) or high density of isopotential or equipotential lines across the channel wall in its central region. On the other hand, if V_m is not equal in both the cells, for example, V_{m1}= -100 mV and V_{m2} = 0 mV or it is equal but with different charges, for example V_{m1}= +50 mV and V_{m2}= -50 mV, then a V_j is established and a constant electric field, E along the pore (Fig. 7).



(a) (b) (c)

Fig. 7: Schematic representation of gap junction channel in response to Vj and Vm.
Source: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4276742/figure/F3/(Fig. adapted from Bukauskas & Verselis, 2004 and Oh & Bargiello, 2015)

7.2. Chemical Gating

In addition to the dependence of gap junctions to transjunctional and/or transmembrane voltage, the activity of gap junction channels is also affected by chemical stimuli. These responses are also termed as slow or loop gating. A characteristic feature of slow gating is the formation of series of transient sub-states between the closed and open states of gap junction channel (Bukauskas & Verselis, 2004).

The opening and closing of gap junctions was explained in response to Ca^{2+} concentration (Unwin & Ennis, 1984, 1983; Unwin, 1989) in a model referred to as '*subunit rotation model*'. According to this model, Ca^{2+} induces the open and closed configurations of the gap junction, wherein; all the six subunits in a hemi channel undergo conformational change simultaneously together, i.e., in a concerted order (Fig. 8). This results in tangential

displacement of the subunits as the subunits move inward and narrow the pore near the cytoplasmic membrane surface (Oshima, 2014).

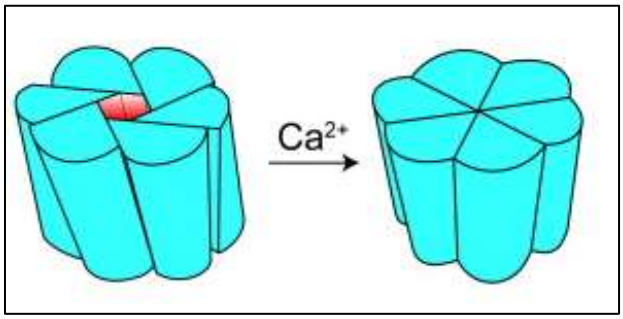


Fig.8: Subunit rotation model, (**Source:** Oshima, 2014, http://onlinelibrary.wiley.com/doi/10.1016/ j. febslet . 2014.01.042/full#feb2s0014579314000714-bib-b0095)

In addition to the response of intracellular Ca^{2+}, gap junction channels also respond to pH changes in a mechanism termed as '*particle receptor model*' or '*ball and chain model*'. In this model, the cytoplasmic tail (CT) acts as a gating particle. At normal pH, the CT or gate is away from the pore of the gap junction channel. Under intracellular acidification, the gating particle moves or swings towards the mouth of the channel and binds non-covalently to separate specific domain acting like a receptor site (Fig. 9). It is proposed that second half of the CL (cytoplasmic loop) acts as the "receptor" for the CT domain. Thus, particle receptor interaction leads to closure of the channel (Werner, 1998, Nielsen et al., 2012).

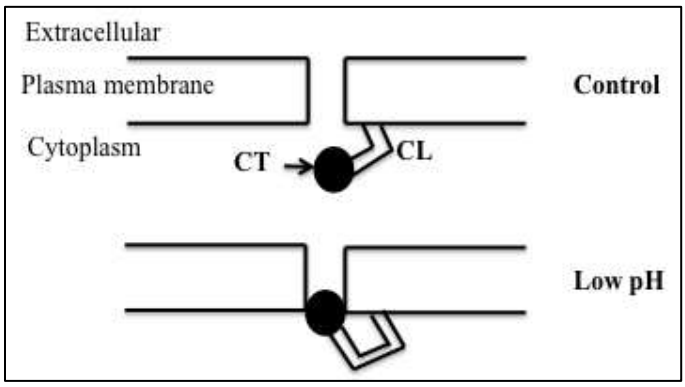


Fig. 9: 'Ball and chain model' of gap junction channel. (**Source:** Author)

8. Role of connexins and gap junctions in cell growth

In addition to their critical role in intercellular communication, connexins and gap junction channels also mediate various physiological processes like cell growth, differentiation and

cell death. Connexins regulate cell proliferation by (a) mediating the direct exchange of cell growth regulators (like cyclin A, cyclinD1, cyclinD2, cdk5, cdk6) between adjacent cells, (b) the paracrine release of homeostatic regulatory proteins (ATP, NAD^+) at the extracellular level and (c) interacting directly or indirectly with the cell cycle regulators and affecting their production. For example, connexins bind with β-catenin (at its cytoplasmic tail) and β-catenin also functions as a regulatory element in Wingless-Int (Wnt) signaling pathway, which in turn controls the G2/M transition of the cell cycle. Thus, connexins also affect cell cycle signaling in an indirect manner. Connexins like Cx43 can also directly affect the cell growth regulators like Skp2 (S phase kinase-associated protein 2) by either interfering with its gene expression or by promoting its degradation. It is also reported that connexins co-localize with cadherins, which maintain cell adhesion and in turn favors prevention of cell growth. Likewise, connexins also bind with scaffolding protein discs-large homolog 1 (Dlgh 1) and nephroblastoma over expressed (NOV) that affect the cell growth (Fig. 10). Also, connexins like Cx43 indirectly regulate the transcription of cell growth regulators by binding to the tight junction components ZO-2 and ZO-1 (zonulaoccludens-1, 2) that interact with transcription factors (TCF/LEF), such as activator protein 1,AP-1 (c-jun/c-fos) and ZONAB (ZO-1-associated nucleic acid binding protein) (Vinken et al., 2011).

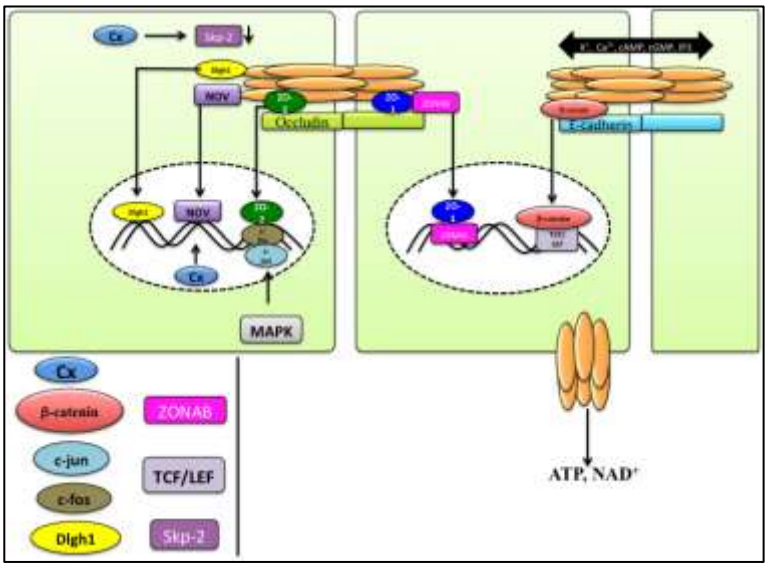


Fig.10: Role of connexins in regulating cell proliferation. (**Source:** Author)

9. Regulation of Gap Junction channels

The intercellular channels of the gap junction can be regulated at various levels of connexin biosynthesis, like transcription, mRNA processing, protein synthesis, post-translational modification, assembly, trafficking, docking and gating of gap junction channels (Chipman et al., 2003, Fig. 11). The transcriptional regulation of connexins is mediated by different promoter response elements and transcription factors, for example, several cAMP and estrogen response elements have been identified in the promoters of Cx43 gene of human, mouse and rat (Chen et al., 1995a; Yu et al., 1994). Also, the methylation of CpG islands present in the promoter of Cx43 gene in MH1C1 rat hepatoma cells has been found to cause gene silencing of Cx43 gene (Piechocki et al., 1999). The post-translational modification of connexin proteins include phosphorylation of the C-terminal domain of the connexin (except Cx26), hence the activity of the gap junction channel can also be regulated at this level. For example, hyper phosphorylation of Cx43 by MAP kinase or protein kinase C can inhibit the cellular communication of gap junction channel (Rivedal & Opsahl, 2001).

Other chemical agents like aliphatic alcohols (octanol, heptanol) and anesthetics like halothane can interfere with membrane fluidity leading to contraction of the intercellular channels and hence functioning of gap junction is impaired. Further, chemical gating of gap junction channels is influenced by changes in calcium ions, pH and other free radicals, thus toxicant-induced changes in cellular calcium levels can lead to inhibition of gap junction channel (Saez et al., 1993, Trosko et al., 1998).

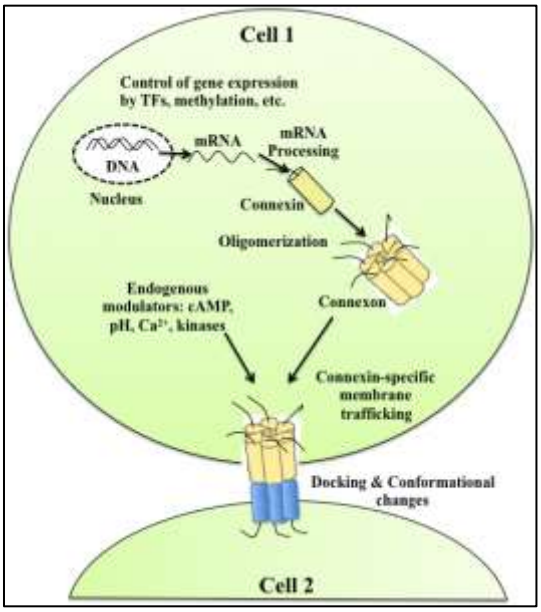


Fig. 11: Regulation of gap junction channels by different biological and chemical factors. (**Source:** Author (Figure adapted from Chipman et al., 2003))

10. Connexin mutations and Human diseases

Connexins play a major role in cellular homeostasis at the tissue or organ level, with the gap junction channels transporting ions, nutrients, chemical messengers and metabolites between the coupled cells. Thus, they play critical roles in various physiological processes and mutations in different connexin genes have been found to be associated with some hereditary human diseases, which have been termed *connexinopathies* by García et al., 2016 (Table 1). The connexin-linked diseases are either congenital or range from mild developmental abnormalities or severe organ failure like hearing impairment or ailments like peripheral neuropathies, cataract, skin diseases, etc. For example, *Charcot–Marie–Tooth (CMT)* disease, an X-chromosome linked disease is caused by more than 270 mutations in the Cx32 gene, majority of which are point mutations. It was the first discovered connexin-linked disease and is clinically manifested by progressive peripheral neuropathy including axon demyelination and limb weakness. The mutation results in misassembly of GJIC or abnormal gating properties and anomalous trafficking of hemichannels. Interestingly, more than 100 mutations in Cx26 (missense, nonsense, frame-shift, insertion and deletion)have been associated with deafness and skin disorders like *palmoplantarkeratoderma* (abnormal thickening of the palms and soles), *keratitis–ichthyosis–deafness (KID) syndrome* and *Vohwinkel syndrome*

('starfish'-shaped thickening over knuckles, tight bands forming around fingers).The symptoms of KID syndrome patients include hearing loss and keratitis (inflammation of the cornea) that can cause photophobia, scarring, and vision loss. The first hereditary skin disease associated with connexin genes was *erythrokeratodermiavariabilis (EKV)*, characterized by red skin areas with sharp borderlines, which tend to shift positions.Then, more than 60 mutations in Cx43 have been tightly correlated with an autosomal dominant syndrome, called *oculodentodigital dysplasia (ODDD)* (Srinivas, 2017, Pfenniger et al., 2010, Mese et al., 2007, Laird, 2006, Sáez et al., 2003, Gerido & White, 2004).

Table 1. Role of connexins mutations in human diseases (Ref: Laird, 2006; Salameh et al., 2013; Srinivas, 2017)

Name of the disease	Causative mutation(s)	Clinical manifestation
X-linked Charcot–Marie–Tooth disease	Cx32 (~270 mutations)	Progressive peripheral axon demyelination and limb weakness
Vohwinkel's syndrome, keratitis–ichthyosis–deafness (KID), hystrix-like-ichthyosis-deafness (HID), Bart–Pumphrey syndrome and palmoplantarkeratodermas (PKKs)	Cx26, Cx30, Cx30.3&Cx31	Abnormal keratinization and hypertrophy of the corneum, particularly in the palmar and plantar surfaces
Oculodentodigital dysplasia (ODDD)	Cx43 (28 mutations)	Syndactyly, craniofacial abnormalities, brittle nails, hair abnormalities, conductive hearing loss, lens defects, cornea defects, abnormalities of the teeth and occasional neurological and heart symptoms
Tetralogy of Fallot (TOF)	C-terminus of Cx43	Congenital heart defect
Craniometaphyseal dysplasia autosomal recessive, Palmoplantarkeratoderma with congenital alopecia, Erythrokeratodermiavariabilis et progressive, Visceroatrialheterotaxia	Cx43	Skin disorders
Cataract	Cx46	
Pelizaeus–Merzbacher-like disease	Cx46·6/Cx47	X-linked hypomyelinatingleukodystrophy, affecting central nervous system
Atrial fibrillation	Cx40	abnormal heart rhythm characterized by rapid and irregular beating
Bart-Pumphrey syndrome	Cx26	sensorineural hearing loss, palmoplantarkeratoderma, knuckle pads, and leukonychia

10.1. Mechanism of Cx mutations

The germ-line mutations in connexin genes can cause diseases, leading to functional defects and disorders. The underlying mechanism of these mutations can be due to (a) accumulation of a Cx mutant in the cytoplasm, (b) dominant-negative effect of a Cx mutant, (c) loss or gain of function mutation, (d) impaired protein trafficking and assembly at the plasma membrane or (e) abnormal or 'leaky' hemichannels (Fig. 12).

An intracellular connexin mutant, which is probably a misfolded connexin protein with trafficking defect is retained either in the ER, ER-Golgi intermediate compartment or the

Golgi apparatus, hence the accumulation of the mutant leads to ER stress or its premature degradation. In addition to misfolding, the Cx mutant can accumulate in these compartments due to the insertion of a retention motif (FF motif) in the connexin polypeptide sequence due to frame shift mutations, wherein the nonsense amino acids are encoded before the stop codon. The other possible mechanism of Cx mutation is the dominant-negative effect of mutated connexin protein over the wild type Cx, thereby affecting hemichannel formation and/or gap junction function.

The loss of function mutations includes the truncating defects of connexin proteins, wherein the deletion of a single base can result in shortened or truncated connexin protein. For instance, deletion of a guanine residue at position 13 results in a frame shift mutation in Cx26, thereby causing non-syndromic hearing loss. Likewise, Cx43 that lacks the CT portion can form gap junctions but their channels have different permeability/electrophysiological properties (Bruzzone et al., 2003, Schulz et al., 2015). Thus a truncated connexin protein is not able to exert its function properly. Another loss of function mutation includes the mutations that prevent connexin to interact or bind with the other proteins that help in regulating the gap junction channel. As discussed in the previous section, calmodulin binds with connexin proteins like Cx43, thus mutations in the calmodulin-binding domain of Cx43 prevent the interaction of Cx43 with calmodulin. This leads to an unregulated gap junction channel acquiring a tendency to be in open state. The last category of loss of function connexin mutation includes defects in the gating and permeability of GJIC. Actually, there is no problem with the trafficking and assembly of the hemichannels, but the fully formed gap junction channels exhibit reduced capacity for the transport of ions and small molecules.

On the other hand, the gain of function of connexin mutations include (a) enhanced permeability of hemichannels and gap junction channels, which can lead to cellular cytotoxicity and cell death, for example mutations in Cx26 leads to increased opening of hemichannels and has been linked with KID syndrome (Fig. 12) and mutations in Cx32 which is linked with CMT disease, a toxic gain in function can lead to severe consequences with respect to collapse of ionic gradients, loss of metabolites, and influx of Ca^{2+} (Abrams et al., 2002), (b) binding with proteins or other connexin proteins, with which they would normally not bind, for instance, Cx43 does not oligomerizes with Cx26, but under mutational

alterations, it may bind and cause trans-dominant effects, (c) shortened or elongated half-life of connexins, thereby either causing their early or delayed internalization and degradation (Kelly, 2014, Scott & Kelsell, 2011).

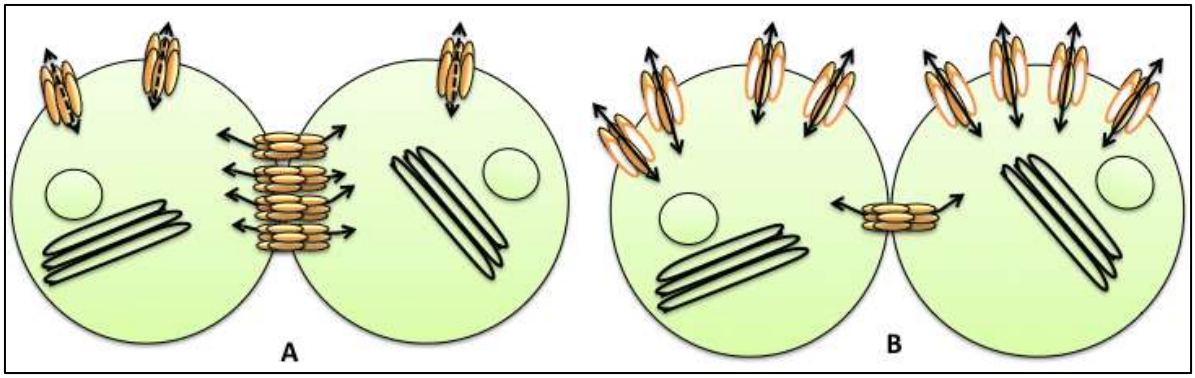


Fig. 12: Effects of mutant connexin 26 (Cx26) on gap junction hemichannels, associated with keratitis-ichthyosis-deafness (KID) syndrome (A) cells expressing wild-type Cx26 (depicted in orange) with gap junction channels (solid arrows) and have minimal hemichannel activity (dashedarrows). (B) Cells expressing mutant Cx26 (white ovals) interact with wild-type Cx26 to inhibit trafficking and formationof gap junction channels. Cx26 hemi channels show increased activity that harms cell function.
Source: Author

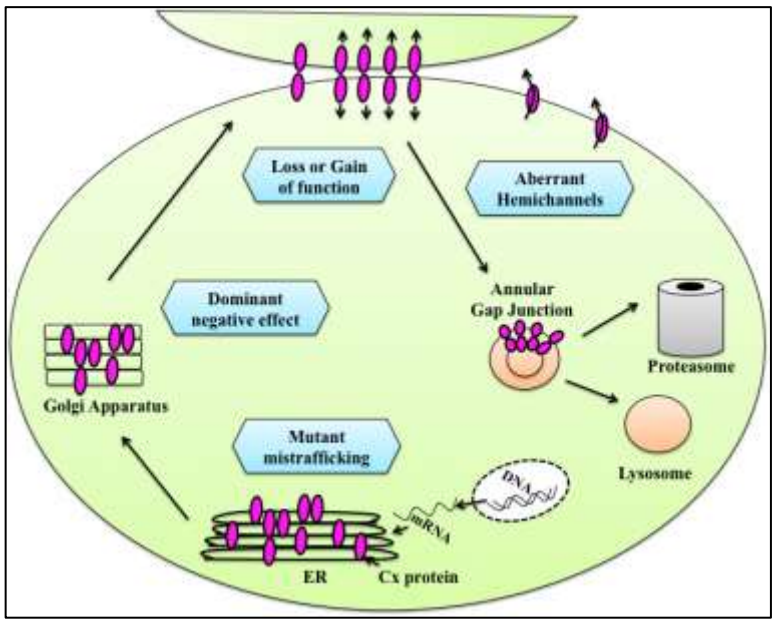


Fig. 13: Possible mechanisms of connexin mutations. (**Source:** Author)

11. Gap Junctions and Cancer

On one hand, GJIC are important regulatory elements for maintenance of tissue or organ homeostasis by transport of important metabolites, ions or small molecules, than on the other hand, they can also spread apoptotic and inflammatory signals between cells, like they can also transport superoxide and calcium ions under conditions of cellular toxicity, as seen in case of cerebral ischemia. Also, when a cell gets damaged, its membrane becomes 'leaky' (to ions like H^+, Ca^{2+}, K^+), so if this damaged cell remains coupled with undamaged or healthy cells, then the spread of toxicity can affect these normal cells as well. Therefore, transient and reversible closure of GJICs is required to protect the nearby healthy cells and control the spread of toxic substances. But, if the closure of GJIC is not reverted back immediately, then the loss of homeostatic control due to sustained inhibition of the gap junction channels can cause pathological conditions like reproductive, cardiovascular or neurological dysfunction and can even contribute to carcinogenesis. Intriguingly, embryonic toxicity or teratogensis can result from 'untimely' inhibition of GJIC during critical stages of embryonic development. Activation of some oncogenes like Ha-Ras, Neu and Src inhibit GJIC and there are different chemicals also which have been reported as modulators of GJIC like tumor promoters, neurotoxins, teratogens, non-genotoxic carcinogens which include pesticides like DDT, dieldrin, heptachlor and lindane and growth factors like epidermal growth factor (EGF), platelet derived growth factor (PDGF), basic fibroblast growth factor (bFGF), and hepatic growth factor/scatter factor (HGF/SF).The mechanism of action of these carcinogens include rapid inhibition of permeability of the gap junction channel followed by its subsequent internalization and degradation in lysosomes, as exhibited by DDT which blocks both Cx43-expressing (lung, pancreas) and Cx32-expressing (liver) tissues or dieldrin, which causes reversible dephosphorylation of Cx43, disaggregation of the gap junction plaques and dispersal of the gap junction particles in the plasma membrane. Other carcinogens like phorbol ester, TPA (12-O-tetradecanoylphorbol-13-acetate) cause alterations in the formation and permeability of gap junction as well as they change the stability of connexins by post-translational modifications. The growth factors can inhibit GJIC by stimulating connexin phosphorylation by the kinases, for example, vascular endothelial growth factor (VEGF) has been reported to disrupt cell–cell junctions and obliterate GJIC by stimulating the

phosphorylation of regulatory amino acid residues located in the C-terminal tail of Cx43.The phosphorylation is carried out by kinases: MAP and PKC (Nimlamool et al., 2015, Ahir & Pratten, 2014, Solan & Lampe, 2009, Chipman et al., 2003).

In addition the regulation of connexins by various factors, the dysregulation of connexin channel mediated cellular communication can either increase or suppress tumorigenesis and metastasis in different cancer types. In this respect, Loewenstein & Kanno (1966), observed that the liver cancer cells were characterized by impairment of gap junction intercellular communication, in contrast to the normal liver cells. Since then, there have been numerous reports of decreased gap junction communication and reduced expression of connexin genes in different tumor types. Moreover, deficient or abnormal connexins have been found in tumor tissues and cancer cells in breast cancer, lung cancer, prostate cancer, etc. For example, reduced expression of Cx26 and Cx43 was found in breast carcinoma cells (Jiang & Penuela, 2016). In case of breast cancer, the epithelial cells and myoepithelial cells, present in the mammary gland, physically detach from each other due to the loss of function of GJIC and reduced connexin expression (Fig. 13). Then in later stages of cancer progression, connexins are up regulated again in the tumor cells, which help the tumor cells to invade and interact with the endothelial cells. This leads to metastasis and extravasation to distant organs (Jiang & Penuela, 2016, Banerjee, 2016).

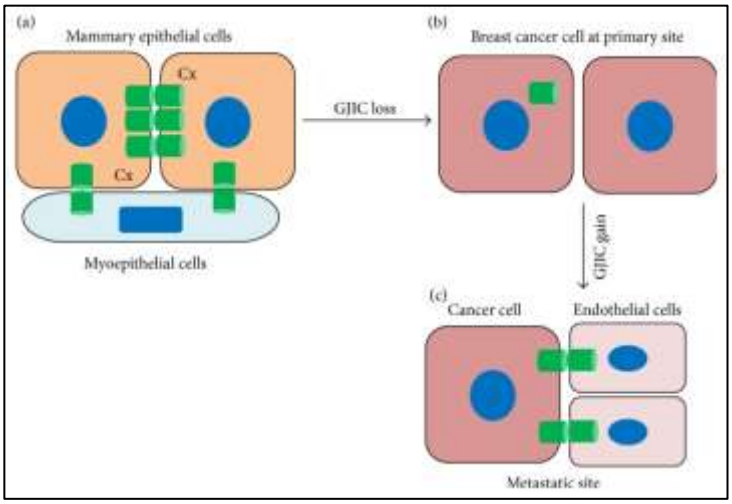


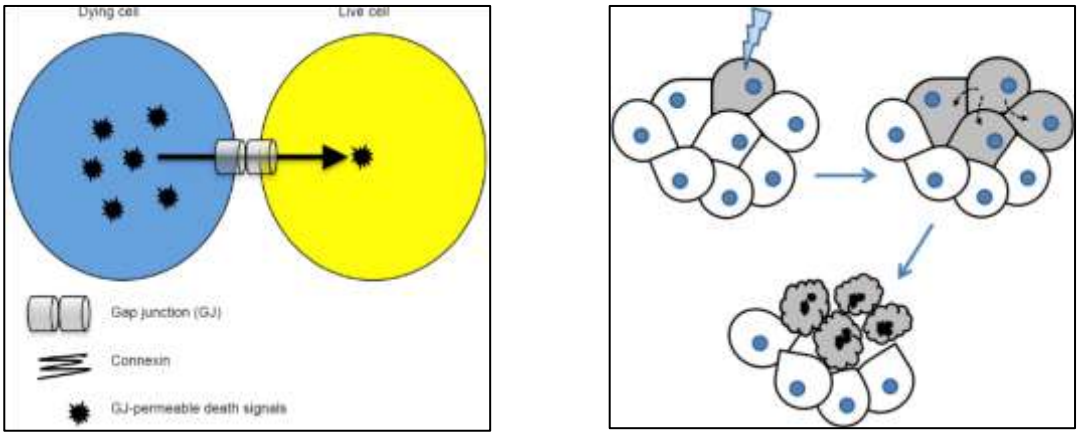
Fig. 14: Role of gap junction connexins in the metastasis of breast cancer. (a) In normal mammary gland, epithelial cells and myoepithelial cells are coupled through gap junction connexins. (b) During breast cancer growth at primary site, impairment of GJIC and low levels of connexins are observed. (c) With progression of cancer, tumor cells connexins expression is upregulated and function of GJIC is restored with endothelial cells that induce extravasation and adherence to the metastatic site.
Source: Banerjee, 2016, https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5011527/figure/fig1/

Thus, it can be concluded that connexins can contribute to both *tumor progression and suppression*. The important point to consider here is that this decisive role of connexins, which largely depends on their degree of expression in specific cells or tissues. For instance, low expression levels of Cx43 are found in the primary stage of tumors obtained from breast carcinoma cells, but as the tumor progresses, its expression becomes high. Due to the differential expression and underlying role of connexins at various stages of metastasis, different cancer therapeutic treatments have been developed in order to regulate the connexin gene expression for antitumor effects (Banerjee, 2016).

12. Gap Junction and Apoptosis

Trosko & Goodman suggested the role of gap junctions in apoptosis over 16 years ago (Trosko & Goodman, 1994), wherein they hypothesized that a "death signal" may be mediated by gap junctions. In this regard, the death of one cell propagates to other cells, which is called "bystander effect" (Fig. 14), in which cell death is mediated by the transport of toxic metabolites or cytotoxins from an infected or damaged cell to the innocent bystanders or non-infected neighboring cells. Such bystander cell death mediated by gap junctions has been described for few diseases like retinitis pigmentosa or HIV-1 infection.

The gap junction connexins like Cx43 can promote apoptosis by transfer of apoptotic signals between cells (Kameritsch et al., 2013, Cusato et al., 2003).



(a) (b)

Fig. 15: Bystander effect, (a) A "death signal" triggered in a single cell is transmitted via gap junctions to neighboring cell, (b) and hence the bystanding cells also die, without being directly targeted by the stimuli.
Source: (a) Belousov & Fontes, 2016,http://www.nrronline.org/article.asp?issn=1673-5374;year=2016; volume=11;issue=1;spage=75;epage=76; aulast=Belousov#
(b) Kandouz, 2011, https://www.intechopen.com/books/gene-therapy-developments-and-future-perspectives/hopes-and-disillusions-in-therapeutic-targeting-of-intercellular-communication-in-cancer-

The bystander effect has been used in treatment of cancer using gene therapy, in which the malignant cells are infected with herpes simplex virus-thymidine kinase gene, and then they are treated with the prodrug ganciclovir. Phosphorylation of ganciclovir forms ganciclovir-triphosphate, which is a cytotoxin that diffuses through gap junctions to the neighboring by standing cells and induces apoptosis in them (Kandouz, 2011).

13. Summary

In this chapter, the literature related to gap junctions has been surveyed in order to provide comprehensive information along with latest developments about the gap junction channels. As can be seen, our understanding of gap junction and connexins and their multifaceted roles has increased tremendously over the past few years. In context of multicellularity, intercellular communication is an important mechanism and gap junctions perfectly demonstrate this role. They are hydrophilic membrane channels, composed of a hexameric

arrangement of polypeptides encoded by connexin (Cx) family of genes. These channels, also termed as gap junction intercellular channels or GJIC mediate the transport of different molecules like ions, metabolites, growth regulators and even cytotoxic substances, which can lead to either maintenance of tissue homeostasis or even cell death. The gap junctions are not just gated channels but play an important role in different non-channel functions like regulation of cell growth and cell death, differentiation and development by interacting with various other proteins like mictotubules, growth regulators, kinases, etc. Mutations in connexin genes have been linked to many human diseases, including deafness, cardiovascular abnormalities, peripheral neuropathies, and cataract and skin disorders. The role of connexin expression has also been linked with tumor suppression as well as tumor induction; hence their therapeutic potential towards treatment of cancer is being explored. In summary, the multiple functions of connexins and gap junctions clearly demonstrate the intricate nature of multicellular biological system and with each new scientific observation; the layers of our understanding about the functioning of cell and an organism as a whole are getting unveiled.