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Gap Junction Intercellular Communication and Connexin Expression Profile in Normal Liver Cells and Hepatocarcinoma

Glaucia M. Machado-Santelli[1] and Marisa Ionta[2]
[1]*Department of Cell and Developmental Biology, Institute of Biomedical Sciences,University of Sao Paulo, Sao Paulo*
[2]*Institute of Biomedical Sciences, Federal University of Alfenas, Minas Gerais*
Brazil

1. Introduction

Gap junction intercellular communication (GJIC) is considered to play a relevant role in homeostasis of multicellular organisms by regulating processes such as cell proliferation and cell differentiation. Specialized membrane structures mediate cell-to-cell communication, the gap junctional channels, which allow the transfer of molecules less than 1000Daltons (Da) between adjacent cells such as ions, aminoacids, nucleotides, metabolites and second messengers. Gap junctional channels consist of the two juxtaposed hemichannels called connexons, each of them constituted of six proteic subunits composed of connexin (Cx). These proteins are codified by multigene family with at least 21 members and their expression is tissue specific. The different isoforms are named according to their molecular mass (kilo Dalton) and they share a similar structure of four membrane-spanning domains, two extracellular loops, one cytoplasmic loop, one cytosolic N-terminal tail and C-terminal region. The transmembrane domains and the extracellular loops are highly conserved among the family members. N-terminus is also conserved, while the cytoplasmic loop and C-terminus show great variation in terms of sequence and length. Furthermore cytoplasmic tail and loop are susceptible to various post-translational modifications (including phosphorylation), which are important to modulate functional activity of connexin (Figure 1).

The liver represents an interesting system to study gap junction intercellular communication (GJIC) and through the years, a wealth of knowledge is available about functional GJIC and connexin expression profile in different physiological conditions which include cell proliferation, cell differentiation and cell death and disruption of GJIC has been associated with hepatocarcinogenesis process.

2. Gap junction channels biogenesis and degradation mechanisms

The synthesis, assembly and turnover of GJ channels follow the general secretory roles for membrane proteins. Connexins are synthesized by membrane-bound ribosomes and they are cotranslationally integrated into the endoplasmic reticulum membrane. The

oligomerization of connexins into connexon (hemichannel) occurs in a progressive fashion starting in the endoplasmic reticulum and ending in the trans-Golgi network, however the exact localization of oligomerization depends on the connexin type. It is thought that both Cx26 and Cx32 oligomerize in the endoplasmic reticulum, whereas Cx43 oligomerizes in the *trans*-Golgi network (Musil and Goodenough, 1993; Martin et al., 2001).

Connexons are then delivery to the cell surface via vesicles transported through microtubules, which fuse to plasma membrane. Upon arrival at the cell membrane, connexons can either reside in nonjunctional regions or docking with an opposing connexon to form fully functional channels.

Connexons can be **homomeric** (formed by a single type of connexin) or **heteromeric** (formed by more than one type of connexin). Functional channels are **homotypic** when formed by identical connexons (homomeric or heteromeric) or **heterotypic**, formed by the interaction between different connexons (Figure 1).

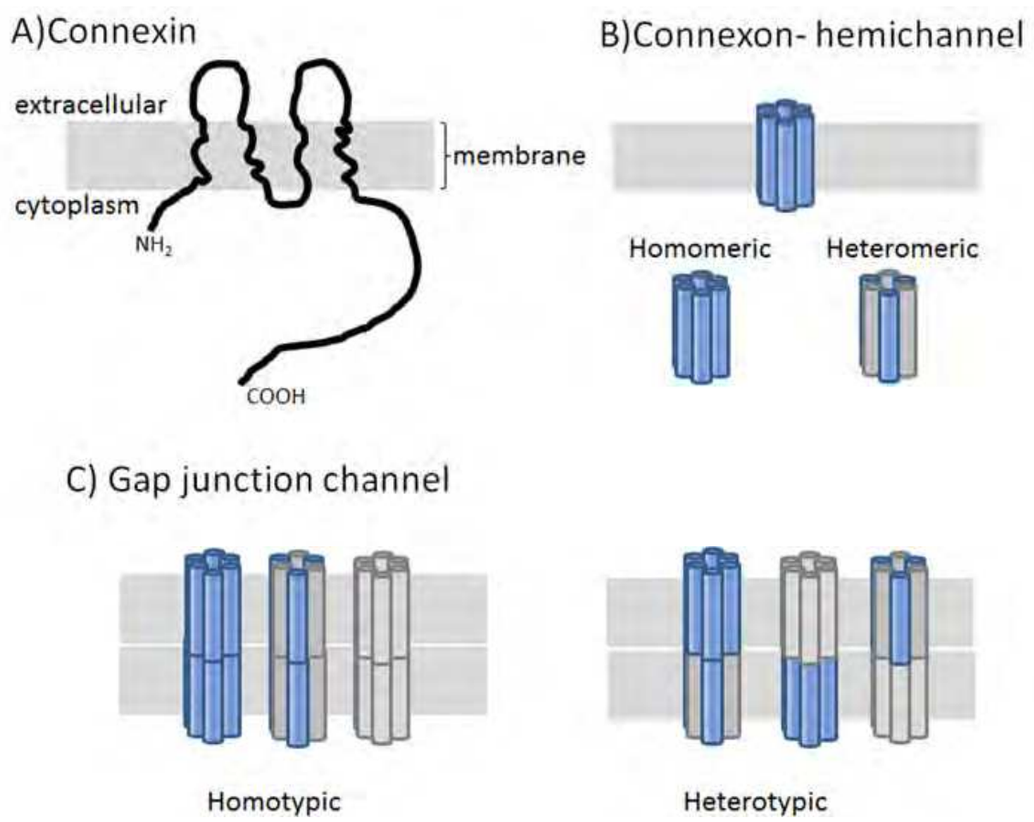

Fig. 1. A) The connexin structure consists of four membrane-spanning domains, two extracellular and one intra-cellular loops, one N-terminal tail and one C-terminal tail; B) Six connexins (represented by cylinder) organize the connexon which can be homomeric (with a single connexin isoform) or heteromeric (formed by more than one type of connexin); C)Two connexons dock together to form a gap junctional channel that can be homotypic if they are identical or heterotypic, if they are different.

The ability to form homotypic and heterotypic channels with homomeric and heteromeric connexons adds even greater versatility to the functional modulation of GJ channels. In

liver, connexin 26 is able to form heteromeric connexon with Cx32 but did not form heteromeric connexon with connexin 43 once the isoforms need to be compatible. It is important to notice that homomeric channels formed by Cx32 or Cx26 present differences in permeability when compared to heteromeric channels formed by just Cxs 26 or Cx32 (reviewed by Mese et al., 2007).

Docking of connexons implies their insertion into gap junction plaques which comprise punctuated regions at membrane juxtaposed area (Figure 2). The formation of functional

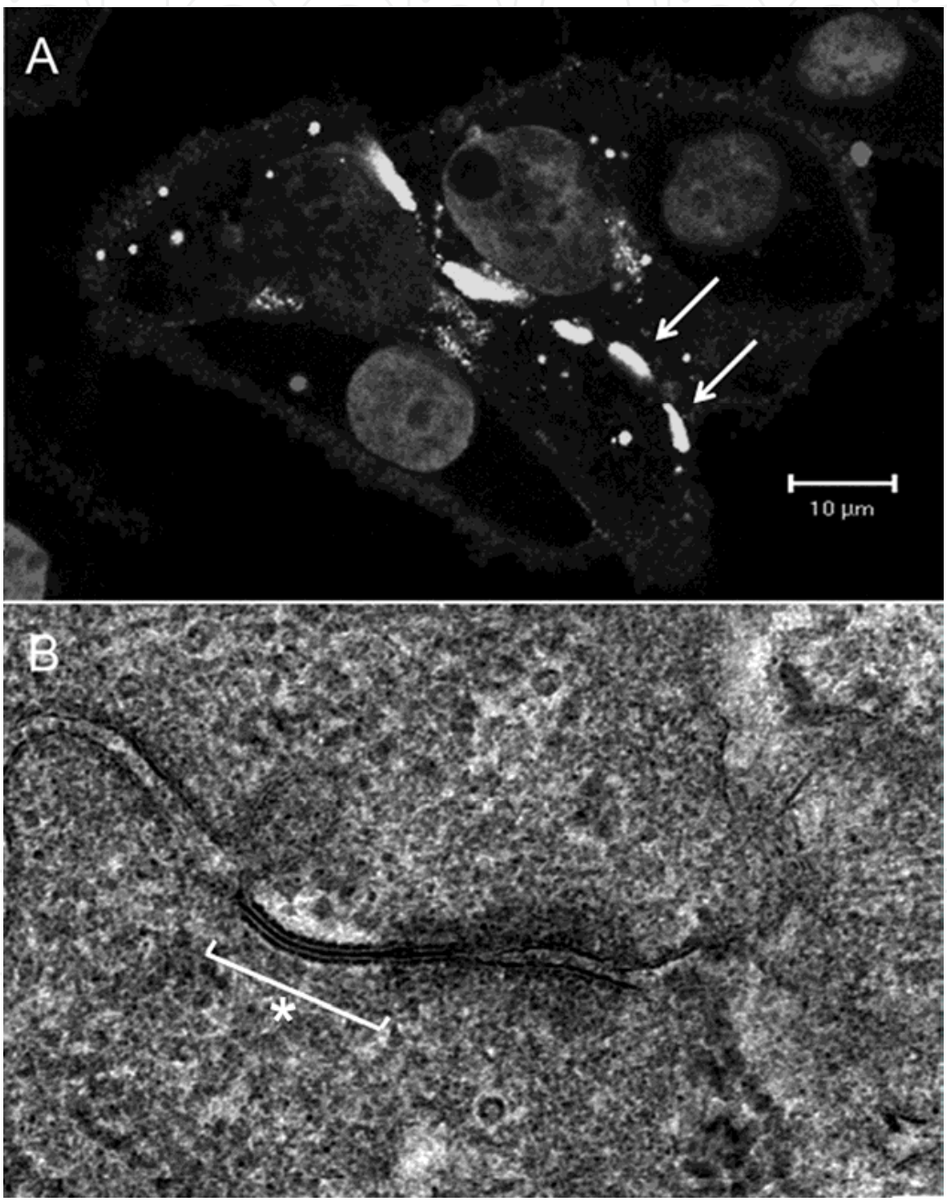

Fig. 2. Gap junctional channels clustered in plaques: A) laser scanning confocal microscopy image of Cx43/GFP-transfected HTC cells showing gap junctional plaques (arrows); B) transmission electron microphotography of gap junctional plaque (asterisk) (courtesy of Prof. Dr.Victor Arana-Chavez, FOUSP).

gap junction requires the appropriate cell-to-cell adhesion. There is evidence that interaction of Cx43 with the tight junction protein (ZO1) may play a role in regulating the size of the gap junction plaque (Hunter et al., 2003).

In general, the turnover rate of connexin is very fast in relation to other plasma membrane proteins. According to studies performed *in vivo* and *in vitro,* the half-lives of Cx26 and Cx32 are respectively 2 and 3 hours (Traub et al., 1983; 1987). The removal of gap junction from the plasma membrane occurs by endocytosis. During this process, both membranes of the gap junction are internalized into one of the adjacent cells and thereby form a double-membrane vacuole called annular gap junction. These structures are further degraded by both lysosomes and proteasomes. The preferential degradation pathway is associated to both cell and connexin type (Laird et al., 2005; 2006). The degradation of Cx32 in the liver occurs mainly via the lysosomal pathway (Rahman et al., 1993). Furthermore the phosphorylation status of connexin is important to regulate its internalization and degradation. *In vitro* studies have been done to understand the mechanism involved in Cx43 internalization and degradation. They showed that the internalization and degradation of Cx43 gap junction is closely related to its hyperphosphorylation (via Mek/Erk pathway) and ubiquitination (Leithe and Rivedal, 2004).

3. Gap junctions and connexin expression in normal liver

The liver is composed of several cell types and consequently it expresses different connexin isoforms. Biliary cells, endothelial cells and oval cells (hepatic stem cells) express mainly Cx43. Hepatocytes express both Cx26 and/or Cx32 depending on the spatial localization in the hepatic lobule. For example, Cx32 is uniformly distributed throughout the liver and therefore it is expressed by hepatocytes in hepatic acinus, while Cx26 is preferentially expressed by hepatocytes localized in the periportal spaces. Thus, most non-parenchymal cells express Cx43 while hepatocytes express Cx26 or Cx32. Considering that hepatic cells establish cell-to-cell communication by channels consisted of different connexins it is not surprising that they present different permeability (permselectivity). It was demonstrated that Cx32 gap junctions had a 10-fold higher relative permeability to adenosine compared with Cx43 channels. By contrast, the phosphorylation status of the adenosine shifted its preferential selectivity from Cx32 channels towards those formed by Cx43. The Cx43 intercellular channels were eight times more permeable to AMP and ADP than Cx32 channels, and the permeability of ATP through Cx43 was more than 300-fold better than that through Cx32 channels. Thus biophysical properties of gap junctional channel depend on the type and phosphorylation status of the connexin that form the channel.

In normal conditions, adult liver display very low proliferative activity. However after injuries or partial hepatectomy, the remaining intact cells start to proliferate. During this proliferation stage, it was observed decreasing of Cx26 and Cx32 expression in hepatocytes. Similar situation was observed when hepatocytes from primary culture were stimulated by mitogen (Kojima et al., 1997). It is important to remember that during liver regeneration process there is not only cell proliferation of hepatocytes but also cell differentiation of oval cells into hepatocytes. Oval cells naturally express Cx43, however along the differentiation program this cell type switch the connexin isoform preferentially express from Cx43 to Cx32

and/or Cx26 (Zhang et al., 1994). Hepatocytes cultured *in vitro* commonly increase Cx43 expression with the concomitant decrease of Cx32 expression (Figure 3). Thus, the immortalized cell lines derived from liver express Cx43 instead of Cx32. Furthermore, hepatocarcinogenesis process leads the hepatocytes to express again Cx43, event that contributes to lose of the differentiated phenotype.

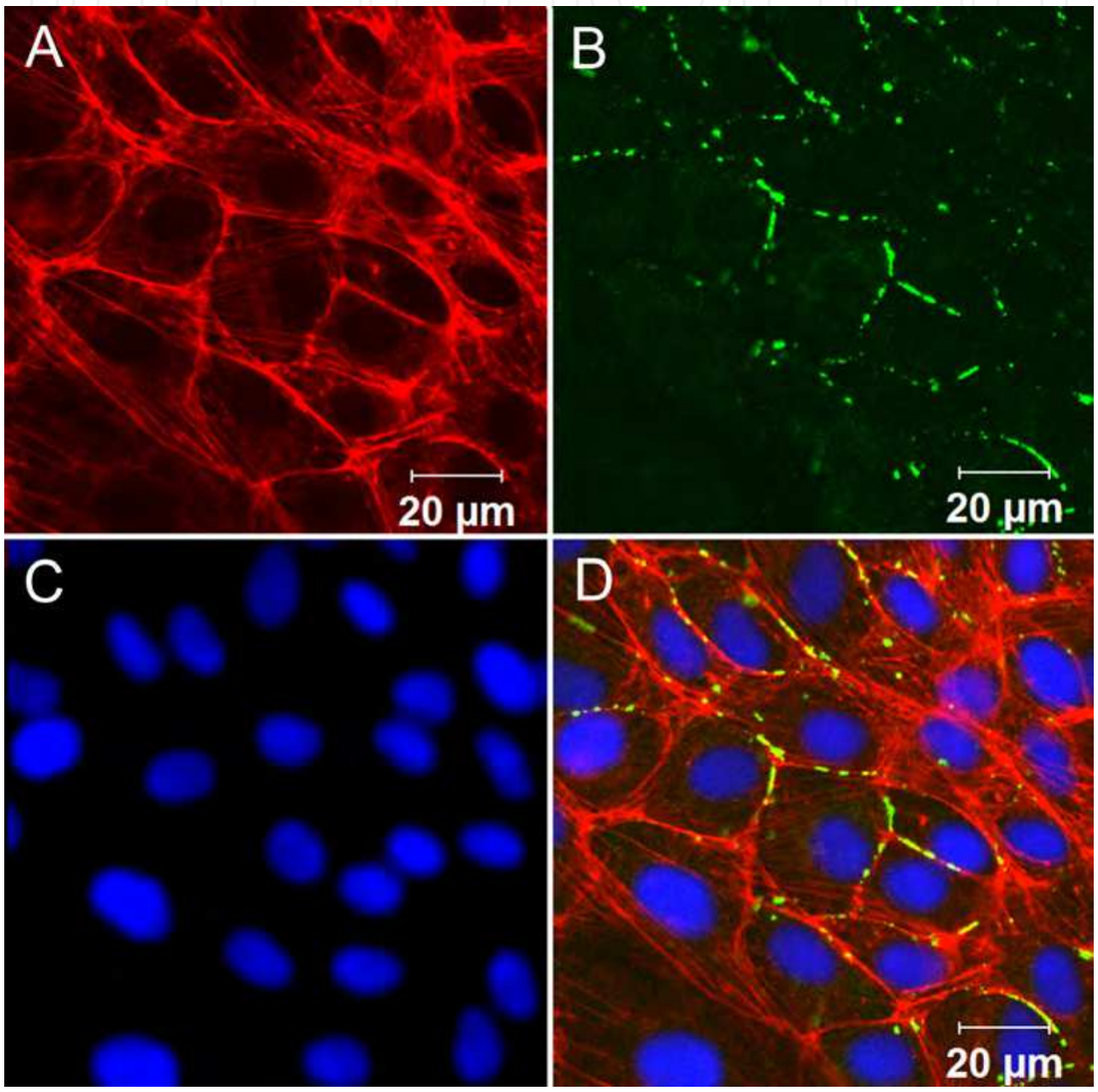

Fig. 3. Laser scanning confocal microscope images of BRL3A (Normal liver cells) submitted to immunofluorescence reaction with anti-connexin43 antibody: A) TRITC-phalloidin stained microfilaments; B) Cx43 is presented in the cytoplasm and gap junctions (green); C) nuclei in blue and D) merged channels .

Although the relationship between GJIC and cell proliferation is well established, its involvement in liver cell apoptosis is not fully understood. It was demonstrated that GJIC is induced in the early phases of apoptosis in serum-deprived rat WB-F344 liver epithelial cells parallel to the increased expression and phosphorylation of Cx43.

4. Modulation of connexin expression, subcellular localization and functional gap junction in HCC cells

Hepatocytes growing *in vitro* change their morphological features losing the liver-cell-specific functions. As early mentioned, the reversion of differentiated phenotype in hepatocytes growing *in vitro* is accompanied by changes in the expression profile of connexin. Under these new physiological conditions, the cells express mainly Cx43, the major isoform expressed by oval cells, instead of Cx32 or Cx26, isoforms typically expressed by hepatocytes.

Similar event occurs during malignant transformation and hepatocarcinoma cells rarely present mutation in connexin genes. Thus changes in connexin expression profile during hepatocarcinogenesis can be related to epigenetic events. For example, it was demonstrated that the reduction of Cx26 expression in hepatocarcinoma was consequence of its promoter hypermethylation. Furthermore, other events have been related to down regulation of Cx32 expression such as inappropriate phosphorylation pattern and aberrant subcellular localization in HCC cells.

Connexin phosphorylation status is very important and essential to regulate several events including intracellular trafficking, connexon assembly and disassembly, insertion in the plasma membrane, degradation and gating of gap junctional channels (Laird et al., 2005). The majority of connexins are phosphoproteins excepting the Cx26 and concerning phosphorylation process, the Cx43 has been widely studied since this molecule has different phosphorylation sites (21 serine and 2 tyrosine residues that are target of different kinase proteins) (Solan and Lamp, 2005). A number of phosphorylated Cx43 variants have been described with different patterns of electrophoretic mobility in sodium dodecyl sulfate-polyacrylamide gel. At least three forms have been described (P0, non-phosphorylated form; P1 phosphorylated form; and P2 hyper-phosphorylated form).

By comparing two rat derived cell lines, one derived from normal liver (BRL3A) and other from hepatocarcinoma (HTC), we could establish association of different phenotype with the phosphorylation pattern of Cx43. Normal liver cells grow in monolayer present contact inhibition and good cell communication capacity via gap junction channels formed by Cx43 (Figure 4). Meanwhile, hepatocarcinoma cell line represents a liver tumor cell line poorly differentiated that grows overlapping, and does not present functional GJIC despite expressing Cx43 (Figure 5). This difference in cell communication capacity is assumed to be due to phosphorylation status of Cx43 and its consequent intracellular localization. The major Cx43 form found in normal cells was the non-phosphorylated (P0) that was correctly inserted in plasma membrane and formed functional GJ. Meanwhile, in hepatocarcinoma cells it was observed predominantly the phosphorylated form of Cx43 (P1) which was concentrated in cytoplasm, unable to form functional gap junction in plasma membrane. These results point out to the importance of phosphorylation status of Cx43 to define its membrane insertion (Ionta et al, 2009).

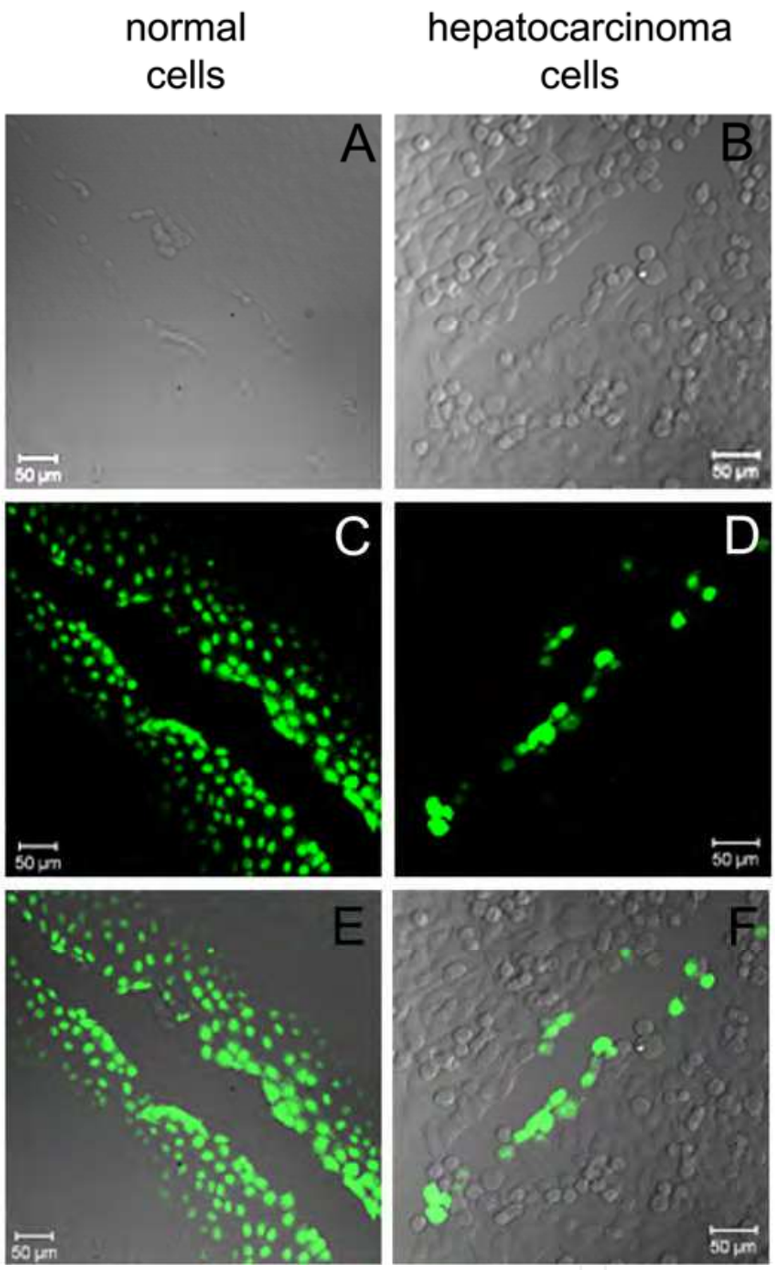

Fig. 4. Scrape loading/dye transfer* assay performed in BRL3A (A, C and E) and HTC cells and HTC cells (B, D and F).Differential interference contrast microscopy images in A and B, fluorescent images in C and D, and merge in E and F. BRL3A presented good communication capacity because it possible to observe at least 5 rows of fluorescent cells from scrape. HTC cells was deficient in GJIC, the fluorescent cells were visualized only in areas near to scrape.

*Scrape loading and dye transfer (SL/DT) is a functional assay widely used to evaluate the level of the intercellular communication and it is based in the introduction of the non-permeable fluorescent dye (Lucifer Yellow, MW= 457,2) into cells of monolayer culture through a transient cut in the cell membrane. Lucifer Yellow does not diffuse through intact membrane but it is transferred into adjacent cells via GJ in competent cells. The transference is monitored with fluorescence microscopy.

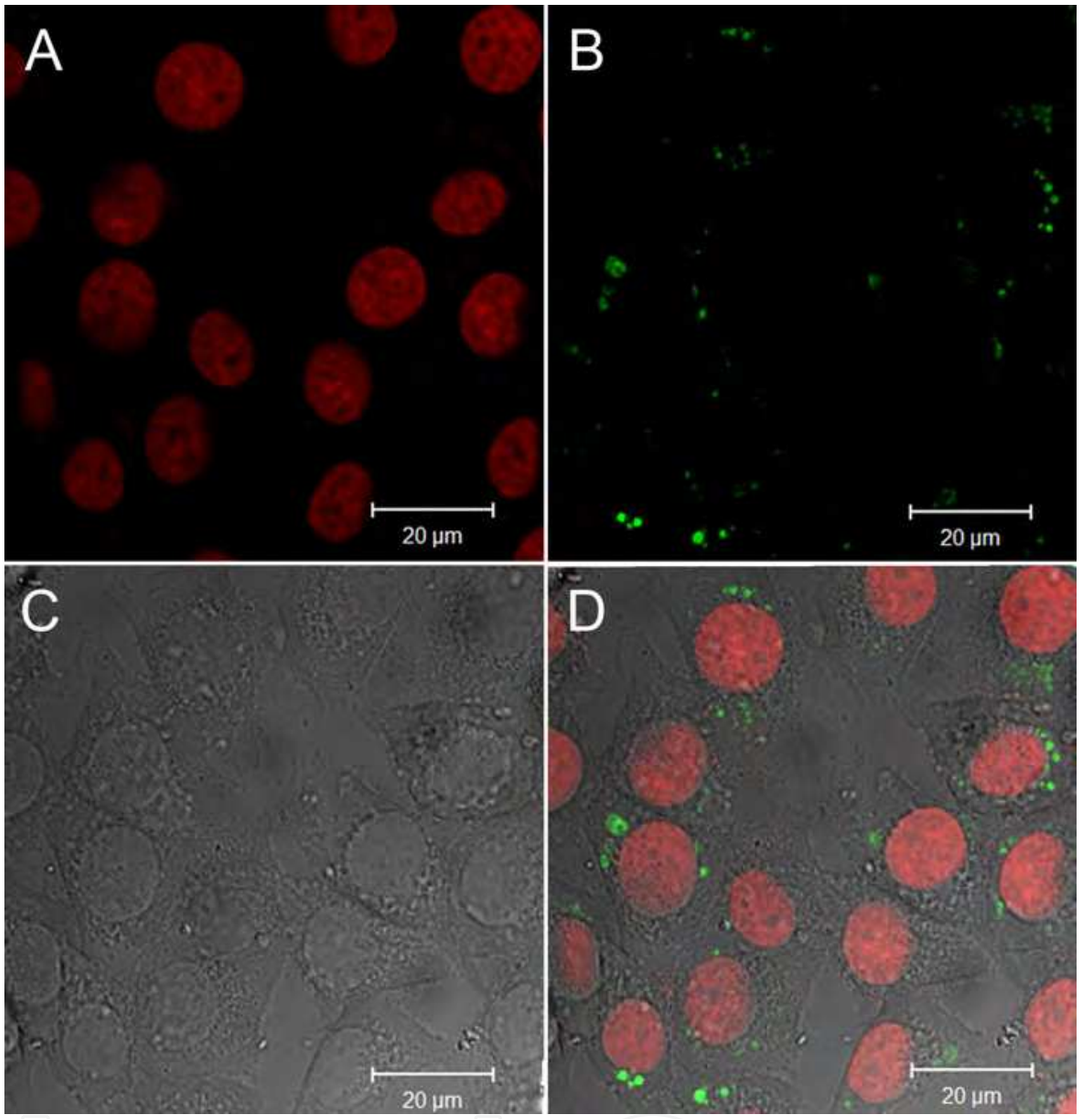

Fig. 5. Laser scanning confocal microscope images of HTC cells submitted to immunofluorescence reaction with anti-connexin43 antibody: A) Nuclei stained with propidium iodide (red); B) Cx43 is presented in the cytoplasm (green); C) differential interference contrast microscopy and D) merged channels.

5. Non-channel functions of connexin and tumor suppressor genes

The deficiency or absence of functional GJIC observed in most of solid tumors led Loewenstein (1979) to propose that the GJ would have a role in controlling cell proliferation. At first this function was associated with the functionality of the channels. There was then an effort to identify if GJIC deficiency was related to a specific stage of chemical carcinogenesis.

The assay performed in well established models permitted to demonstrate the tumor promoter's ability in inhibiting the GJIC. Changes in connexin isoform expressed were

shown in models of chemical carcinogenesis with compounds that induce liver tumors, such as pesticides and phenobarbital. In some cases it was observed the induction of Cx43 expression, connexin isoform usually not expressed by hepatocytes. The molecular mechanism of this process is not well understood, but alterations in the expression and function of connexin are now consistently associated with the hyperproliferative response. In the earlier 1990s, connexin genes have been proposed as tumor suppressor. This idea was reinforced by experimental data that related functional GJ restoration with exogenous Cx expression. *In vivo* studies clearly showed the relevance of Cx32 in maintenance of hepatic tissue normality and in the prevention of hepatocarcinogenesis (Temme et al., 1997).

Although it is not established if there is specific connexin isoforms acting as tumor suppressor in each histological type of cancer, there are some data linking Cx43 and CX32 to liver cancer. Eghbali et al (1991) transfected Cx32 in Sk-Hep1 cells and did not observe growth inhibition *in vitro*, although they detected lower tumorigenic capacity of these cells when they were injected in animals, compared with non-transfected. Sk-Hep1cell line was established from ascitic fluid of a patient with liver hepatocellular carcinoma but it is, in fact, of endothelial origin according morphological, biochemical and immunological markers. Overexpression of Cx32 reverted to normal the transformed phenotype of a rat liver cell line deficient in GJIC derived from a GJIC-competent parental cell line (WB-344).

We studied the effect of exogenous Cx43 expression in rat hepatocarcinoma cell line (HTC) and observed that transfected cells (HTC-Cx43) presented lower proliferation rate than non-transfect cells. The Cx43 was observed forming clusters at plasma membrane in transfected cells in opposition to non-transfected cells that presented Cx43 mainly localized in cytoplasm. Exogenous Cx43 expression induced morphological changes which were compatible with differentiated phenotype. Despite of Cx43 expressed by HTC-Cx43 cells do reach plasma membrane and form cluster there was not GJIC restoration. Therefore, the exogenous Cx43 expression induced a GJ-independent down-regulation in cell proliferation of HTC cell line. We attributed this effect to changes in phosphorylation pattern of Cx43 which was essential to its delivery to plasma membrane. Cx43 expression profile of HTC-Cx43 cells exhibited P1 band intensity similar to HTC cells, P0 and P2 more intense than HTC cells. All together, the data indicate that the exogenous Cx43 was critical for decreasing growth rate in rat hepatocellular carcinoma cells and contributed to reversion of the transformed phenotype. These events were independent of the functional GJIC restoration (Ionta et al, 2009).

Connexin are able to interact direct or indirectly with several molecules related to the regulation of cell proliferation and cellular compounds such as cytoskeleton elements. These interactions were demonstrated by immunofluorescence or co-precipitation studies, approaches that do not distinguish if the interaction is direct or indirect. In fact, the action of connexin as single proteins would affect the production or activity of cell growth regulators, including p27Kip1, cyclin A, cyclin D1, cyclin D2, cdk5, cdk6, ERK1/2, signal transducer and activator of transcription protein 3, Src, human EGF receptor 2, FGF1 and FGF receptor 3 (review in Vinken et al , 2011). Cx43 seems to have a role in cellular

migration by interacting with actin through the zonula occludens. On the contrary of connexin overexpression experiments, these data were observed in an RNA interference knockdown screen. Interactions between Cx43 and ZO-1 or ZO-2, both zonula occludens proteins, were demonstrated in several studies. They bind to the same region of Cx43 in a cell cycle dynamic process, so Cx43-ZO-1 interaction occurs mainly in G0 and while Cx43-ZO-2 in G0 and S phases.

A different kind of interaction with actin seems to occur in HTC-Cx43/GFP cells treated with geodiamolides, natural peptides from marine sponge usually involved with microfilament disruption. We observed that low geodiamolides concentration for short time (2 or 4 hours) treatment did not alter the organization of actin filaments but induces larger GJ plaques (Rangel et al., 2010). The effect could be related to the stabilization of GJ plaques due to both (i) improvement in the connexon exportation or (ii) inhibition of the degradation pathway. To uncouple events leading to GJ assembly from those related to GJ removal, HTC-CX43/GFP cells were treated with geodiamolides in combination or not with fungal antibiotic BrefeldinA, a drug that disrupts the Golgi and protein trafficking to plasma membrane. BrefeldinA drastically reduced the GJ plaques formation, and the same response was obtained when the cells were treated with BrefeldinA and geodiamolides simultaneously. These data indicate that the peptide affects mainly the delivery pathway of Cx43 protein, suggesting that geodiamolide increases the length of GJ plaques mainly because it improves the delivery pathway of Cx43 protein.

6. Acknowledgments

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7. Conclusion

Disruption of GJIC is closed associated to hepatocarcinogenesis process despite of hepatocarcinoma cells rarely present mutation in connexin genes. Epigenetic events can be involved with to lose functional GJ. Change in connexin expression profile (from Cx32 to Cx43) is frequently observed in liver tumor cells. Abnormal localization of connexin (cytoplasmatic instead plasma membrane) also is characteristic de tumor cells. Thus, many studies were employed in the past to verify if connexin could be target of therapeutic approaches. The studies about connexin biosynthesis, traffic, docking at plasma membrane and degradation were very important to understand as functional channels are formed as well as the possible protein partners related with delivery and removal of connexin at plasma membrane. The phosphorylation status of connexin is very critical in relation this issue, likewise the participation of many proteins (signaling pathways, cytoskeleton and cell-to-cell adhesion). The attention for connexins was reinforced when it was observed that functional GJIC was restored in tumor cells by forced expression of connexin. Fortunately along of time a lot of information were collected and today we know that connexins exert tumor suppressor activity on hepatocarcinoma cells dependent and independent-functional GJIC. So, in the future it is possible that connexin will be used as therapeutic target for hepatocarcinoma.

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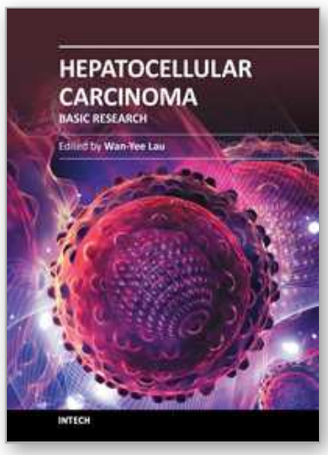

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Hepatocellular Carcinoma represents a leading cause of cancer death and a major health problem in developing countries where hepatitis B infection is prevalent. It has also become increasingly important with the increase in hepatitis C infection in developed countries. Knowledge of hepatocellular carcinoma has progressed rapidly. This book is a compendium of papers written by experts to present the most up-to-date knowledge on hepatocellular carcinoma. This book deals mainly with the basic research aspect of hepatocellular carcinoma. The book is divided into three sections: (I) Biomarkers / Therapeutic Target; (II) Carcinogenesis / Invasion / Metastasis; and (III) Detection / Prevention / Prevalence. There are 18 chapters in this book. This book is an important contribution to the basic research of hepatocellular carcinoma. The intended readers of this book are scientists and clinicians who are interested in research on hepatocellular carcinoma. Epidemiologists, pathologists, hospital administrators and drug manufacturers will also find this book useful.

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