REVIEW OF APOPTOSIS WITH A CONNEXIN EMPHASIS

By

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Abstract:

Gap junction proteins (connexins) are essential components in vital cellular processes such as intercellular communication. Apoptosis is the process through which cells are systematically killed and recycled to maintain healthy tissues and organs. Through a plethora of different signaling cascades connexins can directly influence the incidence of apoptosis in tissue. The relationship between connexins and apoptosis has been recognized by the scientific community, but the factors that govern this relationship remain mysterious. In some tissues connexins have been observed to promote apoptotic activity in combination with cell stressors, while in other tissues connexins prove protective against apoptosis even when cells are stressed. For instance in cancerous tissue and primary culture connexins are often found to be pro-apoptotic, while in healthy tissues connexins often prove anti-apoptotic when cells are subjected to stressors. In effect, connexins are dynamic proteins that have the potential to change from supporting life to supporting death based on a complex interaction between the extracellular environment and internal cellular cues.

Purpose:

The objective of this review is to elucidate the mechanistic relationship between the connexin family, in particular Cx37, and the initiation the apoptotic signaling pathway. Connexins are capable of operating through three different mechanistic pathways, via hemichannels, gap junction channels, and protein-protein interactions. Understanding which of these pathways is involved in apoptotic initiation, and more specifically how, could lead to the development of means to exploit the apoptotic pathway that could have many beneficial
applications. Furthermore, this review will attempt to characterize the circumstances in terms of endogenous expression, tissue type, age of the tissue etc. that lead to the activation or suppression of a given apoptotic pathway. At this time there is an abundance of information about instances of connexin related apoptosis, but little has been done to establish a comprehensive understanding of the way connexins and apoptosis are related under various circumstances. Thus this paper will begin to answer why in some situations connexins enhance apoptosis through hemichannels, gap junctions, or protein-protein interactions, while also inhibiting it through these same processes in different cellular situations.

Summary of Apoptosis:

In order to understand any mechanism of apoptotic initiation or its potential value, a working understanding of apoptosis must first be developed. Apoptosis is the process of programmed cell death that enables the body to develop properly and protect itself from a plethora of abnormalities. It is a vital process because programmed cell death does not compromise the integrity of adjacent cells and therefore provides an organism with a relatively harmless means to deal with internal disruption. Furthermore, apoptosis utilizes several steps including condensation of the nucleus and DNA cleavage resulting in the formation of apoptotic bodies that are shed from the cell and taken up by other cells and phagocytes to be broken down. In fact, cells undergoing apoptosis display signals like phosphotidylserine, which cause macrophages to bind to them and initiate phagocytosis. Furthermore, specific genes regulate this process as can be seen in the model organism, Caenorhabditis elegans, in which CED-6 is necessary for phagocytosis of apoptotic cells, allowing for cell lysis without leaking harmful
chemicals into the extracellular fluid.\textsuperscript{7} Clearly, apoptosis is a complex process that involves numerous interconnected cellular processes; thus, considering every facet of apoptosis is a daunting task and must be approached methodically. To begin to truly understand apoptosis, one must first understand the different mechanistic pathways through which it is activated.

**Pathways of Apoptosis:**

Apoptosis can be activated through three major pathways. The first is the extrinsic pathway, which involves binding of extracellular ligands that induce the cell to activate caspase 8 which in turn can activate BH3 only protein family members that overcome Bcl-2 inhibition and lead to the activation of the destruction caspases 3 and 7.\textsuperscript{8} This pathway is often utilized when a compromising agent is causing necrosis in a tissue and the surrounding cells are designated for programmed cell death instead of enhancing the area damage by undergoing necrosis. The next significant pathway is the Granzyme B pathway which is initiated by cytotoxic T cell assault. In this pathway cytotoxic T cells release granzyme B which enters the cell and can directly activate caspase 3 and caspase 7, as well as initiate a process leading to cytochrome C release from the mitochondria, both of which will lead to apoptosis.\textsuperscript{8} Finally the intrinsic pathway of apoptosis involves activation via numerous instances of significant cell stress or damage that activate the BH3 only protein family.\textsuperscript{9} These proteins proceed to shut down the Bcl-2 apoptotic inhibitors and initialize the formation of BAX/BAK channels on the mitochondria, which in turn enable the efflux of cytochrome C.\textsuperscript{10} Cytochrome C in the cytosol interacts with Apaf-1 and precipitates apoptosome assembly with caspase-9, which then activates caspases 3 and 7 which carry out apoptosis.\textsuperscript{8,11} The mitochondria had been identified as a
pivotal component in several of the apoptotic pathways and as such the stability of the mitochondria is directly related to the level of apoptotic activity in the cell. Due to the wide range of factors that affect the intrinsic pathway and the fact that it is activated by cell stressors, it is the pathway that is most heavily influenced by connexin family activity. However, because so many factors facilitate the initiation of the intrinsic pathway, a more in depth look at connexin involvement in apoptosis is warranted to understand the specific relationships between the apoptotic mechanisms and connexins.

Utility of Apoptosis:

As mentioned previously the causes of apoptosis activation, especially through the intrinsic pathway of apoptosis, are extraordinarily broad. Accordingly, apoptosis serves many roles for an organism and among the most significant are its contribution to development, the immune response, and disposal of damaged cells. For instance, while adult neurons are known for their longevity, developing neurons die in extremely large numbers due to apoptotic activation through the Apaf-1 receptor pathway, and this process is essential for healthy brain development. Apoptosis participates in the immune response in many scenarios, such as when viral particles infiltrate the body and lymphocytes release granzyme B on cells that are in danger of being infected, resulting in apoptosis of a few cells in order to preserve the integrity of a larger tissue. One particularly significant instance of cell stress induced apoptosis is instigated by DNA damage, which prompts p53 to flag the cell for destruction. The protein p53 is a particularly interesting apoptotic initiator because many types of malignant cancer are a direct result of mutations that compromise the ability of p53 to initiate programmed cell death.
Clearly apoptosis is a tool utilized for a plethora of scenarios ranging from development to cancer suppression and observing apoptosis in a population of cells provides valuable information about their compatibility with extracellular conditions. The next question then, is how can apoptosis be detected in a timely and effective fashion?

**Apoptosis Diagnostics:**

Establishing an accurate diagnosis of apoptosis is required before any further steps of analysis or mechanistic determination can be taken, therefore it is worthwhile to consider and understand the most reliable tests for apoptosis. One common method is the DNA ladder analysis, which involves collecting cell DNA, using electrophoresis to run it onto a gel, and observing the pattern that results. Apoptotic cells tend to show distinctive DNA fragmentation at 180 base pair intervals while necrotic cells demolish DNA indiscriminately.\(^{18,19}\) Another way to use the distinctive DNA fragmentation to determine apoptosis is through the TUNEL assay, which utilizes nick end labeling. In nick end labeling terminal deoxynucleotidyltransferases add deoxynucleotidyl-uracil-triphosphates that are secondarily labeled with a marker that can be readily visualized such as biotin.\(^{20}\) Another method of apoptotic detection is phosphatidylserine labeling in which annexin V is used to label phosphatidylinerine that has flipped from the inside of the membrane to the outside during the early stages of apoptosis.\(^{21}\) The fact that the flip happens near the initiation of apoptosis and that the simplicity of the procedure makes it viable for *in vivo* labeling makes this test ideal for diagnosing early apoptotic cells.\(^{22}\) Another popular assay for apoptosis uses the demolition machinery of apoptosis to diagnose a cell state. In this assay the activity of the killer caspases 3 and 7 is measured. Caspase activity is measured by
providing cells with a caspase substrate, usually a nucleic acid-binding dye that contains the DEVD cleavage sequence, that fluoresces when it is cleaved. The caspase 3,7 assay is valuable because these caspases are only activated late in apoptosis and thus their activity is indicative of advanced stages in the apoptotic process. Finally the Ethidium Bromide Acridine Orange diagnostic technique can be utilized to observe apoptotic activity. This technique is useful because acridine orange can enter any cell and make the nucleus appear green, while ethidium bromide can only enter cells after their membrane integrity has been lost and makes the nuclei of early apoptotic cells appear yellow. This method can be utilized to distinguish between late apoptotic cells and necrotic cells because late apoptotic nuclei appear orange and fragmented while necrotic nuclei appear orange but intact. Thus the combination of these two dyes can be used to qualitatively distinguish between apoptosis and necrosis. Now that the tests capable of identifying apoptosis have been established, the distinction between apoptosis and necrosis will be defined.

**Apoptosis vs. Necrosis:**

Confusing apoptosis with necrosis is a common concern among research groups interested in the apoptotic process, so differentiating between the two is a necessary step for those interested in how cells die. The most simple way to distinguish between the two is that while apoptosis is programmed cell death as described earlier, necrosis is accidental cell death, the cell was overwhelmed by a cytotoxic stimulus. Whereas in apoptosis cell death is carried out in stages, including nucleus fragmentation, cell blebbing, and phagocytosis, necrosis occurs violently and suddenly in a much shorter time span and consists of cell swelling followed by
lysis that releases inflammatory cytokines that can potentially damage neighboring cells.\textsuperscript{27} Unlike apoptosis, necrosis does not have easily identifiable pathways through which it acts and is caspase independent.\textsuperscript{28} Necrotic cells generally become extremely ATP deficient or overtaken by an excess of reactive oxygen species (ROS), their mitochondrial membranes are compromised, toxic mitochondrial material is leaked into the cytosol and the cell swells and bursts.\textsuperscript{28} There are clear differences between apoptosis and necrosis, and most of the popular tests for apoptosis distinguish the two. Now that the differences between apoptosis and necrosis have been discussed, it becomes necessary to examine different scenarios in which apoptosis is associated with connexins to gain an understanding of the relationship between the two.

\textbf{Connexin Involvement in Apoptosis:}

As mentioned before connexins maintain three pathways through which they can effect changes in their environment, namely through gap junction channels, hemichannels, and protein-protein interactions.\textsuperscript{1,2} Establishing which circumstances in terms of apoptotic stimulus, cell type, and developmental stage relate to which connexin relationship to apoptosis, stimulatory or inhibitory, along with which mechanistic pathway is involved will comprise the body of this work.

\textbf{Connexin-Connexin Interactions:}

Prior to considering the apoptosis literature however, the potential caveat that connexins are all very closely related and alteration in the function or expression of one type of connexin
can affect the expression of other connexins as well must be noted. Dr. Alex Simon determined that knocking out Cx37 resulted in decreased expression of Cx40, and knocking out Cx40 resulted in decreased expression of Cx37. These types of interactions will not explain possible apoptosis observed in the mutated iRin37 strains within the Burt lab because they do not express connexins other than Cx37, but connexin interaction is still something that should be taken into consideration in vivo. Connexin-connexin interference can be easily overlooked but it must always be remembered that knocking down one type of connexin will often have ramifications on other connexin expression.

**Connexin Based Enhancement of Apoptosis**

**Enhancement through Hemichannels:**

Connexins have been observed to enhance the process of apoptosis in several ways, and in the following section the instances in which connexins enhance apoptosis via hemichannels will be characterized. To begin, in the article "Connexin43 signaling contributes to spontaneous apoptosis in cultures of primary hepatocytes," Vinken et al observe that when primary hepatocyte tissue is isolated from rats it initiates a dedifferentiation process that results summarily in spontaneous apoptosis. Furthermore, they observed that if Cx43 expression is knocked down using siRNA there is a decrease in the activity of caspase 3 and a decrease pro-apoptotic Bid protein implying that Cx43 mediated the enhancement of apoptosis. It was also noted that carbenoxolone, a gap junction inhibitor, also down-regulated the expression of caspase 3 and Bid protein implying that gap junctions or hemichannels were involved in the apoptotic pathway. The researchers determined that hemichannels were involved by measuring an
increase in hemichannel activity as the dedifferentiating hepatocytes progressed through the stages of apoptosis implying that hemichannels were likely the proponents of this apoptotic mechanism. An interesting characteristic of this process was that the dominant connexin in hepatocytes, Cx32, was down-regulated and fetal Cx43 was up-regulated after the cells were isolated and apoptosis commenced.30

This information reinforces work done in another study in which Cx32 is subverted into Cx43 within the liver. Naiki-Ito et al noted that after apoptosis was induced using acetaminophen in vivo within a rat model, Cx43 expression was also induced in parallel to caspase 3 activation resulting in a hepatotoxic state. Furthermore, rats that carried a dominant negative version of Cx32 were protected from apoptotic induction and thus the investigators deemed it reasonable to infer that the channel forming capacity of Cx32 was important for apoptotic initiation. This evidence implies that either hemichannels or gap junctions are important for connexin mediated apoptosis in the liver.31

However, hemichannel mediated apoptosis is not limited to primary cultures, as Cx37 has also been found to enhance apoptotic activity in vitro. In the paper, "Adenoviral delivery of human connexin37 induces endothelial cell death through apoptosis," Seul et al found that transfecting Cx37 into HUVEC cells resulted in extensive apoptotic activity. It was also observed that delivery of Cx37 resulted in HUVEC apoptosis, but the delivery of Cx43 and Cx40 did not, implying that the apoptotic pathway was unique to Cx37. Furthermore this introduction of Cx37 only resulted in apoptosis in HUVECs, N2A and NRK were not affected. The researchers showed that beta glycyrrhetinic inhibits the severity of apoptosis implying that either hemichannels or gap junction channels are involved in the apoptotic process.32
Another instance in which hemichannels are implicated in mediating connexin related enhancement of apoptosis in cell lines can be found in the paper, "A Novel Role for Connexin Hemichannel in Oxidative Stress and Smoking-Induced Cell Injury." This study utilized Marshall cells (MC), which only express Cx43, L2 cells, which express several types of connexins, and N2A cells that express no connexins, to explore the effects of oxidative stress in several different models. The first interesting finding in this paper was that the application of hydrogen peroxide ($H_2O_2$) or cigarette smoke extract (CSE) caused isolated MC and L2 cells to take up lucifer yellow (LY) in the presence of calcium, showing that oxidative stress can "force" open hemichannels. Furthermore, the application of CSE to MC and L2 cells caused them to undergo extensive cell death over a time period of ten hours, and this effect was entirely mitigated by the application of beta-glycyrrhetinic acid. Thus forcing open the Cx43 hemichannels precipitated cell death, but blocking channel function with beta-glycyrrhetinic acid mitigated it. Through YO-PRO/Propidium iodide cell staining the researchers determined that the process by which the MC cells were dying upon being exposed to CSE was apoptosis, which means that the connexin hemichannels were enhancing oxidative stress induced apoptosis.

Another area in which extensive work has been done examining hemichannel mediated connexin driven apoptosis is nervous tissue. In the paper, "Connexin 43 hemichannels contribute to the propagation of apoptotic cell death in a rat C6 glioma cell model," Decrock et al determined that Cx43 hemichannels were responsible for propagating cytochrome C induced apoptosis in C6 glioma cells. Interestingly, they found no evidence of Panx1 mRNA in a RT-PCR assay and thereby eliminated confounding pannexin hemichannels. They confirmed apoptotic activity by transfecting Cx43 into the glioma cells and loading them with cytochrome C using electroporation in divalent cation free media. When researchers pre-incubated their cells
with gap junction blockers like gap 26, and gap 27 for a short 30 minute interval and then put those peptides in the culture medium for 6 hours after exposure to cytochrome C, the amount of apoptotic cell death was significantly decreased. The researchers confirmed that the short incubation period did not affect gap junction coupling, implying that Cx43 hemichannels, which are known to close after short term exposure to gap 26 or 27, were responsible for apoptosis in these cells.\textsuperscript{34} They knew that Cx43 was responsible because in non transfected WT C6 glioma cells there was significantly less apoptosis post induction with cytochrome C.\textsuperscript{34}

In summary, hemichannels appear to play a significant role in several modes of apoptosis, particularly within the liver mediated by Cx32 and Cx43, in HUVEC cells mediated by Cx37, in MCs mediated by Cx43, and in C6 Glioma cells mediated by Cx43. In hepatocytes, Cx43 upregulation is essential for apoptosis, HUVECS must be transfected with Cx37, and the glioma cells had to be transfected with Cx43 to increase the amount present. Only in the MCs were the endogenous connexins sufficient to mediate the hemichannel related apoptotic response, suggesting that in these tissues connexins must be artificially upregulated or introduced to promote apoptosis.

**Enhancement through Gap Junction Channels:**

There have also been some experiments in which it was determined that connexin associated apoptosis is mediated through gap junctions specifically. A variety of cellular signals have been used to induce apoptosis, for example in the paper, "Transfer of IP\textsubscript{3} through gap junctions is critical, but not sufficient, for the spread of apoptosis," Cx43 and Cx26 were both transfected in order to transfer IP\textsubscript{3} amongst cells *in vitro*. It was determined that the IP\textsuperscript{3} was involved in the apoptotic pathway and the connexins were necessary to enhance the apoptotic
signal.\textsuperscript{35} IP\textsuperscript{3} is but one of many hypothetical pro-apoptotic messages propagated through gap junctions and the broad spectrum encompassed by this group includes molecules as esoteric as viral particles, and as clinically relevant as anti-cancer signals.\textsuperscript{36} For instance, Wang et al transfected Cx43 into the prostate cancer cell line LNCaP and observed that the LNCaP cells became very sensitive to the concentration of Tumor Necrosis Factor alpha (TNF\textalpha{}), and would commit to apoptosis, verified through a phophotidylserine flipping assay, if a certain concentration of TNF\textalpha{} was reached.\textsuperscript{37} The researchers in this study noted that more densely plated cells were more likely to exhibit sensitivity to TNF\textalpha{}, leading them to conclude that gap junctions and not hemichannels were responsible for the apoptotic enhancement.\textsuperscript{37}

Other cancer based research has led to the belief that Cx32 gap junctions are responsible for significantly increasing PP1 induced apoptosis in the renal cell carcinoma cell line, Caki 1. In the Caki 1 research it was determined that transfecting cells with Cx32 made them much more susceptible to the Src inhibitor PP1, and apoptosis was detected via a Caspase 3 activity assay. Furthermore, when 18-glycherritinic acid was applied to Cx32 transfected Caki 1 cells, PP1 induced apoptosis was diminished, implying that gap junctions or hemichannels (they didn't do the requisite experiments to rule these out) are the effectors of the Cx32 enhancement of apoptotic activity.\textsuperscript{38}

Gap junction channel based apoptosis has been found to occur in tissue as well as cell culture, and due to the prevalence of apoptotic data associated with nervous tissue it is a good place to start. In the article, "Gap Junctions Are Required for NMDA Receptor–Dependent Cell Death in Developing Neurons," Vaccari et al determined that Cx36 gap junctions were necessary for NMDA based apoptotic activity in primary rat hypothalamic cultures.\textsuperscript{39} This research was carried out based on earlier observations that as development progressed gap junction coupling
was decreased in a manner mediated by NMDA receptors. When the hemichannel soluble dye calcein AM was loaded into neurons that stimulated with excess NMDA, no change in fluorescence was observed, as they believed would occur if hemichannels were opened, leading them to believe that the apoptotic response was due to gap junctions alone. They were confident that the apoptotic activity was gap junction mediated because when 18-GA was applied the effects of hyper-activating NMDA were completely mitigated. Distinguishing gap junction based apoptosis from hemichannel based apoptosis is possible, but the protocols necessary are rarely carried out and thus apoptotic activity mediated through channel activity is difficult to accurately characterize mechanistically.

One of the most common recurring scenarios in which connexins prove to be apoptotic enhancing agents arises from their transfection into cancerous cell lines. Researchers hypothesize that the reason for this is that pro-death signals, such as calcium ions, are propagated through the gap junction channels. Furthermore when connexins are transfected into cancer cells the basic level of intercellular communication is significantly enhanced, thus enabling the pro-death signals to have greater impact. In fact, it is well established that gap junctions mediate a sort of "bystander effect" through which cells adjacent to cancerous cells injected with a cytotoxic agent undergo the same fate as the injected cell. In the case of gap junction mediated apoptosis connexins are often not the direct cause of apoptosis, but greatly enhance the level of apoptosis achieved by pro-apoptotic agents, such as the anti-cancer agents TNFα and Src inactivating PP1.

Enhancement through Protein-Protein Interactions:
Finally, work has been done relating connexins' capacity to enhance apoptosis through the third means of connexin function, protein-protein interactions. For instance in the article, "Connexin 43 (cx43) enhances chemotherapy-induced apoptosis in human glioblastoma cells," Huang et al observed that transfecting Cx43 into human glioblastoma cells made them much more susceptible to chemotherapeutically induced apoptosis. This specific drug was designed to disable topoisomerase, which leads to DNA destabilization during transcription or translation, and apoptosis thus results. Two significant observations were made during this study, the first being that when Cx43 is transfected the levels of anti-apoptotic Bcl-2 are decreased. The second observation was that when the researchers applied beta glycyrrhetinic acid there was no significant alteration in the level of cell apoptosis post induction with the chemotherapeutic agent. This implies that gap junctions and hemichannels are not responsible for the pro apoptotic action of Cx43 in this instance. Taken together it appears that the introduction of Cx43 causes a down-regulation of anti-apoptotic proteins and this action appears to be taking place through a mechanism other than channel function, thus implying protein-protein interactions. Protein-protein interaction mediated apoptosis again appears to be dependent on transfection of Cx43, and it seems likely that the decreased communicative capability of the cancerous cell line made this mechanism more significant.

Connexin Based Inhibition of Apoptosis

Inhibition through Hemichannels/ Gap Junction Channels:

Connexins also have the capacity to inhibit apoptosis through pathways similar to those utilized to induce it. The following section will be devoted to the description of instances in
which connexins utilized hemichannels or gap junctions in order to inhibit apoptosis. In, "Connexin 43 and Bone: Not Just a Gap Junction Protein," Plotkin et al determined that mice lacking Cx43 in their osteocytes experienced enhanced apoptosis in these cells. Furthermore, they noted that bisphosphonates open Cx43 hemichannels that in turn lead to the activation of the ERK survival kinases. Therefore Cx43 hemichannels in osteocytes are vital in the anti-apoptotic mechanistic pathway and damaging their function leads to apoptosis. Osteocytes are known to express Cx43 endogenously at higher levels than any other connexin and the other connexins present in these cells are Cx45 and Cx37.

Furthermore in the article, "Connexins protect mouse pancreatic β cells against apoptosis," Klee et al determined that Cx36 was vital for anti-apoptotic signaling in mouse pancreatic beta cells. The researchers did not determine the exact mechanism but they did note that the beta cells that were the least damaged by the cytotoxic drugs they introduced were very well connected to surrounding beta cells via gap junctions indicating that the enhanced communication was probably cyto-protective.

Another notable instance in which gap junctions proved to be anti-apoptotic is documented in the paper, "Cardiac mitochondrial connexin 43 regulates apoptosis." This paper contains data from neonatal rat cardiomyocytes showing that endogenous Cx43 co-fractionates with VDAC, a protein marker for the outer mitochondrial membrane revealing that Cx43 is located on the mitochondria. They further noted that Cx43 in the outer mitochondria was in a phosphorylated form. Then when the researchers applied beta-glycyrrhizic acid there was a concentration dependent increase in apoptosis that was paralleled by mitochondrial release of cytochrome C. These results indicate that Cx43 channels, or some other connexin channel in cardiomyocytes, are necessary to the cell and blocking them can result in apoptosis. These data
are corroborated by another study done in neonatal ventricular myocytes. By applying antisense Cx43 to primary cultures of ventricular myocytes and observing the resulting decrease in Cx43, Yasui et al successfully decreased gap junction coupling as measured by decreased lucifer yellow transfer. This observation in conjunction with increased apoptosis, determined via nick-end labeling (DNA fragmentation assay), led Yasui et al to conclude that decreases in endogenous Cx43-mediated gap junction activity induced apoptosis in myocytes.46

Another instance in which connexins may utilize gap junction channels to prevent apoptosis was discovered in the study, "Increased Apoptosis and Inflammation after Focal Brain Ischemia in Mice Lacking Connexin43 in Astrocytes.47 In this study Nakase et al induced focal brain ischemia in mice by occluding the middle cerebral artery. In order to observe the effects of ischemia the authors utilized mice with floxed Cx43 genes and an astrocyte specific promoter to drive Cre recombinase expression, thereby depleting astrocytes of Cx43. The authors reported that in the Cre positive mice (where Cx43 was knocked out) there was a significant increase in apoptosis after ischemic insult. The researchers speculate that the lack of gap junctions in these cells is the causal agent for the apoptosis, although they provide no definitive evidence for this assertion.47

In the study, "Loss of connexin 26 in mammary epithelium during early but not during late pregnancy results in unscheduled apoptosis and impaired development," Bry et al noted a relationship between apoptotic inhibition and Cx26 expression that can be most readily explained by protein-protein interactions. The authors observed mammary apoptosis in mice they bred to have floxed Cx26 genes and a promoter for Cre recombinase designed to express Cre recombinase in the mammary epithelium at the time of birth. The researchers noted that mice bred with floxed Cx26 genes and a promoter designed to express Cre recombinase during late
stage pregnancy did not exhibit apoptosis in their breast tissue, which implies that this form of apoptosis is related to early development. The data showing apoptosis revealed a significant difference between control and floxed mice.\textsuperscript{48}

In summary, gap junctions or hemichannels likely play a role in preventing apoptosis in osteocytes via Cx43, β cells via Cx36, ventricular myocytes via Cx43, astrocytes via Cx43, and mammary tissue via Cx26. This anti-apoptotic activity can be characterized further as either developmental (if the connexin is not present at a certain point the cells will undergo apoptosis) or functional (if the connexin channels are blocked or nonexistent, cells will undergo apoptosis upon being exposed to stressors.) Developmental anti-apoptotic activity can be seen in the osteocytes, and mammary tissue, while the β cells, ventricular myocytes, and astrocytes appear to be protected by connexin channels but not dependent on them for survival. In most of these cases apoptosis is actually induced by blocking or eliminating the connexin channel, not introducing an outside agent, which makes the initiation of this anti apoptotic activity starkly different from the pro apoptotic activity characterized earlier.

\textbf{Inhibition through Protein-Protein Interactions:}

The inhibition of apoptosis by connexins has also been documented as a process that takes place via protein-protein interactions. A convincing example of connexins utilizing protein-protein interactions to inhibit apoptosis can be found in the article, "Connexin 43 confers resistance to hydrogen peroxide-mediated apoptosis."\textsuperscript{49} In this experiment Giardina et al first observed that when they exposed C6 glioma cells to hydrogen peroxide, a cell line that did not express Cx43, the cells underwent apoptosis, while a cell line that did express Cx43 was protected. The researchers then applied siRNA to knock down endogenous Cx43 in primary
cultured astrocytes and observed that Cx43 silenced cells were more susceptible to hydrogen peroxide induced apoptosis at all concentrations they used. The researchers then transfected Cx43 EGFP into HEK cells and observed that Cx43 co-immunoprecipitates with apoptosis signal-regulating kinase 1 (ASK1), and further that it inhibited the phosphorylation of a specific ASK1 threonine residue post application of hydrogen peroxide. Thus this article provides some evidence that Cx43 is involved in preventing apoptosis via modulating the phosphorylation of ASK1, thus utilizing protein-protein interactions to mediate apoptosis.\textsuperscript{49}

Another scenario in which Cx43 proves neuroprotective is documented in the study, "Connexin mediates gap junction independent resistance to cellular injury."\textsuperscript{50} In this study C6 glioma cells are transfected with Cx43 and as a result obtain an enhanced resistance to many modes of induced cell death including through calcium ionophores, UV radiation exposure, and tamoxifen.\textsuperscript{50} Interestingly Lin et al also noted that Cx43 did not protect against all toxic agents, and staurosporine and dexamethasone still killed glioma cells effectively. The most interesting point of this work was that treatment with alpha glycyrrhetinic acid didn't reduce Cx43 effectiveness in preventing cell death induced by ionophores specifically lasolocid, which implies that protein-protein interactions were responsible for the observed protection.\textsuperscript{50}

Synthesizing the protein-protein mediated anti-apoptotic connexin activity data, which is heavily focused on C6 glioma cells, provides insight into the relationship between cancerous cells and apoptosis. First it is notable that C6 glioma cells do not appear to utilize gap junctions to prevent apoptosis, which is not surprising considering the tendency of cancer cells to communicate poorly with surrounding cells. Earlier it was determined that C6 glioma cells do utilize channels to undergo apoptosis mediated by cell loading with cytochrome C.\textsuperscript{34} It was also observed that glioblastoma cells, which are also a cancer based cell line, utilized Cx43 to
enhance apoptosis in conjunction with chemotherapy.\textsuperscript{42} Cancerous cells clearly have an increased tendency to utilize the protein-protein pathway to mediate their pro/anti apoptotic effects, and it appears that Cx43 protects C6 glioma cells in a pathway dependent fashion (C6 glioma cells are not protected from all possible inducers of apoptosis, only some.)

**Possible Connections to Apoptotic Induction in Mutated iRin37 Cells:**

It may be worth considering that when the iRin37 S321D or S7D mutants are induced, the iRin37 cell experiences cell stress linked with to their action. It is possible that Cx37 expression is decreased and Cx43 expression is up-regulated as is observed in hepatocytes when apoptosis is induced with acetaminophen, and then Cx43 serves as the catalyst for apoptosis. Another possibility is that Cx37 hemichannels are the primary facilitator of apoptosis, because although little data has been collected on Cx37 hemichannels, Cx43 hemichannels have frequently been linked to apoptotic activity, especially when channel activity is enhanced.\textsuperscript{30,33,34} Another possible avenue is that the mutated Cx37 interacts with anti-apoptotic protection proteins like Bcl-2 and down regulates them in some fashion.\textsuperscript{42} It is very commonly observed that cancer cells, such as iRin cells, transfected with connexins are significantly more susceptible to an apoptosis inducing stimulus.\textsuperscript{34,35,37,42} Therefore it seems reasonable to infer that a Cx37 mutation such as S321D or S7D which are thought to mimic the more active states of Cx37 are mediating apoptosis by activating/spreading anti-cancer signals.

**Conclusion:**
Clearly, connexins play key roles in both stimulating and inhibiting apoptosis, and by considering the data accumulated in this paper some distinctions can be made regarding connexin promotion of life and death. Connexins promote cell survival in healthy cells by activating the ERK survival kinases, providing for necessary homeostatic ion transfer, enhancing beneficial ion transfer after traumatic incidents, diffusing apoptotic signals via gap junction activity, and playing a vital role in tissue development. Furthermore, connexins can prevent apoptosis in aberrant cell lines like C6 glioma cells when they are exposed to certain apoptotic agents like UV radiation by participating in anti-apoptotic signal cascades via protein-protein interactions. In contrast, when C6 glioma cells are exposed to cytochrome C and Cx43 expression is artificially enhanced, apoptosis is stimulated. Similarly, connexin upregulation is required to stimulate apoptosis in primary hepatocyte cultures and HUVECs, leading to the conclusion that connexin upregulation precipitates apoptosis when cells are placed in difficult conditions such as primary culture. Furthermore gap junctions enhance the activity of anti-cancer agents such as TNF-α, resulting in an enhancement of apoptosis within cancerous cell lines due to the expression of connexins. Ultimately, the question of whether connexins will enhance or inhibit apoptosis comes down to the signaling cascades that are activated in the cell, and the stimulus that activated them. In healthy cells connexins usually promote cell survival so this can be considered their natural function. However, when cells are placed in untenable conditions like primary culture and connexins are upregulated they play an active role in enhancing cell death, and when connexins in cancerous cell lines are activated by a signaling cascade begun by an anti-cancer stimulus they enhance cell death. Interestingly, even when connexins are present in cancerous cell lines they can promote survival if these types of cells are challenged by general apoptotic stimuli like UV radiation, although the literature on this point is
conflicting because the effectiveness of other general apoptotic stimuli can be enhanced by the presence of connexins in different types of cancerous cells. However, it is clear that when cancerous cell lines are targeted with anti-cancer signals connexins shift function and become agents of death that promote apoptosis. Naturally connexins often promote survival, but when certain conditions are met, either connexin expression and channel activity are upregulated or apoptotic protein signaling cascades utilize connexins to enhance apoptosis. As a result connexins switch function and become instrumental components of programmed cell death.
References


27. Festjens N, Vanden Berghe T, Vandenabeele P. Necrosis, a well-orchestrated form of cell demise: Signalling cascades, important mediators and concomitant immune response. *Biochimica et Biophysica Acta (BBA)-Bioenergetics.* 2006;1757(9):1371-1387.


