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Book of Abstracts
LOCALIZATION OF PANNEXIN 1 TO PLASMA MEMBRANE CAVEOLIN-1 SUPPORTS ATP RELEASE AND BLOOD PRESSURE REGULATION

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Sympathetic nerve innervation to vascular smooth muscle cell (VSMCs) is a major regulator of arterial vasoconstriction and peripheral resistance. Importantly, the localization of α1-adrenergic receptors (α1-ARs) to the plasma membrane scaffold protein, Caveolin-1, is crucial for efficiently activating downstream signaling molecules important for vasoconstriction. Our lab has previously demonstrated that Pannexin 1 channels, which are highly expressed in VSMCs of resistance arteries, functionally couple with α1-AR, but not other vasoconstriction pathways, through extracellular release of ATP. We hypothesize that a unique Pannexin1 signaling domain exists on the plasma membrane of VSMCs that utilizes Caveolin-1 to localize Pannexin1 to areas of sympathetic innervation. We find here that Pannexin1 and αα1-ARs co-localize using proximity ligation assays at areas of sympathetic nerve innervation with VSMC plasma membrane. Importantly, these areas are enriched for caveolae. Using live cell imaging in primary VSMC culture and proximity ligation assays in intact resistance arteries, we also observed a novel interaction between Pannexin1 and Caveolin-1, but only after adrenergic stimulation. This was confirmed by immunoprecipitation from Caveolin-1 containing membrane fractions. To assess the functional consequences of this transient interaction, we generated an inducible VSMC-specific Caveolin-1 KO mouse. Deletion of Caveolin-1 from VSMC of resistance arteries significantly blunted adrenergic induced ATP release and vasoconstriction. Using radiotelemetry to assess changes in systemic blood pressure, we observed a significant reduction in mean arterial pressure only after Caveolin-1 deletion, which occurred at night when murine sympathetic activity is high. These findings closely recapitulate our published observations in VSMC specific Pannexin1 knock out mice. Moreover, with VSMC Caveolin-1 deletion, we demonstrate resistance to acute blood pressure lowering following treatment with the Pannexin1 inhibitory peptide PanX. Thus, the localization of Pannexin1 to Caveolin-1 at the plasma membrane could facilitate AR-mediated ATP release and vasoconstriction necessary for blood pressure regulation.
MODULATION OF CX37-CONTAINING GAP JUNCTIONS BY SHP-2 AND ITS FUNCTIONAL CONSEQUENCE FOR MYOENDOTHELIAL COMMUNICATION

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We have shown that nitric oxide (NO) inhibits communication via connexin 37 (Cx37)-containing gap junctions (GJ). Here we investigated the NO-induced post-translational modification in the C-terminus of Cx37 and its functional consequence for myoendothelial calcium signal transfer and endothelium dependent dilation of isolated small resistance arteries. Calcium (Fura-2) was increased by mechanical stimulation of HeLa cells expressing Cx37 variants, HUVEC transfected with SHP-2 variants or single ECs of isolated mouse resistance arteries and the signal propagation was analysed respectively (n=8-29). The phosphorylation status of a synthetic Cx37-peptide (AA 324-333) with a phosphorylated tyrosine (“P-Tyr”) equivalent to Tyr 332 was measured after incubation with EC or HeLa cell lysates (MALDI-TOF, n=5-15). The effect of NO-treatment on ACh-induced endothelial calcium increases and dilator responses of isolated vessels (pretreated with 10 µM ODQ to inhibit cGMP) were studied. (n=4-6).

NO significantly reduced the gap junction dependent calcium signal propagation only in cells in which Tyr 332 of Cx37 was present. Impairment of the tyrosine phosphatase SHP-2 by dominant negative mutants or pharmacologic inhibitors abolished the NO effect. Lysates of control cells but not of NO-treated cells or cells lacking functional SHP-2 de-phosphorylated the synthetic peptide (con: 1.03±0.18 Tyr/P-Tyr; NO: 0.55±0.05 Tyr/P-Tyr; SHP-2-deficient con: 0.49±0.12 Tyr/P-Tyr; SHP-2-deficient NO: 0.43±0.08 Tyr/P-Tyr). NO-treatment increased the calcium signal propagation within the endothelial layer of isolated vessels, whereas it significantly decreased the signal propagation from endothelial into smooth muscle cells. The increased endothelial calcium went along with a significant leftward shift of the ACh dose effect relation. We conclude that NO, inhibits Cx37 dependent calcium transfer by increasing its phosphorylation due to a blockade of SHP-2 activity. As a result, in myoendothelial gap junctions of intact vessels, the endothelial calcium loss into smooth muscle is reduced leading to an enhanced, probably EDH-mediated, dilator response of arterial resistance vessels.
TARGETING CONNEXIN40 REDUCES ANGIOGENESIS IN THE DEVELOPING MOUSE RETINA

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Connexin40 (Cx40) forms intercellular channels that coordinate the electrical conduction in the heart and the vasomotor tone in large vessels. We previously documented that altering Cx40 expression or function in endothelial cells (EC) impairs their ability to vascularize tumors. Using Cx40 deficient mice (Cx40\textsuperscript{-/-}) and the neovascularization model of the mouse post-natal retina, we found that Cx40 contributes to physiological angiogenesis. Genetic deletion of Cx40 leads to a reduction in vascular growth and capillary density in the vascular network of the developing mouse retina. At the angiogenic front, vessel sprouting is reduced, and the mural cells recruited along the sprouts display an altered phenotype. These alterations can be attributed to disturbed EC functions as selective re-expression of Cx40 in these cells restores normal angiogenesis. In vitro, targeting Cx40 in microvascular EC, by silencing its expression or by blocking gap junction channels, decreases their proliferation, modifies their secretion of PDGF and promotes the chemoattraction of mural cells. In vivo, an intravitreal injection of a Cx40 inhibitory peptide phenocopies the loss of Cx40 in the retinal vasculature of wild type mice. Collectively, our data show that endothelial Cx40 regulates vessel growth and maturation in the developing retina and may represent a novel therapeutic target for treating pathological ocular angiogenesis.
3D ELECTRON MICROSCOPY COMBINED WITH MULTIPLEX IMMUNOGOLD LABELING TO LOCALIZE PHOSPHORYLATED FORMS OF CONNEXIN 43 IN OVARIAN GRANULOSA CELLS

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While correlative links between Cx43 phosphorylation and gap junction internalization are well recognized, specifically phosphorylated forms of Cx43 have not been localized at the resolution needed to discern individual gap junctions from invaginating gap junctions or from connexosomes near the plasma membrane. To do this, we have established a method for immunogold labeling of serial ultrathin sections collected on tape and imaged with scanning electron microscopy. The ovarian follicle is well suited to studying Cx43 phosphorylation and internalization because gap junctions that connect granulosa cells are modified when meiotic resumption is stimulated with luteinizing hormone (LH). Specifically, Cx43 phosphorylation increases on MAP kinase sites S262 and S279/282, gap junction permeability transiently decreases, and the number of connexosomes found in granulosa cells is increased.

Using specific Cx43 antibodies against phosphorylated S262, S279/282, S368 and S373, we labeled gap junctions and connexosomes in ovarian follicles before and during meiotic resumption. Before LH stimulation, we find that Cx43 is phosphorylated on S368 in all gap junctions and connexosomes examined while phosphorylation on S262 only occurs in about half of the connexosomes and in none of the gap junctions. Thirty minutes after LH stimulation, we see decreased S368 phosphorylation of Cx43 in both gap junctions and connexosomes. Conversely, phosphorylation on S262 is present in most connexosomes and in some gap junctions. Since gap junctions and connexosomes span several ultrathin sections, we labeled individual structures with two or more phosphospecific antibodies by applying different antibodies to separate serial sections. With this technique, we find that Cx43 in individual connexosomes can be phosphorylated on all the sites we examined. Now that we can detect individual gap junctions or connexosomes and label them with multiple antibodies, we will advance our understanding of the spatiotemporal phosphorylation events that regulate gap junction turnover.
INTRAMOLECULAR SIGNALLING IN A CARDIAC CONNEXIN: ROLE OF CYTOPLASMIC DOMAIN DIMERIZATION

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Gap junctions, composed of connexins, mediate electrical coupling and impulse propagation in the working myocardium. In the human heart, the spatio-temporal regulation and distinct functional properties of the three dominant connexins (Cx43, Cx45, Cx40) suggests non-redundant physiological roles for each isoform. There are substantial differences in gating properties, expression and trafficking among these isoforms, however, little is known about the determinants of these different phenotypes. To gain insight regarding these determinants, we focused on the carboxyl-terminal (CT) domain because of its importance in channel regulation and large degree of sequence divergence among connexin family members. Using in vitro biophysical experiments, we identified a structural feature unique to Cx45: high affinity (KD \(~100\) nM) dimerization between CT domains. In this study, we sought to determine if this dimerization occurs in cells and to identify the biological significance of the dimerization. Using a bimolecular fluorescence complementation assay we demonstrate that the CT domains dimerize at the plasma membrane. By inhibiting CT dimerization with a mutant construct we show that CT dimerization is necessary for proper Cx45 membrane localization, turnover, phosphorylation status, and binding to protein partners. Furthermore, we show that CT dimerization is needed for normal intercellular communication and hemicchannel activity. Altogether, our results demonstrate that CT dimerization is a structural feature important for correct Cx45 function. This study is significant because discovery of how interactions mediated by the CT domains can be modulated would open the door to strategies to ameliorate the pathological effects of altered connexin regulation in the failing heart.
STRUCTURAL STUDIES OF HETEROMERIC CONNEXIN 26/30 HEMICHANNELS VIA ATOMIC FORCE MICROSCOPY IMAGING

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Biochemical and functional assays indicate that different connexin (Cx) isoforms can form heteromeric hemichannels with diverse gating and permeability properties. The subunit stoichiometry and molecular arrangements, however, within heteromeric hemichannels remain essentially unexplored. To address this, we used heteromeric connexin hemichannels formed by Cx26 and Cx30 proteins expressed in HeLa cells that contain a haemagglutinin (HA) tag in one isoform (Cx26-HA/30 or Cx30-HA/26 hemichannels). The presence of the heteromers was confirmed by mass spectrometry and western blot analysis. To assess heteromeric hemichannels, purified samples were imaged using air tapping mode atomic force microscopy (AFM). Molecular volumes of the Cx26-HA/30 and Cx30-HA/26 hemichannels showed a particle population centered at 357+/- 9nm³ and 420+/- 11nm³ respectively, which are within the volume range expected for heteromeric Cx hemichannels predicted from their molecular weights. The subunit arrangement and stoichiometry were further evaluated via AFM imaging of hemichannels decorated with Fab fragments derived from anti-haemagglutinin antibodies (Fab-HA). Double bindings were visualized for both samples, Cx26-HA/30 and Cx30-HA/26, and the distribution of angles between Fab-HA and Cx subunits displayed three peaks approximately of 62, 125 and 194 degrees. However, when triple bindings were visualized, measured angles were predominantly at 67 and 121 degrees. Angle analysis indicate that the subunit stoichiometry is 3Cx26:3Cx30 and that the subunit arrangement around the receptor rosette is Cx26-Cx26-Cx30-Cx26-Cx30-Cx30. Funded by Fondecyt postdoctoral 3160568, DPI-Conicyt 20140080 and NIH GM101950 to ALH and JEC grants.
UBIQUITINATION MEDIATES THE SELECTIVE SORTING OF CX43 INTO EXTRACELLULAR VESICLES

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A fine-tuned exchange of substances and signals between cells, tissues and organs is vital to ensure the homeostasis of multicellular organisms. Intercellular communication can occur between adjacent cells, through gap junctions (GJs), or at long distances, via extracellular vesicles (EVs). According to their size and origin, EVs can be divided in Exosomes, Microvesicles and Apoptotic Bodies. EVs convey biological information in the form of lipids, proteins and genetic material, including mRNA and miRNA. Although the mechanisms that regulate the GJs-mediated transmission of information are relatively well known, the signals that govern EVs-mediated communication, namely the processes underlying the selection of the proteins to be sorted into EVs, remain largely unknown. Previous studies from our lab showed that Cx43 channels are present at the EVs surface, where they mediate the release of vesicle content into target cells. However, the signals that regulate the selective sorting of Cx43 into EVs have not been assessed. The main objective of this study was to evaluate the role of ubiquitin as a sorting signal to direct Cx43 into EVs. For that, we assessed the amount of Cx43 present in EVs in experimental conditions known to modulate the amount of ubiquitin bound to Cx43. The results obtained in this study demonstrate that inhibition of Cx43 ubiquitination, through the silencing of Nedd4 (an E3 ligase known to catalyze Cx43 ubiquitination), treatment with Heclin (an inhibitor of HECT domain E3 ligases), overexpression of AMSH (a deubiquitinating enzyme that was shown to remove ubiquitin moieties from Cx43) and ubiquitination-deficient mutants of Cx43, all result in a decrease of the Cx43 levels in EVs. Furthermore, incubation with PKC activator, known to promote Cx43 phosphorylation and ubiquitination lead to an accumulation of Cx43 in EVs. Overall, our results show that ubiquitination promotes the targeting of Cx43 into EVs.
P2X7 RECEPTOR CROSSTALK REGULATES ATP-INDUCED PANNEXIN 1 INTERNALIZATION

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Dynamic modulation of extracellular ATP levels regulates signalling through ATP-activated ionotropic P2X and metabotropic P2Y purinergic receptors in a variety of cells within the nervous system. ATP is released by several mechanisms into the extracellular space, including channels formed by pannexin 1 (Panx1). Notably, we recently reported that a rise in extracellular ATP, in fact, induces endocytosis of Panx1, suggesting a form of negative feedback regulating ATP release. Because this effect was sensitive to inhibitors of the P2X7 receptor, here we investigated the molecular determinants underlying ATP-dependent crosstalk between P2X7 receptors and Panx1. In a series of experiments in Neuro2a cells using exogenous ATP, apyrase (hydrolyzes extracellular ATP) and ARL 67156 (preserves extracellular ATP), we demonstrate that extracellular ATP triggers and is necessary for physical interaction of P2X7 receptors with Panx1. Using in vitro and in cellulo binding assays, we further demonstrate that this interaction occurs within the Panx1 first extracellular loop (EL1). Moreover, mutation of tryptophan 74 within the Panx1 EL1 disrupted the P2X7 receptor-Panx1 cell surface interaction and inhibited ATP-induced endocytosis. Notably, modulation of P2X7 receptor-associated intracellular signalling pathways (Ca2+ influx and Src activation) had no effect on the ATP-induced P2X7 receptor-Panx1 interaction and endocytosis. However, cholesterol-disrupting agents (1 µg/mL filipin III or 10 mM methyl-β-cyclodextrin) impaired ATP-stimulated clustering at the cell periphery consistent with their block of ATP-induced Panx1 endocytosis. Together these observations suggest that ATP induces an interaction between the Panx1 EL1 and P2X7 receptor and is required for their cholesterol-dependent endocytosis. These findings provide important new insights into the regulation of Panx1 trafficking and interactions with P2X7 receptors.
DECREASED EXPRESSION OF CONNEXIN43 PROTECTS AGAINST PODOCYTE DAMAGE AND BLUNTS THE PROGRESSION OF GLOMERULONEPHRITIS

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Glomerulonephritis (GN) refers to a wide variety of renal pathologies that often progress to end-stage renal disease. We observed an increased expression of Cx43 within injured glomeruli of mice treated with nephrotoxic serum (NTS). Consequently, the aim of our work was to study the role of Cx43 in this model of GN. To this end, we compared the evolution of the disease between Cx43WT and Cx43 heterozygous mice (Cx43+-).

Cx43+- mice showed improved renal function, two weeks after the induction of NTS-GN, as proteinuria, blood urea nitrogen and serum creatinine levels were significantly decreased compared to WT animals. In addition, Cx43+- mice showed less histological lesions as crescent formation, tubular dilation, monocyte infiltration and interstitial renal fibrosis were highly decreased. Colocalization experiments indicated that Cx43 was de novo expressed in damaged podocytes, a glomerular cell type which plays a major role in renal filtration. Furthermore, western blotting and immunofluorescence demonstrated that the expression of podocyte markers, such as nephrin and WT1, were preserved in Cx43+- mice, 2 weeks post NTS-GN, confirming the beneficial effect of Cx43 deletion in the integrity of podocytes during the progression of the disease.

In vitro studies showed that treatment of podocytes with TGF-β increased expression of Cx43 and induced either apoptosis or a swift to a migratory phenotype. Blockade of Cx43 function with the Gap26 peptide blunted the TGF-β-induced effects. These effects could be linked to the purinergic signaling activation, since Gap26 blocked the TGF-β-induced P2Y2 increase in these cells.

Finally, in a therapeutic protocol, Cx43-specific antisense in mice suffering from NTS-GN, improved both functional and structural renal parameters. Given that this protein is highly induced in individuals with glomerular diseases, Cx43 may represent a novel target for therapeutic treatment of GN.
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CONNEXIN 26 PLAYS A ROLE IN REGULATING PRO-INFLAMMATORY EVENTS IN THE EPIDERMIS

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Up to 10 connexin (Cx) isoforms are differentially expressed throughout the stratified layers of the epidermis. Their deregulation is associated with psoriasis, chronic non-healing wounds and other inflammatory skin conditions. We characterised the Cx26 and Cx43 expression and function in the skin under pro-inflammatory mediated events. HaCaT cells, a model human keratinocyte cell line, and primary neonatal keratinocytes were exposed to 10µg/mL peptidoglycan (PGN) isolated from the opportunistic skin pathogen Staphylococcus aureus for 15 minutes (min) to 24 hours (h) in the presence or absence of the Cx blockers Gap27 (100µM) or carbenoxolone (CBX) (50µM). Cx26 and Cx43 gene and protein expression levels and hemichannel activity were studied by RT-PCR, western blot and ATP release assays. Supernatants were analysed ELISA for IL-6 release. RT-PCR and ELISA assays confirmed that PGN exposure induced the expression of pro-inflammatory mediators IL-6 and IL-8. RT-PCR analysis determined that Cx26 mRNA expression rapidly increased upon exposure to PGN, peaking at 6 h, followed by return to basal levels by 24 h. Increased Cx26 protein expression was observed 3 h following PGN challenge by immunocytochemistry. In contrast, Cx43 mRNA expression levels remained unchanged; however, western blot analysis revealed that Cx43 protein expression was reduced following 24 h PGN challenge. Immunocytochemical examination showed relocalisation of Cx43 to perinuclear regions 15 min post PGN challenge. Acute exposure (15 min) of keratinocytes to PGN induced ATP release that was inhibited by co-incubation with Gap27 or CBX. ELISA assays determined that co-exposure of the cells to Gap27 or CBX, reduced the PGN-induced IL-6 release emphasising a role for Cx channel activity in induction of the pro-inflammatory response. We conclude that Cx signalling plays a role in the innate immune response in the skin and that maintaining the correct Cx43:Cx26 expression ratio in the epidermis is important for epidermal integrity.
MODULATION OF (hemi) CHANNELS IN THE ENTERIC NERVOUS SYSTEM BY INFLAMMATORY MEDIATORS

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The enteric nervous system (ENS) is a peripheral network of enteric neurons and glia that regulates gastrointestinal (GI) reflexes. Glial connexin-43 (Cx43) hemichannels and neuronal pannexin-1 (panx1) channels play important roles in the regulation of normal GI functions, but also contribute to dysmotility in conditions such as the inflammatory bowel diseases (IBDs) and irritable bowel syndrome (IBS) through increased channel opening (Gulbransen et al. 2012, Brown et al. 2016). Concentrations of key inflammatory mediators are altered during IBD, but it is unknown how these changes affect Cx43 and panx1 function within the ENS.

We measured cytokine levels using a multiplex array and immunohistochemistry in colon tissue and the ENS, respectively. We measured channel opening by the uptake of fluorescent dyes including ethidium bromide (EtBr), and modulated channel function with inflammatory mediators [nitric oxide (NO), IL-1β, IL-17A and IFN-γ], purinergic mediators (ADP and BzATP) and mimetic peptides. We induced colitis in mice with 2,4-dinitrobenzene sulfonic acid.

Colitis increased colonic IL-1β (p<0.005) and the glial expression of IL-1β (p<0.005). Total IL-17A levels were decreased (p<0.01) and no change was observed in whole tissue IFN-γ levels. NO enhanced the ADP-driven uptake of EtBr through Cx43 (p<0.0001). Increased dye uptake in ADP stimulated glia was also observed in the presence of IFN-γ and IL-17A (p<0.001) but was insensitive to blockade by 43Gap26. IL-1β doubled dye uptake in activated glia (p<0.0001) but was sensitive to blockade by 43Gap26. Interestingly, treatment with IL-1β, IFN-γ, IL-17A or NO alone was sufficient to increase channel opening (p<0.0001, p<0.005, p<0.05, p<0.05 respectively).

These results, along with ongoing studies with panx1 permeable dyes, show evidence for modulation of (hemi) channels by inflammatory mediators in the ENS. Specifically, increased glial expression of IL-1β during inflammation, and its significant effects on Cx43 activity, suggests novel, cell-specific mechanisms for channel modulation during inflammation.
PANNEXIN1 LINKS LYMPHATIC FUNCTION TO LIPID METABOLISM AND ATHEROSCLEROSIS

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Atherosclerosis, the leading cause of mortality worldwide, is a progressive disease of the arterial wall arising from an unbalanced lipid metabolism and a maladaptive inflammatory response. Extracellular ATP is a central signaling molecule in the inflammatory cascade and receives increasing attention in the fight against cardiovascular disease. Pannexin1 (Panx1) channels release ATP in a controlled manner and have been implicated in a plethora of inflammatory pathologies. However, the role of Panx1 in atherogenesis remains elusive. Using atherosclerosis-susceptible mouse models with ubiquitous deletion of Panx1 (Panx1⁻/⁻-Apoe⁻/⁻) or with Cre recombinase-mediated deletion of Panx1 in endothelial and monocytic cells (Tie2-CreTgPanx1fl/flApoe⁻/⁻; Panx1delApoe⁻/⁻), we identified a novel role for Panx1 in the lymphatic vasculature. Atherosclerotic lesion development in response to high-cholesterol diet was enhanced in Panx1delApoe⁻/⁻ vs. Panx1fl/flApoe⁻/⁻ mice (14.2±3.6% vs. 6.9±0.9, n=10, P<0.05), pointing to an atheroprotective role for Panx1 in endothelial and/or monocytes. Unexpectedly, atherogenesis was not affected in mice with the ubiquitous deletion of Panx1, but Panx1⁻/⁻-Apoe⁻/⁻ mice displayed reduced body weight (24.8±0.5 vs. 29.2±0.6 g, n=10, P<0.0001), serum cholesterol (727.3±36.3 vs. 921.5±67.4 mg/dL, n=10, P<0.05) and triglycerides (133.3±16.2 vs. 284.2±47.6 mg/dL, n=10, P<0.01) as compared to Apoe⁻/⁻ controls, suggesting altered lipid metabolism in these Panx1-deficient mice. Mechanistically, Panx1⁻/⁻-Apoe⁻/⁻ mice showed impairment of lymphatic vessel function with decreased drainage of interstitial fluids (P<0.05) after footpad injections with Evans Blue and reduced dietary fat absorption compared to control (P<0.05) in an oral lipid tolerance test. Thus, the deleterious effect of Panx1 deletion in endothelial and/or monocytic cells during atherogenesis is counterbalanced by an opposite effect, which results from impaired lymphatic function in ubiquitous Panx1-deficient mice. Collectively, our findings unveil a pivotal role of Panx1 in linking lymphatic function to lipid metabolism and atherosclerotic plaque development.
ENDOTHELIAL PANNEXIN1 CAN REGULATE CEREBRAL MYOGENIC TONE DEVELOPMENT, INFLAMMATION, AND SEVERITY OF ISCHEMIC STROKE

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Pannexin1 (Panx1) regulates adrenergic dependent vasoconstriction in peripheral arterioles; however, unlike peripheral arterioles, regulation of cerebral arteriole vasoconstriction is predominantly due to myogenic tone (MT). Because ATP can elicit potent vasoconstriction of cerebral arterioles, we hypothesized that ATP release from Panx1 channels was involved in MT development in cerebral arterioles. Initially we found that MT in posterior cerebral arteries (PCAs) from wildtype mice was significantly inhibited when treated with apyrase. Next we treated PCAs with pannexin pharmacological inhibitors (carbenoxolone, 10Panx1, and spironolactone), which also significantly reduced MT. For a genetic approach to pannexin inhibition, we used either mice lacking Panx1 in smooth muscle cells (SMC; SMC-Cre Panx1fl/fl) or endothelial cells (EC; EC-Cre Panx1fl/fl). MT was not altered in the SMC-Cre Panx1fl/fl mice, but was significantly reduced in the EC-Cre Panx1fl/fl mice. In peripheral arteries (i.e, third order mesenteric arteries), MT is independent of Panx1 as evident by no change in MT when treated with spironolactone, which we found does not inhibit Cx43 hemichannels, or in EC-Cre Panx1fl/fl mice. Since MT regulates cerebral blood flow and is suggested to play a role in stroke outcome, we investigated the stroke infarct volume in our mice. Experimentally we used a 90 min intra-luminal filament occlusion to induce ischemia of the middle cerebral artery followed by 24 hr reperfusion upon removal. We found that stroke infarct volume was significantly reduced in EC-Cre Panx1fl/fl mice, while SMC-Cre Panx1fl/fl mice showed no protection. EC-Cre Panx1fl/fl mice show reduced TNFα-stimulated leukocyte rolling and adhesion, therefore, we examined infiltration of leukocytes following ischemic stroke. EC-Cre Panx1fl/fl mice have reduced number of infiltrated leukocytes in the ischemic hemisphere, predominately due to reduced neutrophil infiltration. Our data demonstrate that endothelial Panx1 may regulate ischemic stroke outcome, possibly through MT of cerebral arterioles and/or the extent of leukocyte infiltration. Funding: HL088554, HL120840, HL007284, HL131399.
ANTIDROMIC-RECTIFYING GAP JUNCTIONS IN MIXED ELECTRICAL-CHEMICAL SYNAPSES AMPLIFY CHEMICAL TRANSMISSION AND PROMOTE ESCAPE BEHAVIOR IN C. ELEGANS

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Neurons communicate through chemical synapses and electrical synapses (gap junctions/GJs). Although these two types of synapses often coexist between neurons, little is known whether they interact, and whether any interactions between them are important to controlling circuit functions. In C. elegans escape circuit, a pair of interneurons (AVA) contacts downstream A-type cholinergic motoneurons (A-MNs) through both chemical and electrical synapses. In this study, we took advantage of our recent success in performing paired voltage- and current-clamp recordings with C. elegans neurons and the high genetic amenability of the worm to investigate how electrical and chemical synapses interact to control synaptic transmission and behavior. We found that chemical transmission from AVA to A-MNs is mediated by acetylcholine and a specific postsynaptic receptor; the electrical coupling between AVA and A-MNs only allows antidromic current; and disrupting either the electrical or chemical transmission causes defective escape response. Importantly, we found that disrupting the GJs inhibits chemical transmission whereas disrupting the chemical synapses has no effect on the electrical coupling. The GJs between AVA and A-MNs are formed by UNC-7 innexin in AVA and UNC-9 innexin in A-MNs. UNC-7 has three isoforms differing in the length and sequence of the amino terminal. In Xenopus oocyte expression system, all UNC-7 isoforms may form heterotypic GJs with UNC-9 but only one UNC-7 may confer unidirectional current flow (from UNC-9 to UNC-7 oocyte). Expression of this specific UNC-7 isoform in AVA interneurons in an unc-7 mutant significantly restored the antidromic junctional currents between AVA and A-MNs whereas the other two UNC-7 isoforms had either no effect or a much weaker effect. Taken together, our results suggest that the GJs between AVA and A-MNs may serve as an amplifier of chemical transmission and probably consist of a specific UNC-7 isoform in AVA and UNC-9 in A-MNs.
TUBULIN-MEDIATED TRANSPORT OF CONNEXIN 36 (CX36) POTENTIATES ELECTRICAL PLASTICITY IN NEURONAL CELLS

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Previous studies have demonstrated a form of plasticity exclusively at connexin 36 (Cx36) electrical synapses, termed the run-up. However, the mechanism and molecular machinery involved in the run-up phenomenon remains elusive. A hallmark of synaptic plasticity is channel recruitment occurring together with a calcium/calmodulin (CaM)/CaMKII-dependent increase in synaptic strength. We hypothesized that a similar mechanism was involved at the Cx36 electrical synapse, potentiating the run-up plasticity. To address this question, we investigated the tubulin-dependent delivery of Cx36 connexons to the plasma membrane in living Neuro2a cells. A putative Cx36-tubulin binding motif was elucidated via sequence alignment to the known tubulin-binding region of Cx43. Using a His-tag pull-down assay and the BioID methodology, we confirmed the direct binding of tubulin with the C-terminal tail of Cx36, an interaction demonstrating isoform bias. TIRF and FRAP microscopy techniques established that Cx36 is transported from the trans-Golgi network to the plasma membrane via interactions with the tubulin-cytoskeleton. Deletion of the tubulin-binding motif resulted in the reduction of gap junction plaque formation, promoting cytosolic redistribution. Similar observations were obtained with pharmacological disruption of the tubulin-cytoskeleton interaction or competitive inhibition of the tubulin-binding region with TAT-peptides. Interactions between Cx36 and tubulin were critically sustained by the conservation of the Cx36-Lys279 residue, as determined by an alanine site-directed mutagenesis. In parallel to LTP, elevation in CaMKII promoted Cx36-tubulin interactions at the juxtamembrane, thus stabilizing the gap junction plaque and promoting connexon recruitment. In contrast, we found that CaM competitively blocked the binding of Cx36 to tubulin, preventing connexon trafficking and subsequent coalescence. Moreover, disruption of the Cx36-tubulin interaction with colchicine reversibly abolished the run-up, as did the expression of a tubulin binding-deficient Cx36 mutant. Our results suggest that tubulin-dependent trafficking of Cx36 to the juxtamembrane potentiates electrical plasticity in a CaMKII-dependent manner.
CALCIUM-DEPENDENT MECHANISMS UNDERLYING LONG-TERM DEPRESSION OF ELECTRICAL SYNAPSES IN THE THALAMIC RETICULAR NUCLEUS

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Long-term depression (LTD) of connexin36-based electrical synapses in the rat thalamic reticular nucleus (TRN) has been demonstrated following paired activity and following activation of metabotropic glutamate receptors by tetanic stimulation of corticothalamic afferents, but the interactions between and downstream mechanisms of these two paradigms for LTD induction remain unclear. Using dual whole-cell recordings in brain slices containing TRN, we demonstrate that these two stimuli induce LTD by separable pathways. LTD following application of the mGluR agonist ACPD occludes LTD from paired bursting in pairs of coupled TRN neurons, and LTD following paired bursting occludes ACPD-dependent LTD. We show that burst-induced LTD depends on calcium influx via T-type channels, that calcium influx into the intracellular environment recruits calcium release from internal stores, and that the calcium-activated neuronal phosphatase calcineurin is required for activity-dependent LTD. In contrast, ACPD-induced LTD can be induced independently of those sources of calcium. Together, these results provide support for a mechanistic model whereby paired activity-induced depression is mediated by calcium entry and dynamics, while afferent activity-induced depression is not; we hypothesize that the two induction mechanisms converge at a shared downstream pathway. With the aid of computational modeling, we discuss the implications for these two different inductors of LTD in thalamocortical processing.
FREQUENCY-DEPENDENT ASYMMETRY OF HOMOTYPIC ELECTRICAL SYNAPSES

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The most prominent communication between neurons in the adult central nervous system is through chemical synapses. Nevertheless, a majority of GABAergic interneurons use a second mode of communication via homotypic – connexin 36 containing – electrical synapses. These are important for a wide range of brain function like hippocampal dependent spatial coding and memory. While it is generally assumed that homotypic gap junctions are symmetric with respect to their conductance, some reports suggested asymmetry not only in heterotypic but also in homotypic gap junctions. Thus, we investigated whether electrical synapses between two electrophysiologically, cytochemically and morphologically defined interneuron subtypes – namely fast-spiking, parvalbumin-positiv, basket cells (FS) and late-spiking, 5HT3A-positiv, neurogliaform cells (LS) – are asymmetric.

To this end we performed dual whole-cell patch clamp recordings in layer II of cerebral cortex slices in 8 weeks old male GAD-EGFP mice, and measured the steady-state and frequency-dependent electrical coupling in both directions of FS-LS pairs. First of all, by obtaining the frequency-dependent properties of electrical synapses we obtained a minimum parameter set to fully describe the synapse. Thus, we can predict any behavior of the second neuron transduced through the electrical synapse: e.g. the shape and size of the postsynaptic spikelet. Next, we observed that the steady-state conductance was always higher in one direction, while at high frequencies this asymmetry reversed. We currently investigate the following two questions: 1) to which extent is asymmetric gap junction coupling activity-dependent, 2) can the asymmetry be reversed/induced, and if so, what is the underlying mechanism thereof.
MODELLING NEURONAL EXCITOTOXICITY WITH A NEW OPTOGENETIC PANNEXIN-1 CHANNEL

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Pannexin-1 (Panx1) forms large pore ion channels that regulate diverse cellular and (patho) physiological processes including ATP release, vascular inflammation, phagocyte chemotaxis and neuronal excitotoxicity. While characterization of Panx1 signalling has expanded over the past decade, ascribing specific functions of these channels in health and disease has been enigmatic due to non-specific pharmacology and genetic compensation by other Panx isoforms. Here, we developed a new optogenetic (light controlled) tool for the isolation and study of Panx1 signalling, called Opto-Panx1. Opto-Panx1 consists of a light activated protease which targets a newly engineered cleavage motif in the autoregulatory C-terminal domains of Panx1 in response to violet light. Proteolytic truncation of the Panx1 C-tail opens the channel pore. Opto-Panx1 activation mimics known characteristics of Panx1 channel opening, including increased dye uptake, ATP release and whole cell ionic currents in transfected mammalian cells. For in vivo applications, we engineered a Cre-recombinase driven adeno-associated virus for cell type specific expression of Opto-Panx1. Viral delivery of Opto-Panx1 AAV to Cre expressing hippocampal neurons induced large “anoxic-depolarization like” currents and dendritic blebbing, mimicking known functions of the channel during neuronal excitotoxicity in ischemic stroke. This first-generation optogenetic tool provides sensitive spatio-temporal control over Panx1 activity and will expand research efforts aimed at defining Panx1-dependent signalling cascades across a broad range of scientific disciplines.
CX43 ROLE IN BREAST CANCER, A MATTER OF CONTEXT

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CX43 has long been considered a tumor suppressor but this dogma has been challenged by recent studies showing CX43 contribution in cancer progression. However, in breast cancer (BC), the context where it acts as a tumor suppressor or promoter has not been defined. Despite recent progresses, BC is still the second cancer taking the most women lives. It is possible to distinguish several BC molecular subtypes with distinct prognostic and response to treatment: luminal A, luminal B, basal-like and enriched for Her2 (Her2e). The hypothesis driving this project is that CX43 role is dependent of the BC subtype. The goal of this study is to use public databases to determine 1) if CX43 expression could be regulated by epigenetic mechanisms; 2) the functions of the genes coexpressed with CX43 in order to better understand CX43’s functions; 3) if genes coexpressed with CX43 in BC are also coexpressed in other cancer types and in cell lines. Our analysis showed that CX43 methylation is inversely correlated with expression in BC luminal subtypes. Our analysis also show that in Her2e tumor, CX43 is coexpressed with a list of genes (called here EMT genes) strongly enriched for genes expressed in the stroma, in invasive parts of BC, in adult mammary stem cells and involved in the epithelial to mesenchymal transition (EMT). In other BC subtypes, although EMT genes are not correlated with CX43, they are determining CX43’s basal level of expression. Similar to BC, our analysis show that CX43 basal level is also determined by EMT genes in CCLE cancer cell lines and in many other cancer types. These results suggest a new functional link between CX43 and EMT. Together, these results suggest that CX43 role and regulation is subtype dependent in BC.
CONNEIXIN HEMICHANNELS IN SUPPRESSION OF BREAST CANCER BONE METASTASIS AND POTENTIAL THERAPEUTIC INTERVENTION

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The skeleton is the most preferred site for breast cancer metastasis and bone metastasis occurs in 70-80% of patients with advanced breast cancer. Connexin (Cx) 43 is richly expressed in osteocytes, the most abundant bone cell type, and forms gap junctions and hemichannels. In this study, we identified an intrinsic self-defense mechanism of bone cells against invasion of breast cancer cells. We showed that Cx43 hemichannels in osteocytes played an important role in the suppression of breast cancer migration, growth and metastasis. Conditioned media (CM) collected from MLO-Y4 osteocyte cells treated with bisphosphonates, commonly prescribed drugs used to treat cancer bone metastasis inhibited the anchorage-independent growth, migration and invasion of both human breast cancer cells and mouse mammary carcinoma cells, and this inhibitory effect was attenuated with the inhibition of hemichannels by Cx43(E2) antibody. These inhibitory effects on cancer cells were mediated by ATP released from osteocyte Cx43 hemichannels and activation of purinergic receptor signaling in breast cancer cells. Furthermore, Cx43 osteocyte-specific knockout mice and osteocyte-specific Δ130-136 transgenic mice with impaired Cx43 gap junctions and hemichannels, showed significantly increased tumor growth. However, R76W transgenic mice, which form functional hemichannels but not gap junctions in osteocytes, did not exhibit a significant difference in intratibial tumor growth. These data point to the specific involvement of Cx43 hemichannels. We recently developed a new antibody that binds Cx43 and opens Cx43 hemichannels in osteocytes both in vitro and in vivo. The treatment of this antibody suppressed breast cancer bone metastasis in a dose-dependent manner. Together, our studies establish the specific role of osteocytic Cx43 hemichannels in inhibition of breast cancer bone metastasis and the antibody developed that activates hemichannels could sever as a potential therapeutic intervention in the treatment of breast cancer metastasis to bone.
Bone metastasis: important role of connexin43 in the dialogue between prostatic cancer cells and osteoblasts

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In its late stages, prostate cancer (PCa) is mainly characterized by a high propensity to form osteoblastic bone metastases. According to Paget’s notion, this organ preference relies on a cross-talk between cancer cells and the targeted organ microenvironment, involving a complex network of molecules produced by both protagonists. Among the different interacting molecules, gap junction proteins such as connexin43 (Cx43), present in PCa cells, may increase their sensitivity to bone microenvironment. Our previous data using Cx43-overexpressing PCa cell lines clearly demonstrated in vitro and in vivo that Cx43, when exported to the plasma membrane, increased the metastatic behaviour of tumor cells and their bone impact by altering both proliferation and differentiation abilities of bone-forming cells (osteoblasts; OB). The goal of the present study was to analyze the reverse relationship to understand how Cx43 influences PCa cells sensitivity and aggressiveness to bone microenvironment.

Our results clearly demonstrate a Cx43-dependent promigratory effect of OB conditioned media on isolated PCa cells while no significant alteration of proliferative capabilities is demonstrated. Effects on migration are associated with an increase of Cx43 expression in PCa cells and its preferential localization within protrusion structures. These effects mostly rely on hemichannel-independent functions, as illustrated by increased activity of Rac1 and greater interactions between Cx43 and PAK1 (downstream effector of Rac1 regulating cytoskeletal remodeling). This docking role of Cx43 will be further investigated by studying other effectors of Rac1-PAK1 axis. Additionally, global characterization of OB secretome will highlight soluble factors responsible for these Cx43-dependent effects. Finally, our preliminary in vivo studies have demonstrated that Cx43 increased PCa cells aggressiveness in intratibially injected nude mice compared to subcutaneous site.

In the metastatic context, our study demonstrates that Cx43 level could modulate the phenotypic response of PCa cells to the osteoblastic environment.
GAP JUNCTION STABILITY MODULATES PANCREAS CANCER PROGRESSION AND METASTASIS

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Pancreatic ductal adenocarcinoma (PDA) is aggressive, highly metastatic and largely untreatable. A hallmark of PDA is the nearly complete replacement of the normal parenchyma with tumor tissue and complex stroma. Connexin proteins have been implicated in tumor suppression for over 40 years. Cx43, the most widely expressed connexin, interacts with and is phosphorylated by multiple kinases to regulate cell behaviors including migration and proliferation. Phosphorylation of Cx43 by Casein Kinase 1 (CK1) regulates gap junction assembly and substitution of these phosphorylated serines with non-phosphorylatable residues can destabilize gap junctions. Given the complex interplay of cell types in PDA, we hypothesized that reduced gap junction stability would affect PDA progression. We interbred the well established KrasLSL-G12D/+;p48Cre/+ (KC) mouse model of PDA with our homozygous “knock-in” (KI) mutant Cx43 mice bearing amino acid substitution at CK1 sites (KC-CK1) and found profound and surprising effects on cancer progression. We observed an extension of lifespan, from 443 to 584 days (p=0.012), a decreased incidence of metastasis (68% vs 37%) and a cystic phenotype in the KC-CK1 compared to KC mice. However, when we examined early stages of disease we found more rapid onset of tissue remodeling in the KC-CK1 mouse. During tumorigenesis, gap junctions are increasingly present in stromal cells of the KC mice but are absent from the KC-CK1 mice. Tail vein injection experiments with tumor cells derived from KC or KC-CK1 mice recapitulated these effects as KC-CK1 cells had a growth advantage but could not generate the stromal gap junctions that seem to facilitate metastasis. Together these results show that Cx43 plays a tumor suppressor role early during carcinogenesis but they imply that stromal gap junctions facilitate the early stages of the metastatic process. Thus, reduced stromal cell gap junctions may decrease effective metastasis. Supported by grants GM55632-CA149554 from US NIH.
THE COMPLEXITY OF CONNEXINS AND PANEXINS IN CANCER: RNA AND PROTEIN EXPRESSION IN HUMAN TUMOURS

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The role of connexins and pannexins in cancer is complex and depends on the tumour type or subtype, and disease stage [1]. Parameters such as which connexin isoform is expressed, and its subcellular localization, also influence putative pro- or anti-tumoral effects. We have analysed connexin and pannexin RNA and protein expression in human tumours with the purpose of understanding their role in cancer progression.

We present select results and conclusions mainly related to Cx43, Cx46 and Panx1, in multiple tumour types. Some of the initial unexpected findings include the putative association between Cx46 and vascular invasion in breast cancer. Panx1 is expressed in tumours of various organs including cervix, lung and breast. In colon cancer we observe significantly elevated Panx1 expression in metastatic lesions compared with matched primary tumours.

We have particularly focused on lung tumours. Online Kaplan-Meier analysis of a microarray dataset of 2437 non-small-cell lung tumours suggests that both reduced and increased mRNA expression, in a connexin-dependent manner, can be associated with poor survival. Histological subtype — adenocarcinoma versus squamous cell carcinoma — strongly influences these associations. Immunohistochemical staining of human tumours does not always correlate with in silico array data. Noticeably, we have identified a subset of lung tumours with strong expression of nuclear Cx43 associated with worse prognosis, indicating that not only expression levels but subcellular connexin localization, needs to be considered. Further studies are clearly needed to clarify the exact role of different gap junction proteins in cancer, in which connexin subtype and its subcellular location are major determinants that need to be taken into consideration.

THE MOVING PARTS IN CONNEXIN HEMICHANNELS: COUPLING BETWEEN EXTRACELLULAR AND INTRACELLULAR DOMAINS

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Connexin (Cx) proteins form two types of channels at the plasma membrane, hemichannels and gap junction channels. They share common mechanisms of opening and closing, such as the so-called “loop-gate” mechanism. This mechanism is thought to be a permeability barrier formed by a segment of the first extracellular loop (E1) and the first transmembrane domain (TM1) at the outer entrance of the pore. Combining electrophysiology, mutagenesis and molecular dynamic simulations, we found that the E1/TM1 region is the binding site for calcium, but it does not form a physical and/or an electrostatic gate. Upon calcium binding, this region undergoes conformational changes that triggered closure of the gate somewhere below the inner middle pore. In addition, we found that disease-causing human mutations at the N-terminal region of Cx26 hemichannels significantly delay the pore closure by extracellular calcium. These data suggest that conformational changes at the extracellular pore are coupled with intracellular N-terminal domain to close the pore. Correlation analysis using molecular dynamic simulations showed that residues at the N-terminal region are interacting with the first half of the cytoplasmic loop in the open conformation of Cx26 hemichannels. Functional mutagenesis studies indicate that this interaction is important for gating, but does not stabilize the channel in the open state. We propose hemichannel closure involves a concerted coupling mechanism between the N-terminus and the E1/TM1 region.
A QUANTIZED MECHANISM FOR CASPASE- AND A1 ADRENOCEPTOR-MEDIATED ACTIVATION OF PANNEXIN 1 CHANNELS

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Pannexin 1 (PANX1) is an oligomeric plasma membrane channel that mediates nucleotide release for purinergic signaling, which is involved in clearance of apoptotic cells, vascular contraction in peripheral arteries, neuronal communication, and cancer metastasis. While previous studies have suggested that the C-terminal tail of PANX1 interacts with the channel pore and that removal of this autoinhibitory tail is critical for channel opening, how many of the 6 tails within a hexameric channel complex must be displaced from the pore was not known. By using single particle electron microscopy, we first show that caspase-mediated removal of the C-terminal tails reveals a prominently ‘open’ central channel pore. To explore structure-function mechanisms for PANX1 activation, we examined wild type and concatenated hexameric PANX1 channels, and discovered that PANX1 activation involves sequential stepwise sojourns through multiple discrete open states, each with unique channel gating and conductance properties that reflect contributions of the individual subunits of the hexamer. The progressive increase in whole cell currents and ATP/fluorescent dye permeation suggests a common pathway for both small ions and large molecules. This paradigm of multistep channel activation is also operational in a reversible and caspase cleavage-independent activation mediated by α1 adrenergic receptors. This quantized channel activation with multiple discrete activation states reported here is likely to be broadly relevant for purinergic signaling, as well as for the volume regulated SWELL1 and the calcium homeostasis modulator CALMH1 that share a structural similarity with PANX1.
DIFFERENTIAL ROLES OF THE CARBOXYL TERMINUS IN CX43 CHANNEL GATING AND PERMEATION

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Gap junction channels are crucial for cardiac impulse propagation and coordinated contraction. Connexin 43 (Cx43), the main ventricular gap junction (GJ) protein residing at intercalated disks (ID), is phosphorylated in its carboxyl terminus by casein kinase 1 (CK1) in normoxic tissue. Ischemia or pressure-overload-hypertrophy cause CK1-phosphorylation to fade and Cx43 to relocate from the ID to the lateral borders of myocytes (remodeling); such alterations correlate with myocardial vulnerability to arrhythmia. In accord, it was published that transgenic mouse hearts with CK1-phospho-mimicking mutations of Cx43 resist pathological remodeling and arrhythmia, while those with CK1-dephospho-mimicking mutations are more vulnerable to both remodeling and arrhythmia. To evaluate the mechanistic basis for the arrhythmogenic character of these Cx43 phospho-isofoms, we are examining channels and junctions composed of Cx43 mutants wherein CK1-targeted serine residues (325, 328 and 330) were replaced by aspartate (Cx43-CK1-D) or alanine (Cx43-CK1-A) to mimic phosphorylation and dephosphorylation, respectively. Cx43-CK1-D GJs displayed: i) multiple channel amplitudes, some larger (γj >150pS) than those typical of Cx43 wild type (WT); ii) strong Vj-gating; iii) permselectivity (PNBD/gj) similar to Cx43WT; and iv) frequent hemichannel (HCh) openings. In contrast, for Cx43CK1-A GJs, γj values, Vj-sensitivity and HCh activity were similar to Cx43WT. Surprisingly, GJs from both mutants appear resistant to acidification-induced uncoupling. These data imply that the arrhythmogenic resistance of transgenic mice expressing CK1-phospho-mimicking Cx43 has a more nuanced underpinning than GJ resistance to acidification-induced closure (HCh behavior?). Phosphorylation of S368 by PKC is cardioprotective in ischemic contexts. Because PKC activation reduces macroscopic and channel conductance, but increases permselectivity of Cx43WT, the effects of PKC activation and inhibition on both Cx43-CK1-D and Cx43-CK1-A junctions are of interest, but remain uncertain. Possibly, voltage gating, chemical gating and permselectivity of Cx43 GJ channels involve different, phosphorylation-dependent, structural conformations of the interacting domains of Cx43.
CONNEXIN43 HEMICHANNELS, ATP RELEASE AND THE Ca2+/ROS SIGNALLING AXIS CONTRIBUTE TO THE PROPAGATION OF RADIATION-INDUCED BYSTANDER EFFECTS IN BRAIN MICROVASCULAR ENDOTHELIAL CELLS

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The effectiveness of brain tumor radiotherapy is frequently limited by the sensitivity of normal brain tissue to ionizing radiation. Evidence highlights a critical contribution of brain microvascular endothelial cell (BMEC) dysfunction to radiation-induced normal tissue damage. BMEC injury includes DNA damage, cell death, activation and senescence of BMECs, i.e. cellular processes that can affect BBB integrity. Evidence suggests that intercellular communication pathways can propagate radiation-induced effects, a phenomenon known as the radiation-induced bystander effect (RIBE). We here aimed to explore the role of intercellular communication via connexin (Cx) gap junction channels and hemichannels as a propagation pathway for radiation-induced BMEC damage.

To this end, we optimized an in vitro model in which a well delineated zone of an adherent EC monolayer is exposed to X-rays. Subsequent DNA damage, i.e. double-strand break (DSB) formation, was detected in both the irradiated area and the adjacent non-irradiated ‘bystander zone’, with the number of DSB-positive cells being reduced by Cx43 knockout strategies and hemichannel targeting peptides. Extracellular ATP release and dye uptake experiments revealed hemichannel activation within 5 min post-irradiation. Purinergic receptor blockers, scavenging Ca2+ and reactive oxygen species, and microscopy-based Ca2+ and ROS detection techniques furthermore suggest a link between ROS production and Ca2+ signaling in the generation of DSBs. Moreover, in vivo data demonstrate an opening of the BBB after the exposure of mouse brain to X-rays. Our results thus reveal a novel mechanism for radiation-induced BMEC damage which involves Cx43 hemichannels, ATP release and the Ca2+/ROS signaling axis. Application of agents that interfere with these signaling mechanisms could provide perspectives to protect BMECs and healthy brain tissue against X-ray-induced damage.

L. Leybaert and E. Decrock share senior authorship
CNG CHANNEL-DEPENDENT REGULATION OF GAP JUNCTION COUPLING ACTIVATED BY ADENOSINE RECEPTORS IN CEREBRAL MICROVASCULAR ENDOTHELIAL CELLS

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Gap junction-dependent intercellular communication enables the formation of functional units in tissues and must therefore be integrated in the general signalling of the cells within tissues. Adenosine receptors regulate numerous cellular functions and have been proposed as targets of pathophysiological events in endothelial cells of the blood-brain barrier. We analysed the link between adenosine receptors and gap junctions in human microvascular endothelial cells using the hCMEC/D3 cell line. The agonist of the adenosine receptor subtypes A₂A and A₂B, 2-phenylaminoadenosine (2-PAA) increased the gap junction coupling up to 140% within 1 h, as found in scrape loading/dye transfer experiments. At the morphological level, the 2-PAA-related enhancement of the gap junction coupling correlated with an increase in gap junction plaques consisting of Cx43. At the signalling level, 2-PAA acted via induction of cAMP synthesis, however, inhibitors of the protein kinase A (PKA) did not affect the 2-PAA-induced increase in gap junction coupling and the exchange protein directly activated by cAMP (Epac) was not involved in the signalling cascade either. Instead we found a significant attenuation of the increase in gap junction coupling when 2-PAA was applied in presence of the cyclic nucleotide-gated (CNG) channel blocker L-cis-diltiazem or in absence of extracellular Ca²⁺ or to cells preloaded with the Ca²⁺ chelator BAPTA. Accordingly, the whole-cell patch-clamp technique showed that L-cis-diltiazem antagonised a 2-PAA-induced increase in cell current. Additionally, a 2-PAA-induced increase in intracellular Ca²⁺ was observed using ratiometric Ca²⁺ imaging. In conclusion our results show for the first time that CNG channels integrate adenosine receptor-dependent signalling and gap junctions in endothelial cells of the blood-brain barrier. The signalling pathways responsible for the interaction between adenosine receptors, CNG channels and gap junction coupling are currently under investigation.
Connexin mutations cause a high incidence of nonsyndromic hearing loss. In particular, Cx26 (GJB2) mutations are responsible for more than 50% of nonsyndromic hearing loss in the clinics. However, the underlying deafness mechanisms are still largely unknown. In recent years, we used Cx26 deficient mouse models and investigated the deafness mechanisms underlying Cx26 deficiency induced hearing loss. We found that the long-term hypothesized K+-recycling disruption in the cochlea is not a primary mechanism for Cx26 deficiency induced hearing loss. In the clinic, Cx26 mutation induced hearing loss can divided into two major groups: congenital deafness and progressive, late-onset hearing loss. We found that they have different mechanisms. Cx26 deficiency induced congenital deafness is mainly resulted from the cochlear developmental disorders rather than hair cell loss or cell degeneration, whereas Cx26 deficiency caused late-onset hearing loss is mainly resulted from reduction of active cochlear amplification, even though auditory sensory hair cells have no gap junctions and connexin expression. We further found that Cx26 deficiency disrupted the miRNA-mediated intercellular genetic communication in the cochlea, leading to cochlear developments. Unlike deficiency of Cx30, which is co-expressed with Cx26 in the cochlea and abolishes the endocochlear potential (EP) leading to hearing loss, Cx26 deficiency could reduce but not abolish EP. The EP reduction is also not associated with Cx26 induced hearing loss. Finally, Cx30 deletion had no cochlear developmental disorders and the miRNA-mediated intercellular genetic communication also retained normal in the cochlea.
DIAMETRIC EFFECTS OF A CX30-A88V MUTANT IN CAUSING SKIN ANOMALIES YET PROTECTION AGAINST AGE-RELATED HEARING LOSS

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Connexin30 (Cx30), which we have shown to have a long half-life at the plasma membrane, is strongly expressed in the epidermis and cochlea, and is frequently coexpressed with Cx26. Several autosomal dominant Cx30 mutations are linked to skin pathologies with or without hearing loss. Previously, we showed that overexpression of a Cx30-A88V mutant, linked to Clouston syndrome, resulted in a surprisingly severe cellular phenotype by triggering apoptosis, raising questions as to whether this occurs in mutant mice. To that end, Cx30A88V/+ and Cx30A88V/A88V mice were generated on a CD-1 background which inherently suffer from accelerated age-related hearing loss. These mutant mice exhibited skin pathologies which only became apparent in homozygous mice, but were remarkably protected against high frequency early onset age-related hearing loss. The protection of hearing, yet presence of skin abnormalities is intriguing in Cx30A88V/A88V mice and strikes an inverse similarity to some patients with Cx26 mutations who suffer hearing loss, yet have improved skin barrier function. To investigate the mechanism of hearing preservation in Cx30A88V/A88V mice, we performed auditory brainstem recordings to analyze cochlear function and neural output. Consistent with previous reports, we confirmed that outer hair cells were present in the basal, high frequency turn of adult Cx30A88V/A88V mice cochleae but not in wild-types and heterozygotes. These results likely underlie the elevated neural output we recorded from the cochleae of Cx30A88V/A88V mice in response to auditory stimulation. To investigate further how the Cx30-A88V mutant is protective against age-related hearing loss, we are using organotypic cochlear cultures to decipher gap junction/hemichannel function, expression and localization patterns of Cx26 and mutant Cx30, and the effect the mutant has on hair cell development. Our ongoing studies aim to uncover the mechanisms that dictate the divergent effect of the mutant in causing some skin abnormalities while preventing accelerated age-related hearing loss.
A MIMETIC PEPTIDE RESTORES CX32 HEMICHANNEL GATING INHIBITED BY THE R220X MUTATION THAT CAUSES CHARCOT-MARIE-TOOTH DISEASE

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Mutations of connexin 32 protein (Cx32) cause the X-linked form of Charcot–Marie–Tooth disease (CMT1X), a demyelinating peripheral neuropathy for which there is no cure. Recent findings suggest that ATP release through Cx32 hemichannels in Schwann cells participates in a purinergic signaling pathway that regulates nerve myelination, but it is unknown if CMT1X mutations alter the physiological gating mechanism that controls Cx32 hemichannel opening by cytosolic Ca2+ elevations. The current study made it possible to link Cx32 hemichannel dysfunction to CMT1X pathogenesis since loss of the C-terminus in Cx32 (R220X mutation), which causes a severe CMT1X phenotype, inhibits hemichannel opening during a canonical IP3-mediated increase in cytosolic Ca2+ in HeLa cells. Interestingly, the gating function of R220X hemichannels was completely restored by both the intracellular and extracellular application of a peptide that mimics the Cx32 cytoplasmic loop. Molecular dynamics simulations suggest that the restoration effect mediated by the peptide in the mutant hemichannel involves the thermodynamic stabilization of the cytoplasmic loop that would otherwise abnormally fluctuate in the absence of the C-terminal domain. In accord with previous reports, patch clamp experiments with R220X hemichannels also displayed a reduced sensitivity to transmembrane voltage with respect to wild-type hemichannels. Our single channel recordings explained the defect in terms of a reduced number of subconductance states. Finally, experiments of intercellular diffusion mediated by wild-type or R220X gap junction channels displayed similar values of the unitary conductance of the fully open state as well as of the unitary permeabilities to cAMP and Lucifer yellow. Taken together, our findings supports the hypothesis that paracrine signalling alteration due to Cx32 hemichannel dysfunction underlies CMT1X pathogenesis and suggest a candidate molecule for treating the neuropathy caused by the R220X mutation.
CHARACTERIZATION OF ODDD MUTATIONS IN THE CaM BINDING DOMAIN OF THE
CX43CL

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Oculodentodigital dysplasia (ODDD) is an autosomal dominant human disease caused by
mutations in the gap junction protein Cx43. Defects associated with these Cx43 mutations include
altered expression and impaired trafficking as well as differences in plaque formation and
intercellular communication. Interestingly, about one-third of the >70 identified ODDD mutations are
localized in the Cx43CL domain. Here, we assessed the cellular localization, intercellular
communication, and structural consequences of a subset of ODDD mutations occurring in the
Cx43CL domain (M147T, R148Q, and T154A) that overlap with the CaM binding domain. All Cx43
ODDD mutants showed altered trafficking to the plasma membrane and were unable to transfer
Lucifer yellow. Previously, we identified that one Cx43CL region (residues D119-K144) has a
propensity to form an α-helix. However, analysis of the ODDD mutants identified a reduced ability
to form α-helical structure, suggesting these ODDD mutants are not folding correctly. Additional
studies have confirmed the Cx43CL residues involved in binding CaM and that CaM binding of the
Cx43CL is not static (i.e. slides along adjacent hydrophobic residues). Finally, we discovered in
vitro that CaM is also capable of binding the Cx43CT. Experiments are ongoing to verify if this
interaction occurs in cells.
REVERSAL OF ABNORMAL GAP JUNCTIONAL AND HEMICHANNEL ACTIVITY IN KERATINOCYTES HARBOURING HETEROZYGOUS GJB2 MUTATION BY siRNA – A POTENTIAL THERAPEUTIC INTERVENTION FOR KERATITIS-ICHTHYOSIS-DEAFNESS SYNDROME

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Dominant mutations in the gene GJB2 cause keratitis-ichthyosis-deafness (KID) syndrome. 87% of KID patients carry a p.D50N change in the GJB2 coding protein Cx26. Since the mutant allele has a dominant effect, we hypothesised that suppressing the mutant allele expression with mutation-targeted siRNA may alleviate the disease phenotype. A Cx26+/+-keratinocyte cell line was first generated from diploid human keratinocytes (Cx26+/+) using CRISPR/Cas9. Sanger sequencing of a single clone confirmed a heterozygous frameshift deletion, leading to truncated Cx26 expression in one of the two GJB2 copies. Functional assessment using the scrape loading dye transfer (SLDT) assay showed indistinguishable dye transfer levels between Cx26+/+ and Cx26+-keratinocytes (5.2±0.24 versus 5.45±0.66, p>0.05), indicating functional Cx26 in cells with Cx26+/-.

To test our hypothesis, we immortalised keratinocytes that were derived from a patient who harbours the heterozygous c.148G>A (p.D50N) mutation, to generate a KID keratinocyte line (KID-KC). SLDT dye transfer in the KID-KCs was almost 50% lower than control keratinocytes. Whole-cell patch clamp recordings showed altered hemichannel activity, with the maximum current density in KID-KCs being 80% greater than that in control keratinocytes (9.0±1.3 pA/pF versus 5.0±0.6 pA/pF, measured at +110mV, p<0.05). These functional studies indicate an abnormal Cx26 channel function in the KID-KC cells. Following administration of mutation-targeted siRNA, there was a 47% reduction of GJB2 mRNA expression in treated KID-KCs, whereas no change in GJB2 mRNA was detected in keratinocytes treated with control siRNA. Striking functional improvement was seen, with dye transfer improved by 39% in treated KID-KCs, and maximum membrane current density in KID-KCs corrected by 35% (5.9±0.4 pA/pF) compared to either untreated KID-KCs, or KID-KCs treated with a non-specific siRNA (9.0±1.2 pA/pF, p<0.05). The reversal of aberrant hemichannel activity and impaired gap-junctional intercellular communication in treated KID-KCs suggest that mutation-targeted siRNA is a potential therapeutic intervention for KID syndrome.
INHIBITING GAIN-OF-FUNCTION CONNEXIN HEMICHANNELS WITH HUMAN RECOMBINANT ANTIBODIES

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A combinatorial library of single-chain fragment variable (scFv) antibodies expressed in phage may equal the antibody repertoire in any individual. By screening one such library, we identified a human antibody that binds an extracellular domain of connexin 26. We tested the antibody in HeLa cells, human keratinocyte-derived cells and organotypic cultures of mouse cochleae, and determined that it inhibits wild type connexin hemichannels, as well as gain-of-function mutants implicated in autosomal dominant non-syndromic hearing impairment accompanied by keratitis and hystrix-like ichthyosis-deafness (KID/HID) syndrome. We solved the crystal structure of the antibody, identified residues that are critical for binding and uncovered its mechanism of action. Our study highlights the potential of the method and identifies connexins as therapeutic targets addressable by screening phage display libraries expressing human randomized antibodies.

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ROLE OF CX43 HEMICHannels IN VENTRICULAR ARRHYTHMIA ASSOCIATED WITH ABNORMAL Ca2+ HOMEOSTASIS

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Background: Cx43 hemichannels (HCs) are typically closed, but open in response to electrical or chemical triggers. Evidence for HC-opening by [Ca2+]i-elevation is based on indirect ATP/dye uptake measurements. Here, we provide conclusive evidence for [Ca2+]i-dependent activation of Cx43 HCs using simultaneous single-channel recordings and Ca2+-imaging. We further document their arrhythmogenic potential and the impact of selective Cx43 HC blockade on arrhythmogenic substrates associated with impaired Ca2+ homeostasis.

Methods and results: Left ventricular cardiomyocytes were acutely isolated from C57/B16 mice, inducible Cx43 knockdown mice and adult domestic pigs and voltage-clamped at resting Vm. We provoked Ca2+-release from the sarcoplasmic reticulum (SR) both by local application of caffeine (10 mM) and, spontaneously, by pacing and isoproterenol (1 µM for mouse, 10 nM in pig) superfusion which activated an inward current carried by the Na+/Ca2+-exchanger. Superimposed on this macroscopic current, there appeared microscopic currents with a unitary conductance of ~210 pS and maximum response at ~600 nM [Ca2+]i. Unitary currents were inhibited by Gap19 (selective Cx43 HC-blocker) and Cx43 knockdown. Conversely, CT9 (positive Cx43 HC-modulator) increased unitary currents. [Ca2+]i-involvement was confirmed by inhibition of unitary currents following prior depletion of the SR and intracellular application of EGTA (10 mM). Unitary currents were inhibited by W7 and Calmodulin (CaM) binding peptide, suggesting involvement of CaM-signaling. Interestingly, we observed a linear relationship between the number of Cx43 HCs and isoproterenol-induced arrhythmogenic Ca2+-release. Additionally, Cx43 HC-blockade and knockdown significantly reduced isoproterenol-induced arrhythmogenic Ca2+-release by preventing SR Ca2+-overload. Conversely, CT9 significantly increased arrhythmogenic Ca2+-release. Finally, Gap19 and Cx43 knockdown did not significantly influence global Ca2+ handling, suggesting that selective Cx43 HC blockade may reduce arrhythmogenic Ca2+-release without negative inotropy.

Conclusions: Cx43 HCs can be opened by [Ca2+]i at resting Vm through CaM-dependent signaling. Cx43 HCs may promote arrhythmogenic events by providing inward depolarizing currents and/or modulating Ca2+-homeostasis.
NaV1.5 TRAFFICKING IS REGULATED BY A NOVEL TUBULIN-LIKE DOMAIN IN THE C-TERMINUS OF CONNEXIN43

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Aims: We recently showed that the C-terminus (CT) of Cx43 participates in the organization of microtubules at the intercalated disc (ID) and thereby regulates the trafficking of the cardiac voltage-gated Na+ channel, NaV1.5. This explains how loss of Cx43 or deletion of its tubulin-binding domain reduce the sodium current (INa), whereas it is less clear why truncation of a PDZ-binding domain in the last five amino acids of the Cx43-CT (D378stop) has a similar effect. Therefore, the aim of this study was to identify the molecular mechanism by which the tubulin- and PDZ-binding domains regulate NaV1.5 localization and INa.

Methods & Results: INa was measured in HL-1 cells using whole-cell patch clamp and localization of EB1 (microtubule end marker) and NaV1.5 by super-resolution fluorescence microscopy. Microtubule end density at the cell membrane was reduced in Cx43-D378stop-expressing HL-1 cells, which was associated with smaller NaV1.5 clusters and reduced INa. Truncation of Cx43 at Q361 also reduced INa whereas the larger truncations Y301stop and K258stop were associated with normal INa. We therefore speculated that an element between Q361 and Y301 interfered with the tubulin-Cx43 interaction. The region A315-Q333 resembles beta-tubulin and deletion of this region in the D378stop mutant completely restored INa. Furthermore, adding the PDZ-binding domain to D378stop-expressing HL-1 cells as a separate peptide increased INa, whereas a scrambled peptide did not.

Conclusion: The Cx43-CT contains a tubulin-like domain located between amino acids 315-333, which may bind and block the tubulin-binding domain when the last five amino acids of the CT are lacking (D378stop). Similar events may occur when the PDZ-binding domain binds other partners (e.g. ZO-1), thereby regulating transport of NaV1.5 to the ID.

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**AGE-DEPENDENT DECREASE OF CX43 EXPRESSION IS IMPLICATED IN VENTRICULAR FIBROSIS IN Scn5a+/- MICE**

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Progressive Cardiac Conduction Disease (PCCD) alters cardiac conduction during ageing and has been associated with fibrosis. Genetic forms of PCCD have been linked to loss-of-function mutations of SCN5A gene product, the NaV1.5 cardiac voltage-gated Na+ channel. To understand the pathophysiology of PCCD, we studied a Scn5a heterozygous knockout (Scn5a+/-) mouse model, which exhibits deterioration in conduction and development of ventricular fibrosis between 45 and 60 weeks secondary to TGF-β pathway activation. Furthermore, an age-dependent decrease of connexin43 (Cx43) expression and phosphorylation appears between 30 and 60 weeks.

We hypothesized that the decrease of Cx43 expression induced by ageing associated with the decrease of NaV1.5 participates to the age-dependent fibrotic remodeling observed in Scn5a+/- mice.

To validate this hypothesis, Scn5a+/- mice have been treated with Gap-134 ([2S,4R]-1-[2-aminoacetyl]-4-benzamidopyrrolidine-2-carboxylic acid), a gap-junction activator. Gap-134 (5mg/kg/d, p.o.) was chronically administered to Scn5a+/- mice from the age of 45 weeks to the age of 60 weeks. Electrocardiography studies performed at 45 and 60 weeks showed that Gap-134 did not prevent the amplification of conduction defect. But after 15 weeks of treatment, Gap-134 increased the expression and phosphorylation of Cx43 on serine368. Furthermore, Gap-134 inhibited the occurrence of fibrosis, especially patchy fibrosis and collagen I production at the age of 60 weeks with no change of TGF-β pathway activation. The expression of matrix-degrading enzymes (MMPs) and their inhibitors (TIMPs) were unchanged. Moreover, it seems that Gap-134 increases NaV1.5 expression in insoluble fraction.

To conclude, our study demonstrates the causal role of ageing-induced decrease of Cx43 on fibrosis remodelling in Scn5a+/- mice.
SODIUM CHANNEL COMPLEXES LOCALIZED WITHIN THE CONNEXIN43 GAP JUNCTION PERINEXUS MEDIATE EPHAPTIC CONDUCTION IN THE HEART

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Cardiac impulse propagation is thought to occur by direct cell to cell flow of current via connexin43 (Cx43) gap junctions (GJs). We recently demonstrated that cardiac sodium channels (Nav1.5) localized within the perinexus, an intercalated disk (ID) nanodomain adjacent to Cx43 GJ, may enable ephaptic coupling between cardiac myocytes. We hypothesized that β1-mediated adhesion may closely approximate membranes within ID nanodomains, facilitating ephaptic coupling. Super-resolution STochastic Optical Reconstruction Microscopy-based Relative Localization Analysis (STORM-RLA) and immuno-electron microscopy identified two NaV1.5 populations within the ID in guinea pig ventricles (GPVs): A perinexal population, accounting for 47% of ID-localized NaV1.5 and a plicate population, co-distributing with N-Cadherin, accounting for 29%. β1 was preferentially localized to the perinexus (48% of ID-localized β1) over N-Cadherin-rich plicate regions (8%). βadp1, a novel peptide inhibitor of β1 adhesion, selectively and dose-dependently inhibited barrier function in β1-overexpressing 1610 cells in electric cell-substrate impedance spectroscopy studies. Neither βadp1 nor a scrambled control peptide (βadp1-scr) affected INa or action potentials in isolated GPV myocytes. However, βadp1 reduced peak current recorded from NaV1.5 clusters adjacent Cx43-EGFP at cell-cell contacts using scanning ion conductance microscopy-guided patch clamp. In GPVs, βadp1 (100 μM) compromised the diffusion-resistance of the ID as assessed by perfusion of fixable fluorescent dyes. βadp1 (48±4 μm) but not βadp1-scr (22±1 μm) increased perinexal intermembrane spacing compared to control GPVs (17±1μm). Optical mapping revealed that βadp1 but not βadp1-scr slowed conduction in GPVs and iPSC-derived cardiomyocyte monolayers. Importantly, in GPVs, βadp1 increased conduction anisotropy and precipitated spontaneous tachyarrhythmias in a dose-dependent manner. Thus, β1-mediated adhesion generates close apposition between NaV1.5-rich perinexal membranes next to GJs, facilitating ephaptic conduction in the heart. These data identify novel roles for Cx43, NaV1.5 and β1 in cardiac conduction and could prompt a significant revision in our understanding of the phenomenon.
ABSENCE OF N-GLYCOSYLATION AFFECTS PANX2 SUBCELLULAR LOCALIZATION AND ITS INTERACTION WITH THE MITOCHONDRIAL PROTEIN ATAD3A

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Pannexins (Panx1, Panx2 and Panx3) are channel-forming glycoproteins known to be conduits for ions and metabolites with a function in paracrine signaling. N-glycosylation is a common modification of pannexins and is known to modify the localization of Panx1 and Panx3, but the glycosylation site of Panx2 and its significance have not been determined. Based on the predicted N-linked glycosylation consensus site for Panx2, a glycosylation-deficient mutant (Panx2N86Q) was generated. Panx2 and Panx2N86Q constructs were ectopically expressed in human embryonic kidney (AD293) and normal rat kidney (NRK) cells. Substitution of Asparagine (N) 86 for a Glutamine residue (Q) generated a mutant protein with a faster-migrating electrophoretic band, insensitive to enzymatic de-glycosylation compared to Panx2. Immunolabeling and confocal microscopy showed intracellular localization of Panx2 and Panx2N86Q overlapping with the chaperone protein disulfide-isomerase (PDI) at the endoplasmic reticulum (ER). The glycosylation-deficient Panx2N86Q formed punctate aggregates at ER compartments, suggesting impaired protein folding. Cell surface biotinylation showed a decrease in Panx2N86Q detection in the NeutrAvidin bead pull-down compared to Panx2, yet low levels of both proteins were still able to traffic to the plasma membrane. Co-immunoprecipitation (co-IP) and MALDI-TOF-MS identified the mitochondrial membrane ATPase family AAA domain-containing protein 3A (ATAD3A) as a new interacting partner of Panx2. Panx2 and ATAD3A partially co-localize intracellularly, based on confocal microscopy of FLAG-tagged Panx2 and endogenous ATAD3A in AD293 cells. However, the aggregated profile of Panx2N86Q affects the co-localization of both proteins and presumably its function. ATAD3A is implicated in embryonic development, cholesterol transport, and in functional mitochondria-ER cross-talk. Our findings demonstrate that lack of glycosylation reduces cell-surface localization of Panx2, alters its proper folding and intracellular distribution. This is the first report of an interaction between Panx2 and a mitochondrial protein, suggesting novel biological functions that might take place within the mitochondria-ER interface through Panx2 channels.
ALTERED PROTEOMIC AND RESPIRATORY CHARACTERISTICS OF CX32KO MITOCHONDRIA

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Converging evidence points to a role for gap junction proteins in mitochondrial membranes. Indeed, Cx43 is present in cardiac subsarcolemmal mitochondria, and is involved in stress preconditioning and maintenance of normal respiratory function at inner mitochondrial membrane (IMMs). We have previously reported that Cx32 is also present in hepatic mitochondrial IMMs, and interacts with mitochondrial proteins at adhesive plasma membrane -mitochondrial contacts, or within mitochondrial organelles themselves. These data led us to profile global proteomic alterations in membranes from Cx32KO mice using label-free quantitation. Of the 1002 membrane proteins identified, 30 proteins (3%) were significantly upregulated, while 7 proteins (0.7%) were significantly downregulated in Cx32KO tissue compared to WT. Interestingly, half of the significantly upregulated proteins were mitochondrial, and REACTOME pathway analysis indicated that the TCA cycle and respiratory electron transport was the most likely pathway ascribed to the set of upregulated proteins. Following this observation, we assessed the bioenergetics of isolated hepatic mitochondria using high-throughput Seahorse XF Analyzer technology. We identified that Cx32KO mitochondria consume significantly more oxygen during state 2 (basal), state 4o (leak), and state 3µ (uncoupled maximal) respiratory conditions. Cx32KO mitochondria were also more polarized (possessed higher membrane potentials) under conditions of substrate starvation and substrate excess, and produced more reactive oxygen species than WT mitochondria. These findings provide evidence that Cx32 intrinsically restricts rates of mitochondrial oxidative phosphorylation, and may help explain why loss of Cx32 predisposes transgenic mice to tumor generation, or why humans carrying mutated channel-null versions of Cx32 are subject to central myelin abnormalities and peripheral neuropathy. Overall, these data provide the first direct evidence of Cx32 involvement in mitochondrial function, and represent exciting avenues of future investigation into the many roles of gap junctional signaling in cell growth, apoptosis, and tumorigenesis.
STRUCTURAL AND FUNCTIONAL PLASTICITY OF CX36 GAP JUNCTIONS DEPENDENT ON THE ACTIN CYTOSKELETON

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Electrical synapses made of Connexin36 (Cx36) are capable of striking functional plasticity that depends on dynamic phosphorylation and dephosphorylation of the connexin. This signaling drives more than an order of magnitude change in functional coupling of neural networks in minutes without changes in Cx36 expression. We now show that Cx36 gap junctions are integrally connected to the actin cytoskeleton and also show structural plasticity on a minutes time scale. Using Cx36 with an internal HaloTag and covalent labeling with fluorescent ligands, live imaging with Bessel Beam Plane Illumination Microscopy revealed that large Cx36 gap junctions extend dynamic finger-like projections, which we termed filadendrites. These filadendrites quickly changed length, broke apart, fused with each other, or were resorbed by the gap junction. Small gap junctions similarly moved, fused, and split apart in minutes. In fixed cell imaging it was evident that filadendrites were always associated with thin actin filaments. Structural stability of gap junctions depended partially on actin filaments, but the presence of filadendrites also depended on active elongation of actin filaments. Furthermore, the integrity of functional plasticity driven by the protein kinase A-protein phosphatase 2A (PP2A) signaling pathway depended on the elongation of actin filaments, and was disrupted by a Cx36 mutation that prevents binding of scaffolds to the tip of the Cx36 C-terminus. This disruption could be replicated by pharmacological inhibition of PP2A, suggesting that altered regulation results from disruption of PP2A anchoring in the connexin complex and localized activity. We conclude that the actin cytoskeleton plays a critical role in both structural and functional plasticity of Cx36 gap junctions. Actively remodeling actin drives structural plasticity and provides the link between protein phosphatase 2A and the connexin to maintain normal functional plasticity.
A PROTEIN NETWORK ASSOCIATING CONNEXINS TO THE CYTOSKELETON

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Mutations in the GJB2 gene in homozygosity or compound heterozygosity in GJB2 and GJB6 are the most common inherited causes of non-syndromic hearing loss. GJB2 encodes connexin (CX) 26 (CX26), which is expressed in the cochlea, liver, and embryonic brain. An affinity capture assay searching for CX26 cytoplasmic C-terminus binding partners in the mouse liver and brain identified 23 proteins by mass spectrometry analysis. We show that, as previously reported for other CX family members, CX26 distributes to molecular complexes containing tight junction protein 1 (TJP1), vinculin, arginine succinate synthase (ASS1) and microtubule-associated protein RP/EB (MAPRE2). These and other eight proteins are contained in a single protein-protein interaction (PPI) network further corroborating they are part of the same molecular complex, including the adaptor protein cingulin. Cingulin immunoprecipitation from mouse liver confirmed its association with CX26 and other proteins formerly captured in the complex, comprising TJP1, ASS1 and MAPRE2. Moreover, CX30, CX31 and CX43, respectively encoded by Gjb6, Gjb3 and Gja1 genes, co-immunoprecipitate with cingulin, indicating they share with CX26 a motif that mediates binding to cingulin or to a putative direct ligand. CX26 C-terminus shares limited similarity with group-B connexins, but not with other groups. Conversely, we show that CX from most groups display enrichment in basic residues in their initial C-terminal segment. We ruled out a direct interaction between CX26 and TJP1, vinculin, ASS1 or MAPRE2 by the yeast two-hybrid trap. This assay has been employed to clarify if a direct association takes place between cingulin and connexins. In conclusion, we provide evidence to further understanding the cytoskeleton PPI network in which hub proteins, associated with actin, microtubule filaments, tight- and adhering junctions, connect with connexins in gap junctions.
EARLY ADENOVIRAL PROTEINS TARGET INTERCELLULAR COMMUNICATION TO FACILITATE VIRAL REPLICATION

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Adenoviruses are causative agents in several pathologies including acute respiratory disease, gastroenteritis, conjunctivitis, and myocarditis. During adenoviral infection, viral proteins uncouple the cell cycle, initiating S-phase for viral genome replication to occur. Gap junction intercellular communication (GJIC) and the connexin43 (Cx43) protein are known to regulate cell cycle progression. Mechanisms of connexin-mediated cell cycle regulation remain elusive however and the relationship between adenoviral infection and connexin protein regulation is largely unexplored. Given that Cx43 (gene name GJA1) alters cell cycle progression, and that adenovirus uncouples cell cycle regulation, we hypothesized that adenovirus would target Cx43 to facilitate viral replication. In primary and immortalized human cells we find reduced GJA1 expression at the mRNA and protein level within 24 hours of infection with adenovirus type 5 (Ad5). To understand the mechanism of Cx43 targeting by Ad5, we investigated early adenoviral proteins which activate growth factor signaling in the cell, and have identified E4-ORF1 to be sufficient in suppression of Cx43 expression and gap junction formation. Manipulation of Cx43 protein expression by transient overexpression or siRNA-mediated knockdown reveals that Cx43 negatively regulates viral genome replication and synthesis of late viral proteins. To test if GJIC is necessary to inhibit viral replication or if Cx43 protein expression alone is sufficient, we employed known trafficking- and channel-dead Cx43 point mutations. We find that these dominant-negative Cx43 mutants rescue viral genome levels, confirming functional Cx43 channels are required to inhibit adenoviral replication. These findings provide novel insight into disruption of intercellular junctions during adenoviral infection to promote replication. It is our hope that revealing viral mechanisms of gap junction dissolution will contribute not just to the development of novel antiviral therapeutics, but also provide insight into fundamental Cx43 biology and contribute to better understanding the role of Cx43 in cell cycle regulation and cancer progression.
HUMAN PAPILLOMAVIRUS TYPE 16 (HPV16) E6 ONCOPROTEIN CONTROLS THE GAP JUNCTION PROTEIN CX43 IN CERVICAL TUMOUR CELLS

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Human papillomavirus type 16 (HPV16) E6 oncoprotein binds the cell polarity regulator hDlg (human homologue of Drosophila Discs Large) through its C-terminal binding motif X-T-X-V. Previously we showed in vitro, and in vivo, that hDlg binds Connexin 43 (Cx43). Like the Cx43 trafficking regulator, ZO-1, hDlg is a PDZ domain-containing scaffolding protein. However, whereas the Cx43 C-terminal domain binds the second of three PDZ domains in ZO-1, it interacts with the N- and C-termini of hDlg. We have demonstrated that hDlg maintains a cytoplasmic pool of Cx43 that is capable of trafficking to the plasma membrane in HPV16-positive cervical tumour cells.

To investigate a role for HPV E6 and hDlg in Cx43 regulation we examined location of the three proteins in HPV16-positive non-tumour cervical epithelial cells (W12NT: low E6 levels, good gap junctional communication) and in HPV16-positive tumour cells (W12T: high E6 levels, poor gap junctional communication). In W12NT cells Cx43 localised to the plasma membrane, hDlg was located adjacent to the membrane and E6 was found in the cell nucleus. In contrast, in W12T cells Cx43 was found co-located with hDlg and E6 in the cytoplasm. Moreover, using co-immunoprecipitation, E6 could be detected in complex with Cx43 and hDlg in W12T cells. siRNA depletion of E6 resulted in relocation of Cx43 to the cell membrane suggesting that E6 can control Cx43 trafficking. C33a cervical tumour cells are HPV-negative and display membrane Cx43-positive gap junction plaques. Under conditions of stable expression of HPV16 or 18 E6 in C33a cells, Cx43 was located primarily in the cytoplasm. Conversely, mutation of the E6 C-terminal hDlg-binding motif resulted in appearance of Cx43 on the plasma membrane. Therefore, E6 alters Cx43 trafficking and recycling to the membrane. This suggests a novel E6/hDlg-associated mechanism for changes in Cx43-mediated cell-cell communication in HPV-positive cervical tumour cells.
CONNEXIN43 HEMICHAEL-MEDIATED ATP RELEASE AMPLIFIES THE INFLAMMASOME PATHWAY IN AN AUTOCRINE MANNER

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Background: Connexin43 hemichannels have been implicated in many inflammatory diseases including retinal ischaemia and diabetic retinopathy in the eye. However, exact molecular mechanisms remain to be fully elucidated. Using an in vitro model, we have evaluated the role of connexin43 hemichannels in diabetic retinopathy and propose a mechanism by which connexin43 hemichannel opening perpetuates inflammatory disease.

Methods: Retinal pigmented epithelial (ARPE-19) cells were exposed to high glucose (HG), 10 ng/mL pro-inflammatory cytokines IL-1β and TNF-α, or a combination of both. Quantitative Cytometric Bead Array (CBA) analysis was carried out to measure the release of inflammatory cytokines, IL-6, IL-8, MCP-1 and sICAM-1. Immunohistochemistry was utilized to detect connexin43 expression and localization at gap junctions. Relative ATP release levels under different treatment conditions were also measured. To examine the role of connexin43 hemichannels in the disease process, changes in cytokine, ATP release, and gap junction localization, were evaluated following treatment with Peptide5, a blocker of connexin43 hemichannels.

Results: Co-application of both HG and pro-inflammatory cytokines increased the secretion of IL-6, IL-8, MCP-1 and sICAM-1 to higher levels compared to the cytokines alone. Connexin43 protein localization at gap junctions, seen in basal conditions, was disrupted in cells exposed to HG and cytokines. Peptide5 prevented the increase in IL-6, IL-8, MCP-1, and sICAM-1 release, and restored gap junction localization of connexin43. Furthermore, Peptide5 prevented the increase in ATP release following co-application of HG and cytokines. However, adding exogenous ATP into the culture medium negated peptide5-mediated protection against inflammatory cytokine release in injury conditions.

Conclusions: Our findings show that connexin43 hemichannels play an important role in cell damage processes by regulating an ATP autocrine feedback loop in the inflammasome/inflammation cycle. Targeting connexin43 hemichannels thus offers a potential therapeutic strategy to break the self-perpetuating inflammasome cycle in chronic diseases such as diabetic retinopathy.
REDUCING NETosis BY TARGETING PANNEXIN1 CHANNELS

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Neutrophil chemotaxis involves the autocrine purinergic activation of surface receptors and release of ATP by Pannexin1 (Panx1) channels. Neutrophils are the first responders in inflammation resulting from tissue injury or infection. One of the defense mechanisms employed is the Neutrophil Extracellular Trap formation (NETosis), which leads to the expulsion of nuclear contents into the extracellular environment as a trap for pathogens and foreign particles. Prolonged recruitment of neutrophils and delayed clearance of NETosis leads to tissue damage, which makes this process deleterious in inflammatory and autoimmune diseases. Here, we examined the contribution of Panx1 channels to NETosis. Bone marrow-derived neutrophils (BMDNs) were obtained from wild type (WT) and Panx1−/−-mice. Panx1 expression was analysed by RT-qPCR and immunofluorescence, which revealed significant and specific expression in these cells. In addition, Panx1 channel function in neutrophils was confirmed using YO-PRO-1 dye uptake assay. As NETosis is a dynamic process, we developed a fluorescent assay to measure extracellular DNA release over time using Sytox green. NETs were induced with calcium ionophore A23187 (1µM), phorbol 12-myristate 13-acetate (10nM) or Staphylococcus aureus strains (MOI 10), to evaluate the various mechanisms of activating NETosis. NETs' induction was fit by a sigmoid relationship with a reduced slope in Panx1−/−-BMDNs as compared to WT BMDNs (49.34 AU ± 10.07, 80.36 AU ± 8.765 respectively, p<0.05, Student t test). Thus, Panx1 is expressed in mouse BMDNs and blocking Panx1 channel function decreases NETosis. Ongoing studies should provide detailed insight into the specific role of Panx1 channels in the signaling pathway leading to NETosis. Understanding Panx1 channel function might point to a new therapeutic target for inflammatory diseases in which NETosis contributes to the pathophysiology.
The concerted action of calcium, calmodulin (CaM), and calmodulin kinase II (CaMKII) with the NMDA receptor is at the core of synaptic plasticity, learning and memory formation mediated by chemical synapses. Previously, our research has shown that connexin 36 (Cx36), the major electrical synaptic protein in the central nervous system, interacts with CaMKII in a way resembling binding to the NR2B subunit of the NMDA receptor. Recently, we have demonstrated that the direct interaction between CaM and Cx36 serves as a critical priming step before membrane insertion. Both CaM and CaMKII interact with Cx36 in a Ca2+-dependent manner at gap junction plaques (GJP) in the mouse neuroblastoma cell line Neuro2a, and they compete for an overlapping binding region in the carboxyl-terminal domain of Cx36. Here, we advance previous findings presenting live cell imaging techniques including Fluorescence Resonance Energy Transfer (FRET) and dynamic Ca2+ imaging showing the temporal and spatial dimensions of interactions between Cx36 and CaMKII at single cell resolution. Mutant CaM, CaMKII and Cx36 proteins were used to determine protein interactions, together with pharmacological interventions modeling neuronal activation or inhibition. The inhibition of AMPA, NMDA receptors and Src kinases with antagonists such as DNQX, MK801, kynurenic acid, or PP2 altered CaMKII interaction at the GJP providing evidence that activities of electrical and chemical synapses are linked and that calcium signaling and Src kinases play a role in this process. Our investigations support a modern concept in which electrical and chemical synapses within mixed synapses are functionally interdependent units sharing a core molecular machinery.
ASTROGLIAL AND OLIGODENDROGLIAL CONNEXINS DIFFERENTIALLY INFLUENCE BOTH ACUTE AND CHRONIC PROGRESSIVE EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

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Objective: We previously reported that astroglial connexin (Cx)43 and oligodendroglial Cx32 and Cx47 were frequently lost in acute and chronic lesions of multiple sclerosis (MS) and neuromyelitis optica spectrum disorder (NMOSD) and that loss of glial Cxs was associated with more grave clinical course and earlier death in these conditions. However, the roles of glial Cxs in these demyelinating diseases remain to be established. In the present study, we aimed to elucidate glial Cxs’ roles in demyelination using experimental autoimmune encephalomyelitis (EAE), an animal model of MS, in Cxs-deficient mice.

Methods: Tamoxifen was administrated into Plp1-CreERT;Cx47 fl/fl mice and Glast-CreERT2;Cx43fl/fl mice to allow tamoxifen-inducible oligodendroglia-specific Cx47 knock-out (Cx47 iKO) mice and tamoxifen-inducible gray matter astroglia-specific Cx43 knock-out (GM Cx43 iKO) mice, respectively. Cx30 knock-out (Cx30 KO) mice, Cx47 iKO mice and GM Cx43 iKO mice were used together with their control littermates for induction of EAE by myelin oligodendrocyte glycoprotein (MOG) peptide 35-55.

Results: EAE was markedly attenuated in the chronic but not acute phase of EAE in Cx30 KO mice, which showed less demyelination and diffuse activation of microglia. These microglia had down-modulation of Toll-like receptor 4 and an M2 phenotype by microarray analysis and immunohistochemistry. In GM Cx43 iKO mice, acute and chronic EAE were significantly ameliorated with less inflammatory cell infiltration and less demyelination. By contrast, Cx47 iKO mice showed more severe EAE at the acute as well as chronic phases.

Interpretation: Absence of astroglial Cx30 and Cx43 ameliorate EAE while absence of oligodendroglial Cx47 exacerbates EAE, suggesting that astrogial and oligodendroglial Cxs differentially influence EAE severity.
A CONNEXIN43 MIMETIC PEPTIDE THAT INHIBITS c-Src EXERTS NEUROPROTECTIVE EFFECTS THROUGH THE INHIBITION OF GLIAL HEMICHANNEL ACTIVITY

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c-Src is an important mediator in several signalling pathways related to neuroinflammation. Our previous study showed that cortical injection of kainic acid (KA) promoted a transient activation of c-Src in reactive astrocytes surrounding the neuronal lesion. As a connexin43 (Cx43) mimetic and cell-penetrating peptide, TAT-Cx43266-283, inhibits Src activity, we investigated its effect on neuronal death promoted by cortical KA injections in adult mice. KA promoted neuronal death and reactive gliosis. Interestingly, TAT-Cx43266-283 injected with KA diminished neuronal death and reactive gliosis compared to KA or KA + TAT injections. In order to get insight into the neuroprotective mechanism, we used in vitro models. In cultured neurons, TAT-Cx43266-283 did not prevent neuronal death promoted by KA, while when neurons were grown on top of astrocytes, TAT-Cx43266-283 prevented neuronal death. These observations demonstrate the participation of astrocytes in the neuroprotective effect. Furthermore, the neuroprotective effect was also present in non-contact co-cultures, suggesting the participation of soluble factors released by astrocytes. As glial hemichannel activity is associated with the release of several factors, such as ATP and glutamate, that causes neuronal death, we explored the participation of these channels. Our results confirmed that inhibitors of ATP and NMDA receptors prevented neuronal death in co-cultures treated with KA, showing the participation of astroglial hemichannels in neurotoxicity. Furthermore, TAT-Cx43266-283 reduced hemichannel activity, assessed by ethidium bromide uptake, promoted by KA in neuron-astrocyte co-cultures. In fact, TAT-Cx43266-283 and dasatinib, a specific c-Src inhibitor, strongly reduced the activation of astroglial hemichannel promoted by LPS in astrocyte cultures. In conclusion, our results suggest that TAT-Cx43266-283 exerts a neuroprotective effect through the reduction of hemichannel activity probably mediated by c-Src in astrocytes. These results unveil a new role of c-Src in the regulation of Cx43-hemichannel activity that could be part of the mechanism by which c-Src participates in neuroinflammation.
TARGETING MAPK PHOSPHORYLATION SITES OF THE CONNEXIN43 C-TERMINAL TAIL PROVIDES NEUROPROTECTION IN STROKE

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Astrocytic connexin43 (Cx43) gap junction (GJ) and hemichannel activity affect neuronal survival in ischemic conditions. Cx43 activity is influenced by intracellular kinases that phosphorylate specific sites located on its C-terminus. Given that ischemia is a powerful inducer of kinase activity, their effect on Cx43 function could be critical in stroke. Therefore, using wild-type (WT) and Cx43 null phosphorylation mutant mice that include serine to alanine mutations in the protein kinase C (PKC) site Cx43S368A, the casein kinase 1 (CK1) site Cx43S325A/328Y/330A, and the mitogen-activated protein kinase (MAPK) site Cx43S255/262/279/282A we investigated the impact of these Cx43 mutants in a permanent middle cerebral artery occlusion (pMCAO) model of stroke. No significant differences in infarct volume were measured in the PKC Cx43S368A or CK1 Cx43S325A/328Y/330A transgenic mice, compared with WT controls. However, a significant decrease in infarct volume and apoptosis was measured in the MAPK Cx43S255/262/279/282A (MK4) transgenic animals following stroke. This was also correlated with significant improvement in behavioural performance. In the penumbra, an increase in astrocyte reactivity along with a concomitant decrease in microglial reactivity was observed in the MK4 mice. In contrast to WT astrocytes, MK4 astrocytes displayed a reduction in Cx43 hemichannel activity in both dye uptake assay and single channel recordings. This decrease in activity is associated with the neuroprotective phenotype of MK4 animals. This was confirmed by injecting ischemic mice with TAT-Gap19, a nonapeptide derived from the cytoplasmic loop of Cx43 and previously shown to inhibit Cx43 hemichannel activity without affecting GJ coupling. Collectively, this study provides new molecular insight and potential new avenues for therapeutic intervention associated with Cx43 channel function in stroke. Funded by a Canada Research Chair (CN), a Heart & Stroke Foundation Fellowship (MFA), NIH GM-55632 (PL), Fund for Scientific Research Flanders, Belgium (LL).
Cardiovascular diseases are the number one cause of premature death worldwide. The major underlying cause, atherosclerosis, results in a blockage of the blood vessel through the formation of plaques for which the most common treatment is percutaneous coronary intervention (PCI) with drug-eluting stent (DES) placement. However, surgical intervention can damage blood vessel walls leading to vascular smooth muscle cell (SMC) migration and proliferation, and re-occlusion of the vessel termed neointimal hyperplasia. Complications following surgery include thrombosis due to reduced endothelial cell proliferation, related to off-target effects of DES. Thus, we aimed to understand how SMCs proliferate in disease and identify potential therapeutic targets. Our previous studies in mice have shown that the protein connexin 43 (Cx43) plays a critical role in SMC proliferation through control of the cell cycle protein, cyclin E. Here we aimed to define whether these processes were analogous in human cells in vitro. Testing interactions between Cx43 and cyclin E in human coronary artery smooth muscle cells in vitro and ex vivo on sections of tissues from CABG surgeries. To determine the impact of Cx43-cyclin E interaction in vitro, we tested proliferation in response to platelet derived growth factor-BB (PDGF) and tested the effects of novel Cx43-cyclin E disruptor-peptides developed in our laboratory. Our results demonstrate for the first time that Cx43 and cyclin E directly interact in human arterial SMC and diseased vascular tissues. In addition, targeted disruption of Cx43-cyclin E interaction in human SMC reduces cell proliferation in response to PDGF. Taken together our findings highlight a novel pathway for regulation of neointimal hyperplasia in humans and highlights key targets for future therapeutic development.
PIVOTAL ROLES OF THIOREDOXIN-INTERACTING PROTEIN IN GAP JUNCTION-MEDIATED REGULATION OF CELLULAR STRESS RESPONSES

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Gap junctions (GJs) regulate many cell functions, including cell growth, differentiation, survival, and metabolism. Currently, the molecular mechanisms involved are still poorly understood. Given that thioredoxin-interacting protein (TXNIP), an endogenous inhibitor of thioredoxin, regulates a broad range of cellular processes, we tested a possible involvement of TXNIP and explored the underlying signaling mechanisms. (1) Disruption of GJs in NRK cells (a renal epithelial cell line) with several chemical GJ inhibitors or connexin43 (Cx43) siRNA caused a rapid and potent suppression of TXNIP expression, which was preceded by an activation of extracellular signal-regulated kinase (ERK). Inhibition of ERK or its upstream kinase MEK with specific inhibitors prevented, whereas activation of ERK with mitogens or phosphatase inhibitors reproduced the suppressive effects of GJs, indicative of a mediating role of ERK in the observed effects of GJs. (2) Dysfunction of GJs caused an accelerated degradation of TXNIP, which was associated with an increased TXNIP phosphorylation and ubiquitination. Blockade of proteasome protein degradation pathway with MG132 largely blunted the inhibitory effect of GJs on TXNIP. On the contrary, inhibition of ERK delayed TXNIP degradation. (3) Inhibition of GJs elevated GLUT1 and enhanced cell resistance to cell apoptosis induced by oxidative and ER stress inducers. This effect was reproduced by treatment of cells with TXNIP siRNA. Further analysis revealed that inhibition of GJs or downregulation of TXNIP led to an increased activation of AKT, a kinase regulating many cellular functions, including metabolism, growth, and survival. Collectively, our study thus characterizes ERK-mediated suppression of TXNIP as a presently unreported mechanism by which GJs regulate cell behaviors. Cx43/ERK/TXNIP/Akt signaling pathway could be critically involved in the control of responses to multiple stimuli.
CONNEXIN-43 (CX43) REGULATES MITOCHONDRIAL DYNAMICS THROUGH MACROSCOPIC AUTOPHAGY IN DIVIDING HEMATOPOIETIC STEM CELLS

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Understanding of the mechanisms controlling hematopoiesis regeneration upon stress is expected to provide molecular targets for intervention in highly toxic regimens of curative chemotherapy which nowadays frequently result in lethal blood cytopenias. In bone marrow (BM), Cx43 is required for prevention of senescence and apoptosis of hematopoietic stem cells (HSC) and efficient blood formation. HSC but not more mature progenitors express Cx43. Cx43 has been shown to be a negative regulator of autophagy in HeLa cells. Stressed hematopoietic response requires synchronized mitochondrial turnover upon cell division, which in the end depends on mitochondrial fission and autophagy (mitophagy). We hypothesized that Cx43 controls mitochondrial turnover upon forced cell division. To analyze the role of Cx43 in HSC mitochondrial dynamics, we created HSC mitochondrial reporter mice by crossing COX8 mitochondrial signal localization peptide-Dendra2 fusion protein transgenic (mito-Dendra2) mice with Vav-Cre; Cx43\textsuperscript{f/f} mice. We analyzed HSC mitochondrial burden and turnover by ex vivo tracking of photo-convertable mito-Dendra2 by time-lapsed cinematography. In dividing HSC but not in quiescent HSC, mitochondrial length is increased. Dividing Cx43\textsuperscript{KO} HSC contain \textasciitilde 2-fold more large mitochondria and increased co-localization of mitochondria with autophagosome LC3 or autophago-lysosome Lamp1/2, compared to their WT counterparts. Pharmacological inhibition of mitochondrial fission using the Drp1 inhibitor mDivi1 or early autophagy process by bafilomycin A1 on Cx43\textsuperscript{KO} HSC resulted in decreased mitochondrial co-localization with LC3 or Lamp1/2. In dividing Cx43\textsuperscript{KO} HSC, but not in quiescent HSC, inhibition of autophagy but not of mitochondrial fission reversed the effect of Cx43 deficiency on mitochondrial size. Activation of autophagy in WT HSC by the mTOR inhibitor rapamycin however does not fully recapitulate the effect of Cx43 deficiency on mitochondrial dynamics. In summary, Cx43 controls dividing HSC mitochondrial size and turnover through its negative regulatory role on autophagy, and is a key regulator of HSC fate.
DYNAMIC REGULATION OF CX43 AND PANX1 IN HUMAN INDUCED PLURIPOTENT STEM CELL DIFFERENTIATION

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Stem cell reprogramming, self-renewal, maintenance of pluripotency, and differentiation are all thought to be supported by gap junctional intercellular communication (GJIC). We recently reported the first example of the generation of induced pluripotent stem cells (iPSCs) from a patient with a connexin-linked development disorder known as oculodentodigital dysplasia (ODDD). ODDD is linked to primarily autosomal dominant mutations in the GJA1 (Cx43) gene and is characterised by craniofacial malformations involving both bone and cartilage, ophthalmic deficits, enamel hypoplasia and syndactyly. ODDD iPSCs harboring a Cx43 p.V216L mutation exhibit reduced Cx43 mRNA and protein abundance and were less coupled when compared to familial control iPSCs. Osteogenic differentiation involved an early, and dramatic downregulation of Cx43 followed by a slight upregulation during the final stages of differentiation. Interestingly, osteoblast differentiation was delayed in ODDD iPSCs while chondrogenic pellet morphology was altered compared to control iPSCs. Our studies highlight the importance of Cx43 expression and function during osteoblast and chondrocyte differentiation, and establish a potential mechanism for how ODDD-associated Cx43 mutations may have altered cell lineages involved in bone and cartilage development. Given that we have the ability to model cell differentiation along many cell lineages in controlled environments, we are extending our iPSC studies to determine if pannexins are also selectively regulated during cell fate decisions. To that end, early studies have revealed that iPSCs express significantly more Panx1 than the fibroblasts from which they were derived and Panx1 continues to be upregulated during osteogenic differentiation. In summation, our studies have revealed a critical role for Cx43 in guiding cell fate decisions and further raise the possibility that Panx1 may also be of importance in stem cell reprogramming and cell fate decisions. Supported by the CIHR.
EPIGENETICS, TRAFFICKING AND EXOCRINE SIGNALS REGULATE CX43 FUNCTION IN ENDOMETRIOSIS AND ENDOMETRIAL CANCER - GAP JUNCTIONS ARE REQUIRED FOR INVASIVENESS IN ENDOMETRIOSIS

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Intercellular interactions, including gap junction intercellular coupling (GJIC), have been implicated in many invasive processes (e.g. extravasation, metastasis and oocyte implantation). Yet the role of gap junctions in endometriosis, a major invasive medical problem, has not been investigated. Comparisons of the expression profile of endometrial cells (ECs) from endometriosis patients and normal subjects revealed a consistent up-regulation of Cx43 expression. In contrast, GJIC of ECs from endometriosis patients was consistently reduced compared to normal subjects, primarily due to intracellular accumulation of Cx43. However, heterotypic interactions with mesothelial cells induced an increase in GJIC, and enhanced trafficking of Cx43 to the cell surface. Blocking GJIC between endometrial and mesothelial cells dramatically reduced the enhanced invasiveness of endometriosis derived ECs. Finally, GJIC induction by mesothelial cells was mediated by a secreted factor, suggesting a promising strategy for future non-hormonal endometriosis therapies.

We were interested if these transient changes in GJIC associated with endometriosis may have more permanent manifestations in invasive endometrial cancers. A comparison of the methylation patterns in ECs for normal and cancer patients revealed an increase in transcriptional start-site methylation of the GJA1 and GJC2 genes, and even more notable hyper-methylation of CpG islands in known modulators of Cx43 (PKCβ, ERK1 and ZO2). This correlated with markedly reduced GJIC in endometrial cancer cell lines, which in the case of Ishikawa and HEC-1A cells, was significantly enhanced by treatment with de-methylating agents, associated with increased trafficking of Cx43 to the cell surface. This was blocked by PKC inhibition, suggesting a primary effect of hyper-methylation was on the genes regulating Cx43 trafficking. Consistent with the association of obesity with increased incidence of endometrial cancer, similar changes in expression and gene methylation profiles were seen in ECs co-cultured with adipocytes, a PTEN+/-obesity mouse model, and a cohort of young obese patients.
Osteocytes communicate among themselves and with other cells by releasing factors able to regulate bone formation and resorption. One of such factors is ATP, which can be released through pannexin1 (Panx1) channels in conditions of caspase3-mediated apoptosis, as with aging. We examined Panx1 role in adult and aging bone using genetic and pharmacologic tools. First, Panx1fl/fl mice were crossed with DMP1-8kb-Cre mice to generate Panx1ΔOt mice. Bone mineral density became 3.5-7% higher in Panx1ΔOt mice (n=10) at all sites at 2-3 months of age, compared to Panx1f/f littermates (n=9). Vertebra µCT analysis showed 6-9% increase in cancellous bone parameters in 4-month-old Panx1Δot mice. Bone formation also increased by 24%, whereas osteoclast number/activity were unchanged in cancellous bone. Femoral cortical thickness was 3.5% higher, and osteoclastic parameters were 27-30% reduced on the endocortical surface, likely due to lack of osteoclastogenic signals from Panx1-deficient osteocytes; whereas bone formation was unchanged. Thus, osteocytic Panx1 deletion increases bone mass by different cellular mechanisms in cancellous versus cortical bone. Next, Panx1 activity was pharmacologically inhibited using mefloquine (5mg/kg/d, 14d) in 21-month-old (old) and 3.5-month-old (young) mice. Old mice exhibited low spinal bone volume, and femoral bone area, measured by µCT, compared to young mice, and mefloquine increased these parameters only in old mice. Old mice exhibited 40% more active caspase3-positive osteocytes than young mice and elevated apoptosis-related gene expressions, and mefloquine did not alter these values. Serum ATP was increased in old mice, and was reduced by mefloquine. Old mice also exhibited more osteoclasts on femoral endosteal surface and mefloquine, as Panx1 deletion, reduced osteoclasts in young and old mice. In summary, osteocytic Panx1 deletion increases bone mass by different cellular mechanisms in cancellous versus cortical bone; and induces osteoclastogenesis by mechanisms independent and dependent of apoptosis-induced ATP release in young versus old mice, respectively.
MAINTAINING CONNEXIN-36 FUNCTION IN DIABETOGENIC ENVIRONMENT PRECLUDES ISLET DYSFUNCTION AND BETA-CELL DEATH

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Type2 diabetes (T2D) is caused by insufficient secretion of insulin from pancreatic islets upon insulin resistance. Connexin-36 gap junctions (Cx36) maintain coordinated islet electrical activity, pulsatile insulin secretion, and can protect against beta-cell death. Cx36 function is disrupted in conditions associated with the progression of T2D. We tested whether islet dysfunction in T2D could be ameliorated by preventing Cx36 dysfunction.

We synthesized mimetic peptides towards specific Cx36 residues hypothesized to be important in channel function. This was combined with beta-cell specific Cx36 over-expression (Rip-Cx36) or pharmacological activation (modafinil). These were applied in mouse and human islets exposed to pro-inflammatory cytokines or palmitate, as well as islets from db/db mouse model of T2D and human T2D donors. We used fluorescence recovery after photobleaching, calcium imaging and measured glucose-stimulated insulin secretion and cell viability.

Treatment of mouse and human islets with a pro-inflammatory cytokine cocktail or palmitate significantly reduced Cx36 function, which was restored to control levels when incubated with mimetic peptide targeted towards Cx36 serine-293. Ca2+ oscillations were similarly disrupted under each condition and restored by serine-293 mimetic peptide. Glucose-stimulated insulin secretion was disrupted (p=0.013) and cell death increased (p<0.001) by the pro-inflammatory cytokine cocktail, each of which was partially mitigated by the mimetic peptide (secretion p=0.083, viability p=0.031 vs. non-peptide inclusion). Proinflammatory cytokine-induced declines in Cx36 function and insulin secretion were also partially mitigated in islets from Rip-Cx36 mice and by modafinil treatment. Islets from db/db mice showed significantly decreased Cx36 function and insulin secretion compared to non-diabetic controls; which were restored to control levels by the mimetic peptide. Finally the mimetic peptide and modafinil increased Cx36 function and insulin secretion in islets from T2D donors.

These results indicate Cx36 dysregulation plays an integral role in islet dysfunction during T2D, and preventing Cx36 dysregulation may be therapeutically beneficial.
TARGETING CONNEXIN43 TO PREVENT GLIOBLASTOMA PROGRESSION AND RECURRENCE

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Glioblastoma (GBM) is a highly malignant and lethal cancer of the central nervous system. Despite intensive research, a median patient survival time of only 14.6 months is achieved by the current multimodal therapy of surgical resection, radiotherapy, and chemotherapy with temozolomide (TMZ). Obstacles to effective treatment include 1) GBM cells are infiltrative, preventing complete surgical resection, and 2) cellular heterogeneity within GBM tumors, including a sub-population of GBM cancer stem cells (GSCs) capable of self-renewal and radio/chemotherapy resistant. Recent studies, including our own research, have demonstrated increased levels of the gap junction protein Connexin43 (Cx43) correlate with GBM TMZ resistance and poor patient survival. Therefore, altering Cx43 activity may represent a potent strategy in GBM treatment. Through distinct channel-dependent and channel-independent mechanisms, Cx43 can regulate cell proliferation, migration, and apoptosis. We isolated GSCs from patient tumors and, using super-resolution microscopy, observed intracellular Cx43 decorating microtubules, localized in proximities that indicate direct interaction in addition to microtubule-based Cx43 vesicular transport. Regulation of Cx43 function is associated with multiple sites of protein–protein interaction within the Cx43 carboxy-terminus (Cx43-CT), including a tubulin binding domain. We have developed a peptide named JM2 (juxtamembrane 2) composed of the Cx43-CT amino acids encompassing the tubulin-binding sequence, and a cell penetration domain to promote cellular uptake. We confirm JM2 specific interaction with microtubules concomitantly with a loss of Cx43 microtubule interaction and decreases in Cx43 gap junction plaque size and intercellular communication in GSCs derived from patient tumors. Importantly, JM2 decreases cell survival in TMZ-resistant GSCs, limits GSC neurosphere formation in vitro, and perturbs GSC-derived tumor growth in vivo. We are currently developing JM2-loaded biodegradable nanoparticles for JM2 sustained delivery in preparation for future clinical trials. The Cx43 mimetic peptide JM2 represents a novel and potent therapeutic to target chemoresistant GSCs in GBM treatment.
GJA1-20K PROTECTS THE HEART FROM ISCHEMIC INJURY BY INDUCING MITOCHONDRIAL METABOLIC QUIESCENCE

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The mRNA of the GJA1 gene that encodes gap junction protein connexin 43 (Cx43) undergoes alternative translation, producing N-terminal truncated smaller protein isoforms. Unlike the well-characterized full-length Cx43 (GJA1-43k), little is known about the function and regulation of the smaller isoforms. Here, we report that global ischemia/reperfusion injury in Langendorff-perfused mouse hearts upregulates endogenous GJA1-20k, the most abundant isoform of alternative translation. Biochemical fractionation indicates that the induced GJA1-20k is preferentially enriched in cardiomyocyte mitochondria. When introduced in vitro through adenovirus-mediated gene expression, exogenous GJA1-20k, but not full length protein GJA1-43k, localizes to mitochondria and improves the survival and viability of adult cardiomyocytes when subjected to oxidative stress. In vivo gene transfer of GJA1-20k through retro-orbital injection of AAV9 virus results in lower mitochondria-dependent basal oxygen consumption as well as maximal respiratory capacity in cardiomyocytes, unlike GFP or GJA1-43k controls. Thus GJA1-20k, but not GJA1-43k, protects the heart against ischemic injury induced by permanent LAD ligation. As compared to GFP control group, myocardial infarct size in GJA1-20k treated hearts is reduced by 30% at 72 hours post LAD ligation. These results indicate that endogenous GJA1-20k is induced upon stress and has strong tropism to mitochondria. Increased GJA1-20k induces mitochondrial metabolic quiescence and affords cardioprotection in vitro and in vivo. Alternatively translated GJA1-20k acts as a novel mitochondrial stress protein and demonstrates therapeutic potential against ischemic injury.
THE LIPIDATED CONNEXIN MIMETIC PEPTIDE, SRPTEKT-HDC, IS A POTENT INHIBITOR OF CX43-MEDIATED INTERCELLULAR COMMUNICATION WITH SPECIFICITY FOR THE PS368 PHOSPHO-FORM

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Connexin mimetic peptides derived from extracellular loop II sequences (e.g. Gap 27: SRPTEKTIFII, and Gap36: KRDPCHQVDCFLSRPTEK) have been used as reversible, Cx-specific blockers of gap junction intercellular communication (GJIC). These blockers typically require high concentrations (100-200 µM with IC50s of ~20-30 µM) to achieve inhibition. We have shown that addition of a hexadecyl (Hdc) lipid tail to the conserved SRPTEKT peptide sequence, SRPTEKT-Hdc, results in a novel, highly efficacious and potent GJIC inhibitor. Mechanically-induced Ca²⁺ wave propagation in Madin-Darby Canine Kidney cells expressing mCx43 (MDCK43) was significantly inhibited following a 60-90 min incubation in SRPTEKT-Hdc; IC50 of SRPTEKT-Hdc inhibition was 66.5 pM (95% CI: 31.8-138.9 pM). Lipidated reverse-sequence (TKETPRS-Hdc) and scrambled sequence (EPTKRTS-Hdc) peptides had no effect. SRPTEKT-Hdc also decreased NBD-m-TMA, but not Alexa350, dye coupling, but not to the same extent as inhibition of Ca²⁺ waves. Reduction of plaque-associated Cx43 (an indicator of channel number) in cells treated with SRPTEKT-Hdc could not explain the loss of Ca²⁺ wave propagation. However, we did find that SRPTEKT-Hdc inhibition of Ca²⁺ wave propagation depended on the functional configuration of Cx43 in which the S368 site is phosphorylated (pS368). Ca²⁺ wave propagation was enhanced in MDCK cells expressing single site mutants of Cx43 that favored or mimicked phosphorylation at S368 (MDCK43-S365A and MDCK43-S368D). Further, Ca²⁺ wave propagation in MDCK43-S365A and –S368D cells was inhibited by SRPTEKT-Hdc whereas Ca²⁺ wave propagation in MDCK43-S365D and –S368A cells, mutations that favor or mimic dephosphorylation at S368, was largely unaffected by SRPTEKT-Hdc. Together, these data indicate that SRPTEKT-Hdc is a potent inhibitor of physiologic Ca²⁺ wave signaling mediated specifically by the pS368 phospho-form of Cx43.
A NOVEL CONNEXIN 43 PEPTIDOMIMETIC INFLUENCES WOUND CLOSURE RATES AND PRO-INFLAMMATORY MEDIATED EVENTS IN SKIN MODEL SYSTEMS

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SiRNA targeting Cx43 and Cx43 peptidomimetics, including Gap27, improve in vitro wound closure rates. However controversy between channel and non-channel functions exists. We studied the impact of a panel of novel Cx43 peptidomimetics on hemichannel activity, cell migration rates and pro-inflammatory events in fibroblasts sourced from juvenile foreskin (JFF), human neonatal fibroblasts (HNF), adult dermal fibroblasts (ADF) and adult keratinocytes (AK).

ATP release following acute calcium deprivation was attenuated by Gap27 (100nM) and the novel Cx43-peptidomimetic (Compound A (100nM)) in JFF and HDF cells. Dose response curves in HeLa43 and HeLa26 expressing cells revealed that Compound A was Cx43 specific with an optimum response at 100nM. Exposure of JFF and AK to Gap27 (doses 100µM -100nM) and Compound A (doses 100µM-10nM) significantly enhanced scrape wound closure rates. By contrast in HNF and ADF the peptides had minimal influence on cell migration rates. Exposure of JFF and AK cells to Cx43SiRNA enhanced scrape wound closure rates by 50% compared to non-treated cells. In ADF cells this was only marginally improved.

To simulate a pro-inflammatory environment ADF and AK cells were challenged with 10µg/ml peptidoglycan isolated from S. aureus. ELISA assays determined IL-6 induction following 6 hr exposure. In ADF and AK cells co-treated with Gap27 or Compound A (100nM), release of IL-6 and a panel of pro-inflammatory cytokines was reduced.

We conclude that Compound A is a versatile and stable tool for development as a specific Cx43 channel inhibitor. Our data suggests that the role for Cx43 in wound healing significantly differs between JFF and keratinocytes with those derived from neonatal fibroblasts and deeper adult tissue. Hemichannel signalling plays an important role in keratinocytes and JFF cell migration events. In neonatal and adult fibroblasts this role is reduced and Cx43 hemichannel signalling in adult fibroblasts plays a role in inflammatory events.
SEVERE LOSS-OF-CX43 FUNCTION LEADS TO HEARING LOSS IN A MUTANT MOUSE MODEL OF OCULODENTODIGITAL DYSPLASIA

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Cx26 and Cx30 are expressed in the epithelial and connective tissue gap junction networks of the cochlea and are essential for auditory function. Currently, children with hearing impairments are routinely screened in developed countries for over 100 different GJB2 gene (encodes Cx26) mutations that are linked to either loss- or gain- of Cx26 function. Cx43 is also expressed along the auditory tract, although its functional role in hearing is not well understood. To test our hypothesis that Cx43 plays a role in auditory function, we utilized Cx43I130T/+ (courtesy of Dr. Glenn Fishman) and Cx43G60S/+ (courtesy of Dr. Janet Rossant) mutant mouse models of oculodentodigital dysplasia that harbor approximately 50% and 15-25% Cx43 channel function, respectively. Immunofluorescence revealed Cx43 plaque staining in the cochlear nerve of Cx43I130T/+ mutant mice and wild type (WT) littermates, while Cx43G60S/+ mutant mice had decreased Cx43 expression compared to littermate controls. To assess hearing, auditory brainstem responses were recorded in male mice in response to both a broadband click stimulus and tone pips at specific frequencies (4-, 8-, 16-, and 24kHz) in young and adult mice. Interestingly, while 2 or 6 month old Cx43I130T/+ mice did not harbor any hearing deficits, 1-3 month old Cx43G60S/+ mice had severe hearing loss, which correlated with a decrease in cochlear Cx43 protein levels as revealed by western blotting. Precisely dissected organotypic cochlear cultures (P0-P2) revealed no hair cell loss in either mutant mouse line suggesting that the mechanism of hearing loss in Cx43G60S/+ mutant mice was not due to postnatal hair cell loss. We propose that mutant mice exhibit hearing loss when Cx43 channel function falls to approximately 25% or below, and furthermore, this finding may explain why only a subset of patients with Cx43-linked oculodentodigital dysplasia have reported hearing loss. Supported by the CIHR to DWL and BLA.
Intercellular communication is vital to ensure tissue and organism homeostasis. This communication can occur directly between neighboring cells via gap junction’s channels (GJ), formed by connexin (Cx) or indirectly, at longer distances, through extracellular vesicles, including exosomes. Exosomes are small, lipid bilayer membrane vesicles of endocytic origin; they contain nucleic acid and proteins that are functional when transferred into recipient cells. In pathological condition the tumor environment is characterized by oxygen concentration around 1%, in this pathological condition cancer cells release ten times more exosomes than normal cells. Exist evidence that lens cells increased expression of Cx46 in low oxygen conditions, other studies show increased Cx46 upon exposure of promoting agents tumors. Our research focused on establishing whether Cx46 is involved in the resistance to hypoxia by the tumor cells, low or high invasiveness tumor phenotypes, and determine if Cx46 is present in exosomes release from cancer cells, and if exosomes containing Cx46 are able to modulate the interaction and transfer information between donor and acceptor cells, in this way we deliver important information about the role of Cx46 in the local and long distances tumor communication. The results obtained show that the over expression of Cx46 change the cellular phenotype from low to high invasiveness, the exosomes release by breast tumor cells contain Cx46 on their surface and this exosomes are incorporated by Cx46 positive cells. This result would provide important information about the role of Cx46 in the intra or extracellular tumor cells communication.

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MUTATIONS IN GJB2 (CONNEXIN 26; F142L AND G12R) ARE ASSOCIATED WITH MITOTIC INSTABILITY RESULTING IN CELL DEATH

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A 6-year-old Caucasian girl who presented with recurrent skin rashes and profound bilateral sensorineural hearing loss (SNHL) requiring cochlear implants was found to harbour a heterozygous point mutation in GJB2 (424T>C; Cx26F142L). In the present study we compared the effect of this Cx26 mutation with that of G12R, associated with Keratitis Ichthyosis deafness syndrome (KID) and D66H, associated with Vohwinkel syndrome. F142L was fused in frame with mCherry, WT CX26 with mCherry or eGFP, and G12R and D66H mutations with eGFP. Constructs were transiently transfected into HeLa or HaCaT cells, a model keratinocyte cell line, in the presence or absence of the microtubule stabiliser paclitaxel [0.25µM] for 20 hrs. Cells were fixed and stained with cytoskeletal markers and protein localisation determined by confocal microscopy. mRNA was harvested and qPCR performed to monitor any changes in gene expression of GADD153, a marker for ER stress. Hemichannel function was monitored by dye uptake assays. F142L did not assemble into gap junction plaque like structures giving diffuse cytoplasmic staining and perinuclear localisation with limited co-localisation with WTCx26. The nuclei of the transfected cells were severely compromised suggestive of apoptosis and necrotic events. This was also evident for the G12R mutation, however D66H remained co-localised with the p58 Golgi marker with intact healthy nuclei. In cells expressing F142L and G12R the microtubular network was profoundly disrupted suggestive of mitotic catastrophe. Co-treatment with paclitaxol rescued microtubule structure and the localisation of F142L but not G12R. Analysis of GADD53 expression, confirmed that despite altered Cx localisation none of the mutations evoked an ER stress response. We conclude that expression of the F142L mutation results in collapse of the microtubule network that is rescued by stabilisation of the microtubules. The data adds to the spectrum of GJB2 mutations associated with epidermal keratinisation disorders.
Genetically-MODIFIED MICE HARBORING A CX30 A88V MUTATION EXHIBIT A HEALTHY EPIDERMIS WITH NO EVIDENCE OF DEFECTIVE WOUND HEALING

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Clouston Syndrome is a disease that arises from human autosomal dominant mutations in the GJB6 gene encoding connexin30 (Cx30). Patients exhibit clinical symptoms that include hair loss, nail dystrophy, and palmoplantar keratoderma. Surprisingly, Cx30A88V/+ mutant mice that harbor the mutation, A88V, found in Clouston syndrome exhibit little evidence of disease in thin or thick skin and even Cx30A88V/A88V mutant mice have limited skin abnormalities. These findings raise the possibility that other epidermal connexins may be compensating for the reduction in Cx30 function in the epidermis. Immunofluorescent labelling of epidermal connexins revealed no differences in Cx30, Cx26 and Cx43 protein localization in mutant footpad epidermis as compared to its littermate controls. Western blot analysis further revealed no differences in Cx26, Cx30, or Cx43 expression levels. To further assess whether the A88V mutant leads to cellular consequences, we isolated and interrogated primary keratinocyte cultures from control and mutant mice. Preliminary studies revealed a high prevalence of Cx30 gap junction plaques in keratinocytes harvested from Cx30A88V/+ and Cx30A88V/A88V mice, similar to littermate controls. Furthermore, scrape-loading studies revealed no significant difference in dye transfer between mutant and control primary keratinocytes. To assess keratinocyte migration, scratch wound assays were performed and mutant keratinocytes from Cx30A88V/A88V mice demonstrated faster migration into scratch wounds suggesting that there may be an effect in wound healing. Dorsal punch wound assays performed on mutant and control mice; however, revealed no difference in wound healing responses even in Cx30A88V/A88V mutant mice. In summary, these results revealed that Cx30A88V/+ mutant mice do not phenocopy human Clouston Syndrome for reasons that remain elusive. Further analysis is required to determine the underlying pathological mechanisms of this mutant and whether compensation by other epidermal connexins act to alleviate skin phenotypes consistent with Clouston Syndrome. Supported by the CIHR.
HUMAN CX40 DOES NOT FORM FUNCTIONAL HETEROPTYPIC GAP JUNCTION CHANNELS WITH CX45

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Gap junction (GJ) channels form low resistance passages between cardiomyocytes and play a role in the rapid propagation of action potentials in the heart. A GJ channel is formed by two properly docked hemichannels and each hemichannel is a hexamer of connexins. Connexin40 (Cx40) and Cx43 are the dominant connexins in atrial myocytes, while Cx45 is mostly expressed in the sinoatrial (SA) and atrioventricular (AV) nodes. Cardiac action potentials propagate from SA node to atrial myocytes and then to AV node, possibly via heterotypic Cx40/Cx45 and/or Cx43/Cx45 GJs. However, the functional status and channel properties of human heterotypic Cx40/Cx45 or Cx43/Cx45 GJs have not been studied. Here we investigated human Cx40/Cx45 and Cx43/Cx45 heterotypic GJs by recombinant expression. To our surprise, cell pairs expressing human Cx40 in one and Cx45 in the other failed to form functional GJs. Modifications in human Cx40 with designed variants (D55N, P193Q, or a double variant, D55N-P193Q) are sufficient to establish functional heterotypic GJs with Cx45. On the contrary, heterotypic human Cx43/Cx45 GJs are functional similar to that described on rodent Cx43/Cx45 GJs. Detailed characterizations of human heterotypic Cx43/Cx45 GJs revealed a rapid asymmetric Vj-gating and a much slower recovery, which can substantially reduce the GJ conductance in a junctional delay and frequency dependent manner. Dynamic uncoupling in Cx45-containing GJs might contribute not only in a slower action potential propagation in the AV node, but also in preventing high frequency atrial fibrillation from propagating into ventricles. Supported by Heart and Stroke Foundation of Canada.
PUTATIVE PORE-LINING RESIDUES AND [Mg2+]i INFLUENCE CX50 GAP JUNCTION UNITARY CHANNEL CONDUCTANCE

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Gap junctions (GJs) formed by different connexins show different channel properties, including the ability of the GJ channel permeating ions known as the unitary channel conductance ($\gamma_j$). The $\gamma_j$ varies between 300 pS (Cx37 GJ) and too small to be measured (Cx36 GJ). The underline mechanisms determining the $\gamma_j$ level are not fully clear. We hypothesize that the $\gamma_j$ is influenced by the properties of pore lining residues and intracellular magnesium concentration, [Mg2+]i. Protein sequence alignment of Cx50 (its GJ $\gamma_j$ = ~200 pS) and Cx37 ($\gamma_j$ = ~300 pS) revealed residue differences in putative pore-lining domains, specifically G8 and V53 of Cx50 which are E8 and E53 in Cx37, respectively. We generated point mutants in Cx50 (G8E or V53E), or another point mutant (G46E) with large effects on the $\gamma_j$, individually and together (a triple mutant, G8E-G46E-V53E) to study their effects on $\gamma_j$. We used dual patch clamp to study the $\gamma_j$ of these mutant GJs in different [Mg2+]i. Our experimental data indicated that the $\gamma_j$ of the triple mutant, G8E and G46E, but not V53E, were significantly increased, with the triple mutant GJ the highest, 329 pS. Furthermore, when increasing [Mg2+]i, from 0 to 3 mM, Cx50, G8E, G46E, and the triple mutant (but not V53E) GJs showed a significantly decreased $\gamma_j$. Our data are consistent with a model where increase in negative electrostatic potentials in the pore surface of Cx50 GJ channel facilitate the rate of ion flow (the $\gamma_j$) of this cation preferred GJ channel. [Mg2+]i elevation significantly decreased $\gamma_j$ of Cx50 and some of these mutant GJs. This work was supported by the Natural Sciences and Engineering Research Council of Canada.
FUNCTIONAL CHARACTERIZATION OF NOVEL ATRIAL FIBRILLATION-LINKED CX40 MUTANTS

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Atrial fibrillation (AF) is the most common cardiac arrhythmia affecting ~3 million in North America. Both Cx40 and Cx43 are abundantly expressed in the human atrial myocytes forming gap junction (GJ) channels at cell-cell interfaces to ensure fast action potential propagation in the atria. Recently four novel lone AF-linked Cx40 mutations, Q236H, L223M, K107R and I257L were identified (Shi et al., Mol. Med. Reports 7, 767) and were inherited in an autosomal dominant manner in families. We studied these Cx40 mutants in connexin deficient HeLa cells for their localizations and in N2A cells for their functional properties. GFP-tagged Cx40 mutants showed clustered localizations at cell-cell interfaces similar to those observed for wildtype Cx40 in HeLa cells. N2A cell pairs expressing Q236H showed a significantly lower coupling conductance (Gj), while the Gjs of other mutants were similar to that of wildtype Cx40. When the mutants were co-expressed with wildtype Cx43, no change in the Gj was observed comparing to the Gj of cell pairs expressing both Cx40 mutants and Cx43. No apparent changes in Vj-gating in the GJs of these mutants. A significant reduction in the Q236H GJ conductance is consistent with previous findings on the majority AF-linked Cx40 mutants. The rest of these novel AF-linked mutants without any apparent defects in our experimental system may reflect the limitations of our model system or these mutants may not play a role in AF. This work was supported by the Heart and Stroke Foundation of Canada.
PANNEXIN1 CHANNEL PARTICIPATES IN TRYpanosoma CRUZI INVASION OF CARDIAC MYOCYTES

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Introduction: Trypanosoma cruzi (T. cruzi), the etiological agent of Chagas disease, invades the cardiac tissue causing acute myocarditis and heart electrical disturbances. Panx1 is involved in the infection of obligate intracellular pathogens such as Chlamydia and HIV. The aim of our study is to investigate the participation of Panx1 in the invasion of T. cruzi in cardiac cells. Methods: The hemichannel functional state was evaluated by dye uptake measurements in neonatal rat cardiac myocytes or HeLa cells transfected with Panx1 exposed to T. cruzi in absence or presence of probenecid or ¹⁰⁰Panx1 peptide (Panx1 Blockers) or Gap19 peptide (Cx43 Blocker). Intracellular calcium signals were evaluated by Ca²⁺ sensitive dye Fluo-3. T. cruzi invasion was determined by quantification of the number of intracellular parasites with DAPI fluorescence. The Panx1 expression was evaluated by immunofluorescence. Results: Exposure to T. cruzi increased dye uptake in cardiac myocytes (~4 fold) compared to uninfected cells. This effect was completely prevented by ¹⁰⁰Panx1 (100 µM). T. cruzi evoked repetitive Ca²⁺ signal transients with 0.026 ± 0.004 Hz. These responses were blocked by ¹⁰⁰Panx1 (100 µM) or probenecid (400 µM), and were not modified by Gap19 (100 µM). The invasion was significantly reduced by ¹⁰⁰Panx1 1.3 ± 0.5 % and probenecid 2.0 ± 0.5% vs control 16 ± 2.9% conditions. The infected cells showed greater immunoreactivity to Panx1. Discussion: These results provide first evidence that Panx1 participates in the invasion of cardiac cells by T. cruzi. Acknowledges: FONDECYT Nº11130013 (to J.L.V.), FONDECYT Nº1131007 (to J.G.). Barria and Güiza holds a CONICYT PhD Fellowship.
CX43 ISOFORM GJA1-20K PROMOTES MICROTUBULE DEPENDENT MITOCHONDRIAL TRANSPORT

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Connexin 43 (Cx43, encoded by GJA1) is a cell-cell communication gap junction protein expressed in all organ systems. It was recently found that GJA1 mRNA undergoes alternative translation to generate truncated C-terminal isoforms, of which GJA1-20k is the most abundant. Here we report a surprising finding that, unlike full length GJA1-43k, both endogenous and exogenous GJA1-20k have a powerful tropism for mitochondria. Exploring function, we found that GJA1-20k appears to be an organelle chaperone and that overexpression of GJA1-20k is sufficient to rescue mitochondrial localization to the cell periphery upon exposure to hydrogen peroxide, which effectively limits the network fragmentation that occurs with oxidative stress. By high-resolution fluorescent imaging and electron microscopy, we determined that GJA1-20k is enriched at the interface between mitochondria and microtubules, appearing to load organelles for transport. Mutagenesis experiments revealed that although the microtubule-binding domain (MTBD) in GJA1-20k is unnecessary for protein localization to mitochondria, the MTBD is essential for GJA1-20k to facilitate mitochondrial transport and maintain mitochondrial localization at the periphery. These results reveal an unexpected role for the alternatively translated isoform of the Cx43 gap junction protein, GJA1-20k, which is to facilitate microtubule-based mitochondrial transport and to maintain mitochondrial network integrity during cellular stress.
A NEW REGULATORY MECHANISM OF CX43 DEGRADATION IN THE HEART: CMA ENTERS THE STAGE

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Gap junctions intercellular communication (GJIC) between cardiomyocytes, mediated by specialized cell-cell contacts formed by connexins, is vital to ensure an efficient electric impulse propagation throughout the heart, thus allowing a synchronous and coordinated heart contraction. Regulation of GJIC can occur at different levels, including internalization and degradation of Connexin (Cx). We have previously shown that in ischemic heart the degradation of Cx43-containing GJ plaques relies on macroautophagy. Lysosomal degradation can also occur through another and more selective type of autophagy, chaperone-mediated autophagy (CMA) that requires the cytosolic chaperone, hsc70 and the lysosomal LAMP2A. Despite the degradation of Cx43 through the endolysosomal pathway or macroautophagy has already been described; its degradation through CMA has never been addressed.

In this study we demonstrate that CMA activation in cardiac cells, either by nutrient deprivation or with the CMA activator, 6-AN, leads to an increase in the degradation of Cx43 with a concomitant increase in the interaction between Cx43 and CMA components namely hsc70 and LAMP2A. We also identify a putative KFERQ motif on Cx43 and demonstrate that a mutation in this motif increases the half-life of the protein and modulates its interaction with both hsc70 and LAMP2A. Moreover, we show that KD of LAMP-2A partially reverts degradation of Cx43, both in cultured cells and organotypic heart slices. The interaction of Cx43 with CMA machinery was also demonstrated in mouse heart. Altogether, these results strongly suggest that Cx43 is a substrate for CMA degradation.
ISCHEMIC REPERFUSION BRAIN INJURY CAN BE REDUCED IN MICE FOLLOWING TREATMENT WITH DANEGAPTIDE BY INCREASING ASTROCYTIC GAP JUNCTIONAL COUPLING

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The presence of Connexin 43 (Cx43) in astrocytes in the brain has been shown to reduce brain injury following ischemic conditions such as stroke. The hemi-channel (pore) of Cx43 has been observed to be detrimental under stress whereas the gap junctional channels are beneficial. To explore the therapeutic potential of increasing astrocyte gap junctional coupling (GJC) to provide neuroprotection in ischemic stroke, primary cultures of mouse astrocytes were treated with the antiarrhythmic dipeptide ZP1609 (danegaptide) (247nM - 2.47mM) and gap junctional communication was measured. ZP1609 increased astrocyte GJC by 22.2% at 247nM concentration.

For in vivo stroke studies, we used a 60 min transient unilateral middle cerebral artery occlusion (MCAO) followed by reperfusion in 3-4 month old C57BL6 male mice. At 50 min, saline (pH 7.4) or ZP1609 (75ug/kg) was injected into the tail vein. After 60 min, the restriction in MCA was released to allow reperfusion. Mice were treated with subsequent ZP1609 injections at 1, 2 and 3 hr after the initial injection. The mice were euthanized at 48 hrs post-stroke and the volume of the stroke infarct measured. There was a significant reduction (P<0.002) in infarct size following ZP1609 treatment. In saline treated mice, infarct volume was 26.2 + 2.9 mm\(^3\) (n=10), while ZP1609 treatment reduced infarct volume to 14.3 + 1.3 mm\(^3\) (n=11). To assess ZP1609 levels in the brain, 10 µm thick coronal brain sections were processed for imaging mass spectrometry in positive reflectron mode on the 5800 TOF/TOF Sciex instrument. The spatial distribution of ZP1609 was observed throughout the brain tissue, consistent with passage through the blood brain barrier. Our results show that ZP1609 enhances GJC in mouse astrocytes, and provides a protective effect in transient brain ischemia with reperfusion. CCN holds a Canada Research Chair.
ENDOTHELIAL PANNEXIN 1 IS ESSENTIAL IN TNFα-INDUCED VENOUS PERMEABILITY

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During acute inflammation, the recruitment of leukocytes to sites of infection or tissue damage is a tightly controlled process. We previously showed that Pannexin 1 (Panx1) channels open in response to TNFα treatment in venous endothelial cells leading to an increase of leukocyte recruitment. Here, we present a novel role of Panx1 in the regulation of venous permeability. Using ex vivo cannulated veins perfused with fluorescein, TNFα induces an increase in the permeability in veins but not arteries. Pharmacological inhibition or endothelial-specific genetic deletion of Panx1 prevented the TNFα-induced increase in permeability. Panx1-mediated ATP release can activate multiple signaling pathways. PCR expression analysis for key purinergic receptor signaling molecules showed high expression of the ecto-ATPase CD39 in veins, but not arteries. By contrast P2X, P2Y, and adenosine A\textsubscript{1}-A\textsubscript{4} receptors and CD73 were comparable for veins and arteries. We hypothesize that Panx1-released ATP is rapidly degraded to adenosine by the CD39/CD73 complex in venous endothelial cells that activates A\textsubscript{2} adenosine receptors leading to an intracellular signaling cascade that regulates endothelial permeability. Indeed, the increase in venous permeability is blocked by application of the ecto-ATPase inhibitor ARL 67156 and by pharmacological inhibition or genetic deletion of the A\textsubscript{2} receptor, respectively. Additionally, mice deficient in TRPV4, a Ca\textsuperscript{2+}-permeable, nonselective cation channel that is activated by adenosine receptor signaling, were protected from the TNFα-induced increase in permeability suggesting a role of TRPV4 channels in the Panx1-dependent increase in permeability. TNFα-induced changes in permeability were prevented by Panx1 inhibition, inhibiting adenosine receptor signaling and in vessels from TRPV4-deficient mice. Therefore, we conclude that TNFα causes a venous-specific increase in vascular permeability by stimulating Panx1 channels to secrete ATP that is metabolized to adenosine by the CD39/CD73 complex. This extracellular adenosine then activates an A\textsubscript{2} receptor-mediated signaling pathway via TRPV4 channels to increase endothelial permeability.
DIBUTYRL CYCLIC AMP AND ANNULAR GAP JUNCTION VESICLE PROCESSING

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The internalization of gap junction plaques into the cytoplasm to form double-membrane annular gap junctions has been suggested to be a mechanism for degradation. However, it has become clear that in addition to degradation, annular gap junctions may undergo other processing events. While protein kinase A has been demonstrated to decrease internalization, the role of this kinase in annular gap junction processing is unknown. To demonstrate the role of PKA activation in determining the fate of annular gap junctions, adrenocortical cells were treated with dibutyryl cyclic AMP (DbcAMP) or diluent and analyzed with immunocytochemistry, live-cell, and transmission electron microscopy. With live-cell imaging, annular gap junctions in both control and DbcAMP populations underwent fission, reassociated with the plasma membrane, disappeared from view, or fused with other organelles. The number fusing with other organelles was 2 fold higher in DbcAMP treated populations compared to controls. Ultrastructural analysis was used to evaluate the identity of these organelles. As expected, some of the organelles were lysosomes, consistent with annular gap junction degradation. Mitochondria were also associated with annular gap junctions and surprisingly the percentage of annular gap junctions contacting mitochondria (5.2% +/- 1.1) was greater than that associated with lysosomes (2.6% +/- 0.6). Our findings are consistent with annular gap junctions undergoing processing events in addition to those involved in degradation and with cAMP increasing association of annular gap junctions with other organelles. The benefits of these events can only at this point be speculated but it is tempting to suggest that annular gap junctions may undergo fission before being degraded and connexin recycling may facilitate gap junction plaque formation. Further, since mitochondria are known to provide cell energy and to sequester calcium, it is possible that annular gap junction/mitochondria associations facilitate calcium-dependent events needed for annular gap junction processing. NSF grant #MCB-1408986.
A CONNEXIN-DEPENDENT JUXTACRINE SIGNALLING NETWORK REGULATES ADULT HIPPOCAMPAL PROGENITOR NEUROGENESIS

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The adult human hippocampus is capable of significant neurogenesis; however, seizure-induced cell replacement produces ectopic granule neurons that impair functional and behavioural recovery. Here, we show that adult type 1 and type 2a NPCs are part of a pan-glia juxtacrine interactome coupled by heterotypic gap junction subunits Cx32:Cx30, Cx47:Cx43, Cx30:Cx43 and homotypic Cx30:Cx30 and Cx43:Cx43 channels. In uninjured hippocampus, we find that oligodendrocyte (Cx32):NG2-Glia (Cx32) regulates NG2-Glial proliferation while NG2-Glia (Cx32):NPC (Cx30) communication regulates type 1 and type 2a NPC proliferation. Further, we show that Cx30-mediated gap junctional intercellular communication between astrocytes and type 1 and 2a NPCs is sufficient to induce neurogenesis in vitro. In vivo, following kainic acid-induced seizures, we find that Cx32:Cx30-mediated gap junctional intercellular communication between astrocytes and type 1 and 2a NPCs opposes the pro-neurogenic effects of Cx30-mediated astrocyte:NPC gap junction coupling in injured hippocampus. Removing regulatory Cx32 NG2-Glia: Cx30 astrocyte and NPC coupling from this juxtacrine network is sufficient to promote functional neuronal replacement, prevent ectopic granule cell integration into damaged cornu ammonis (CA) fields, and reverse seizure-induced learning and memory deficits. These results describe a novel juxtacrine signalling interactome that can be modulated to instruct functional NPC-mediated cell replacement in the adult hippocampus following acute seizure. Supported by CIHR.
OPTICAL AND PHYSIOLOGICAL ALTERATIONS PRECEDE THE APPEARANCE OF CATARACTS IN Cx46fs380 MICE

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Connexin function is crucial for lens transparency and cell homeostasis. Mutations in the lens fiber connexins, Cx46 and Cx50, have been linked to congenital cataracts. We developed a mouse model expressing a Cx46 mutant, Cx46fs380, corresponding to a human congenital autosomal-dominant cataract. In these mice, levels of Cx46 and Cx50 are decreased at 1 month of age, preceding cataracts by ~1 month in homozygotes and at least 3 months in heterozygotes. To test whether Cx46fs380 causes alterations before cataract detection, we studied lenses from wild type mice and Cx46fs380 heterozygotes and homozygotes at 1-3 months of age.

Differentiating (DF) and mature (MF) fiber cells of Cx46fs380 animals had significantly depolarized membrane potentials and decreased gap junctional coupling (G). In homozygotes, GDF represented 23.1% of the wild type values and GMF was undetectable, which correlated with the residual levels of Cx46 and Cx50 (0.7% and 23.1% of wild type levels, respectively). In heterozygotes, GDF was 46% and GMF was 31%, while the remaining levels of Cx46 and Cx50 were 3.8% and 56.7% of the wild type values. Before cataract appearance, homozygous lenses had slightly decreased size and radius of curvature of the anterior surface, reduced focal lengths, and they significantly distorted the pattern of an electron microscopy grid when photographed through these lenses. Heterozygous Cx46fs380 lenses were similar to wild type lenses in size, shape and focal distance, but distorted more the electron microscopy grid pattern.

In conclusion, all mutant lenses show changes in membrane potential and gap junctional conductance. The pre-cataract alterations in homozygous Cx46fs380 lenses are consistent with an increase in refractive index. Taken together, these data suggest that Cx46fs380 lenses undergo a sequence of changes before the appearance of cataracts: low levels of connexins, decreased gap junction coupling, alterations in lens cell homeostasis, and changes in refractive index.
PANNEXIN-1 MODULATES IN VITRO LYMPHANGIOGENESIS

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The growth of new lymphatic vessels, a process called lymphangiogenesis, has been associated with several pathological conditions including the formation of metastases. Although the deletion of members of the connexin family has been linked to diseases of lymphatic vasculature (most notably lymphedema), the roles of the pannexin family in the regulation of the lymphangiogenesis remain to be elucidated.

We show in this study by qPCR and Western blot analysis that lymphatic endothelial cells predominantly expressed Pannexin-1 (Panx1) rather than Panx2 and Panx3. Using two selective inhibitors of Panx1, probenecid and brilliant blue FCF, we observed an inhibition of the lymphangiogenesis in vitro that occurred spontaneously when LECs are seeded directly on Matrigel®. This result was further confirmed by siRNA-mediated knockdown of Panx1 in LECs. This effect was not the consequence of an inhibition of the LECs proliferation as demonstrated by BrdU incorporation assays. Our interest will be now focused on migratory and invasive capacities of LECs as a link between Panx1, cytoskeletal proteins and migration has already been demonstrated. Interestingly, Panx1 expression in LECs was up-regulated by prostate cancer cells which are known to metastasize preferentially through the lymphatic vasculature and by a treatment with Vascular Endothelial Growth Factor-C (VEGF-C), the major growth factor regulating lymphangiogenesis.

In conclusion, our results identify Panx1 as a protein expressed in lymphatic endothelial cells and suggest that Panx1 might be an important regulatory factor for lymphangiogenesis especially during prostate cancer dissemination.
IN VITRO MODEL TO STUDY EFFECTS OF ENVIRONMENTAL CONTAMINANTS ON GAP JUNCTIONAL INTERCELLULAR COMMUNICATION IN HUMAN AIRWAY EPITHELIAL CELLS

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Gap junctional intercellular communication (GJIC) is a very crucial process for the maintenance of cell and tissue homeostasis. GJIC is commonly dysregulated by environmental contaminants, including diverse airborne pollutants, which can directly expose airway epithelial cells. Although alterations of GJIC in the airway system have been implicated in the development of both acute inflammatory and chronic pulmonary diseases, there is only limited information regarding effects of airborne contaminants on GJIC and connexin function in the respiratory tract. To study the effects of these pollutants on GJIC in human airway system, we used an in vitro model of immortalized human bronchial epithelial cells HBE1, which expressed connexin43 primarily localized to the outer cell membrane and exhibited functional GJIC. Diverse groups of contaminants were evaluated: low molecular weight (LMW) PAHs, ubiquitous air pollutants formed during combustion processes including cigarette smoking; brominated flame retardants (BFRs), relevant contaminants of indoor environment; and cyanobacterial water bloom components found in aerosol particles formed during recreational activities. We observed a majority of these contaminants to elicit a rapid inhibition of GJIC in HBE1 cells at non-cytotoxic concentrations, usually accompanied by activation of MAPK-ERK1/2 and MAPK-p38. The effects were concentration- and time-dependent, as well as structure-dependent (e.g. LMW PAHs). While the most studied cyanobacterial toxins microcystin-LR and cylindrospermopsin did not inhibit GJIC, complex cyanobacterial biomass extracts elicited significant effects indicating the existence of other cyanobacterial metabolites responsible for GJIC dysregulation. Our data clearly show the potential of ubiquitous airborne pollutants to dysregulate GJIC and subsequently induce pro-inflammatory signaling and stress responses in human airway epithelial cells. Modulations of these critical processes might represent an important non-genotoxic mechanism involved in the development of airway tissue damage and chronic diseases in response to air pollution.

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CONNEXIN 43-FORMED HEMICHANELS PLAY A CENTRAL ROLE IN THE HYPERPERMEABILITY-ASSOCIATED INTRACELLULAR CA2+ SIGNAL ACTIVATED BY PAF IN POST-CAPILLARY VENULE ENDOTHELIAL CELLS

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Endothelial cell barrier contributes to keep the tissue environment. In post-capillary venules, inflammatory agents evoke a Ca2+-dependent increase in endothelial permeability to macromolecule (hyperpermeability), which leads to edema. This process depends on extracellular Ca2+ entry by a mechanism that remains to be determined. We analyzed the participation of Cx43-formed hemichannels in the Ca2+ signaling associated to PAF-activated hyperpermeability. We used the intact mesenteric vascular bed of the rat and primary cultures of mesenteric endothelial cells of venules (EC-V) to evaluate hemichannel activation by measuring ethidium uptake, changes in [Ca2+]i by loading the Ca2+ indicator Fluo-4, ATP release by luciferin-luciferase luminiscent assay and Cx43 S-nitrosylation by Proximity Ligation Assay (PLA). In addition, PAF-induced interendothelial cell pore formation was detected using atomic force microscopy (AFM). Stimulation with PAF induced an increase in ethidium uptake in intact venules and in EC-V, which was associated with a rapid rise of [Ca2+]i. Both the hemichannel activation and Ca2+ signaling were inhibited by treatment with the connexin blocking peptide 37,43Gap27 or 43Gap26, but also by blockade of purinergic receptors with PPADS. Consistent with the participation of purinergic receptors in the response, PAF induced a 43Gap26-sensitive increase in ATP release. In addition, PAF stimulation resulted in NO-mediated S-nitrosylation of Cx43 and, in line with this finding, inhibition of NO production with NG-nitro-L-arginine blunted the PAF-induced hemichannel activation and Ca2+ signaling. Interestingly, the treatment with 37,43Gap27 also blocks the PAF-initiated pore formation detected by AFM at the endothelial cell membrane apposition. These results indicate that activation of Cx43-formed hemichannel by NO-mediated S-nitrosylation plays a central role in the increase of [Ca2+]i associated to PAF-induced hyperpermeability. Cx43-formed hemichannels may participate in the response by allowing direct Ca2+ influx and by activation of a purinergic-dependent signaling through ATP release.

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CONNEXIN43 INCREASES THE INVASION CAPACITY OF HUMAN GliOBLASTOMA CELLS BY INDUCING THE FORMATION OF INVADOPODIA

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The resistance of glioblastomas to the treatments is mainly the consequence of their invasive capacities. Their ability to invade the surrounding parenchyma prevents their complete surgical resection and favours tumour recurrence. Therefore in order to treat better these tumours, it is important to understand the underlying molecular mechanisms which are responsible for their invasive behaviour. Previous work suggested that connexins can be involved in regulating the invasion of cancer cells. Here, we demonstrate that Connexin43 (Cx43) is implicated in the formation and function of cellular extensions such as invadopodia which are responsible for invasion capacity in U251 human glioblastoma cells. This was confirmed by using clones exhibiting lower invasion capacity once their levels of Cx43 are reduced by shRNAs. By confocal microscopy, Cx43 was precisely detected in all formation stages of invadopodia which exhibit proteolytic activity. The kinetics of formation of these structures seems to be positively dependent of Cx43 interactions with partners such as Src and Cortactin. On the contrary, Cx43, acting as hemichannel, negatively regulates this kinetics and invadopodia function by decreasing Matrix Metallo-Proteinase 2 (MMP2) activity. Moreover, the formation of gap junction plaques has, on the other hand, an indirect and negative impact on the invadopodia formation. In conclusion, these results show, for the first time, that Cx43 induces the formation and function of the invadopodia in cancer cells.
CONNEXINS IN BONE AND FAT

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Connexin43 (Cx43), the major gap junction protein expressed in bone, modulates cortical modeling. Ablation of the Cx43 gene (Gja1) in the osteogenic lineage causes periosteal expansion and cortical thinning mimicking changes occurring in bone disuse and aging. Such changes are phenocopied by conditional replacement, in osteolineage cells, of one Gja1 allele with the oculodentodigital dysplasia Gja1G138R mutant, which lacks gap junction channel function, but retains “hemichannel” activity; or with a truncated Gja1 mutant, lacking the last 5 aminoacids at the C-terminus (Gja1D378STOP). Since Gja1D378STOP cannot bind ZO-1, our results suggest that both channel and scaffolding / signaling functions are necessary for Cx43 action on cortical bone modeling. At variance with Cx43, conditional ablation of the Cx45 gene (Gjc1) in osteolineage cells results in high trabecular bone mass but no cortical abnormalities. Furthermore, while Cx43-deficient and Gja1G138R –expressing bone marrow stromal cells exhibit accentuated support of osteoclast differentiation, the opposite occurs with Cx45-deficient stromal cells. Mechanistically, Cx43 inhibits osteoclastogenesis via up-regulation of osteoprotegerin, an inhibitor of the pro-osteoclastogenic factor, receptor activator of NFκB ligand (RANKL). By contrast, Cx45 stimulates RANKL expression by bone marrow stromal cells. Thus, Cx43 and Cx45 have distinct, compartment specific functions in regulating bone modeling and opposite actions on osteoclastogenesis. Cx43 and functional gap junctions are also present in brown and white adipose tissue (WAT). Indeed, ablation of Gja1 embryonically in mesenchymal multipotential cells leads to a “lean” phenotype, with decreased body weight, fat mass and WAT. Inactivation of Gja1 selectively in bone cells does not reproduce the lean phenotype, which is instead seen in aged Gja1−/− mice. Thus, Cx43 favors accumulation of WAT via cell autonomous actions in adipocytes. Cx43 may represent a potential new pharmacologic target to modify bone structure and to counteract obesity.
A CELL PENETRATING CONSTRUCT FOR EFFICIENT DELIVERY OF AN INTRACELLULARLY ACTING CONNEXIN HEMICHANNEL MODULATING PEPTIDE IN THE TREATMENT OF OCULAR DISEASE

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Age-related macular degeneration (AMD) is a disease which affects 170 million people globally. Choroidal neovascularisation (CNV) is the hallmark of neovascular AMD, where the haemorrhage of leaky blood vessels in the choroid creates an inflammatory environment resulting in hemichannel mediated cell death including the loss of retinal pigment epithelial (RPE) cells which maintain blood retinal barrier integrity and rod/cone function. Current treatments do not address the self-perpetuating, hemichannel mediated inflammatory and hypoxic environment underlying disease cause. The Cx43 hemichannel specific intracellular acting peptide, Gap19, can be used to reduce hemichannel mediated cell death in response to inflammation and ischemia but has low cell permeability requiring high concentrations to achieve efficacy. By fusing Gap19 to the cell penetrating peptide, Xentry, we have developed an intracellular acting Cx43 hemichannel blocking fusion peptide, XG19, which enables low dose delivery yet treatment efficacy. Xentry interacts with cell surface expressed Syndecan-4, which we have found is highly expressed in hypoxic retinal cells. This means that XG19 preferentially targets hypoxic cells and can be delivered systemically as it is not sequestered by red blood cells. Our results have shown XG19 uptake can be achieved at concentrations as low as 5 to 10 µM in cultured human endothelial and RPE cells while native peptide uptake is undetected up to 100 µM. Furthermore the fusion peptide has shown efficient Cx43 hemichannel inhibition in dye uptake studies as well as in ATP release assays at 5 µM concentration. Scrape-loading dye transfer assays show that XG19 does not interfere with gap junction function. This fusion peptide has therapeutic potential to shut down the inflammatory cycle and repair vascular damage that occurs with CNV as well as other inflammatory and hypoxic-ischemic diseases of the body.
LOSS OF PANNEXIN1 SIGNALING COUNTERACTS LIVER FIBROSIS IN MICE

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Background: A key molecular initiating event in liver fibrosis includes the activation of hepatic stellate cells, which is a process that partly relies on the inflammatory response. The latter, in particular the attraction, activation and migration of immune cells as well as inflammasome activation, is mediated by several signaling cascades. Among those are pannexin1 channels, which support paracrine cellular communication. Pannexin1 channels indeed play a role in inflammatory processes in liver diseases, including acute liver failure and ischemia/reperfusion injury. The present study was set up to specifically investigate the role of pannexin1 signaling in hepatic fibrosis.

Methods: Liver fibrosis was induced in wild-type and pannexin1-/- C57BL/6 mice by treatment with carbon tetrachloride during 8 weeks. Thereafter, a series of read-outs were evaluated based on histopathological examination, measurement of biometric and serum biochemical parameters, collagen deposition, as well as assessment of hepatic stellate cell and inflammasome markers, and oxidative stress scavengers. Results: Pannexin1-/- mice showed lowered relative liver weight in comparison with wild-type, which was accompanied by decreased collagen content and reduced hepatic stellate cell activation. In addition, decreased inflammatory cell infiltration was observed in liver tissue as a result of genetic pannexin1 ablation, which is in line with the downregulation of the inflammasome expression. On the other hand, pannexin1-/- mice displayed reduced hepatic damage as evidenced by diminished serum aminotransferases levels and increased superoxide dismutase activity. Conclusions: Collectively, these results show the importance of pannexin1 signaling in liver fibrosis, in particular by the modulation of the inflammatory response and inflammasome activation. In this context, pannexin1 signaling may therefore represent a suitable target for the development of anti-fibrotic therapies.
DISTRIBUTION OF CONNEXINS 26, 32, 40 AND 43 IN THE EQUINE AGLANDULAR GASTRIC EPITHELIUM

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The scarcity of studies on interference of connexins (Cxs) in integrity of the gastric mucosa of horses motivated this study. The distribution of Cxs 26,32,40 and 43 in different regions and layers of the stomach epithelium of 16 healthy horses were investigated by immunofluorescence. All the Cxs were labeled in the gastric epithelium, however, there were variations in the distribution (basal, intermediate and superficial) and in the regions (M1- cardiac ostium, M2-margo plicatus and M3- fundic). As for the basal layer, 100% of the M3 samples were labeled by Cx26 and Cx32. There was no Cx26 labeling in 50% of the samples in M2 and 20% in M1; And Cx32 in 71.42% M2 and 20% M1. As for the Cx40, there was absence in 100% of the samples in M2; 66% in M3 and 60% in M1. No immunostaining of Cx40 was observed in the M2 (37.5%) and M1 (20%) regions. In the middle layer of the epithelium, staining was observed in all regions (M1, M2 and M3) in 100% of the Cx26 samples; Cx40 and Cx43, only in Cx32 there was no staining in M1 (20%) and M2 (14.28%). In the surface layer, there was no marking of Cx26 in 50% of samples of M2 and M3 and of 40% of M1. Absence of Cx32 labeling in the surface layer occurred only in 14.29% of the M2 samples. Regarding Cx42, the immunostaining was absent in the M2 (37.5%) and M1 (20%) regions. The superficial layer of the epithelium did not show Cx43 labeling in 100% of samples of M2, 80% of M1 and 66,6% of M3. The higher incidence of ulcerative lesions in the M2 region may be correlated with the decrease of expression of Cxs in the different layers of the aglandular gastric epithelium of horses.
Purinergic signaling (i.e. ATP-mediated signals) plays a key role in regulating kidney function by altering renal vascular resistance, tubular glomerular feedback, and renin secretion from juxtaglomerular (JG) cells. However, when systemic blood pressure (BP) is severely challenged, renal vascular smooth muscle cells (VSMCs) re-activate fetal renin expression programs to restore BP homeostasis. The mechanism coordinating these cellular responses remains unclear, but evidence suggests a strong regulatory role for extracellular ATP. Pannexin1 channels have emerged as conduits by which ATP is controllably released from cells. Work from our lab has demonstrated that Pannexin1 localizes to VSMCs and JG cells of the afferent arteriole. We hypothesized that Pannexin1-mediated ATP release from renin expressing cells supports retrograde signaling necessary for the recruitment of renin expression. To test this hypothesis, our lab generated a novel renin cell-specific Pannexin1 knockout mouse (Ren1-Px1 KO). These mice exhibit no gross differences in kidney size or tissue morphology, but do present with increased plasma sodium concentration, reduced urine volume, increased urine osmolality, and enhanced arterial vasoconstriction responses at baseline. Concurrently, we observed a significant elevation in plasma and urinary aldosterone, but interestingly no significant difference in baseline renin levels, indicative of hormonal dysregulation. Using radiotelemetry, we measured systemic blood pressure in Ren1-Px1 KO mice, which we found to be hypertensive at baseline. Finally, we assessed renin recruitment in response to severe blood pressure lowering (ACE, low salt) and determined that Ren1-Px1 KO mouse retain adequate capacity to increase plasma renin levels, but have an impaired ability to upregulate renin expression in the renal vasculature. We conclude that loss of Pannexin 1 from renin expressing cells results in baseline activation of RAAS, dysregulation of fluid/electrolyte balance, impaired blood pressure homeostasis. Thus, Pannexin1-mediated ATP release in the renal vasculature may be a novel regulator of long term blood pressure control.
ANALYSIS OF THE GAP JUNCTION-DEPENDENT COMMUNICATION DURING NEURONAL TRANSDIFFERENTIATION OF HUMAN MESENCHYMAL STEM CELLS

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Mesenchymal stem cells (MSCs) are well accessible and are said to have the capacity to be transdifferentiated into non-mesodermal cell types like neurons. They are therefore very promising cells to work with in the field of stem cell therapy. Concerning the field of cellular physiology, the process of transdifferentiation can be used to gain more insights into cellular and molecular mechanisms driving cell differentiation. In our experiments we analyzed the influences of the transdifferentiation process from human bone marrow-derived MSCs into neuronal-like cells on the gap junction dependent cell-to-cell-communication. qRT-PCR and western blot experiments showed a high expression of different gap junction-forming connexins in undifferentiated MSCs. These connexins were able to build functional gap junction channels, which could be shown by gold nanoparticle-mediated laser perforation / dye transfer (GNOME-LP/DT) and single cell dye injection. To induce neuronal transdifferentiation the MSCs were treated with all-trans retinoic acid for 24 h followed by the application of a neuronal induction medium for additional 24 h. Resulting morphological changes could be observed in brightfield microscopy. qRT-PCR revealed a reduced expression of MSC characteristic markers (CD73/CD90/CD166) while neuronal markers were upregulated, indicating a progress in differentiation. At the same time the expression pattern of connexins was changed. In particular, we observed a down-regulation of the MSC typical connexin Cx43, as well as a higher expression of Cx26, Cx37, Cx40 and Cx45.
18F-FDG PET IMAGING HIGHLIGHTS THE MODULATION OF BRAIN METABOLISM INDUCED BY THN102, A NEW COMBINATION OF MODAFINIL AND LOW-DOSE FLECAINIDE

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Modafinil, a non-amphetaminic psychostimulant, is prescribed as first line therapy in narcolepsy, an invalidating disorder characterised by excessive daytime sleepiness and cataplexy. Although its mode of action remains incompletely known, recent studies indicated that modafinil modulates astroglial connexin-based gap junction communication. We recently demonstrated that administration of a low dose of flecainide, an astroglial connexin inhibitor, enhanced the wake-promoting and pro-cognitive activity of modafinil in rodents and healthy volunteers. We aimed at investigating changes in glucose cerebral metabolism in rodents, induced by the combination of modafinil + flecainide low dose (called THN102).

The impact of THN102 on brain glucose metabolism was non-invasively investigated using ¹⁸F-2-fluoro-2-deoxy-D-glucose (¹⁸F-FDG) Positron Emission Tomography (PET) imaging in Sprague-Dawley male rats under isoflurane (2%) anaesthesia. Animals were i.v. injected with either vehicle, flecainide 1 mg/kg, modafinil 10 mg/kg, or THN102 (n=5 per group). After 30 min, animals were i.v. injected with ¹⁸F-FDG followed by 60 min PET acquisition. ¹⁸F-FDG PET images were co-registered to a ¹⁸F-FDG rat brain template and normalized from the total brain PET signal using Pmod software. Voxel-to-voxel analysis was performed using SPM8 software. Comparison of brain glucose metabolism between groups was performed using a One-way ANOVA followed by Bonferroni’s post-hoc test (p<0.05).

Doses of modafinil and flecainide were chosen not to significantly alter brain metabolism compared to vehicle. THN102 significantly increased regional brain glucose metabolism as it resulted in the activation of large clusters localized in the cortex, striatum and amygdala compared to control or drugs administered alone. These regions, highly involved in the regulation of sleep-wake cycles, emotions and cognitive functions were hence quantitatively modulated by THN102. Data presented here provide the first demonstration of a regional brain activation induced by THN102, currently being tested in a phase II clinical trial in narcoleptic patients.
TARGETING THE CONNEXIN43-EZRIN INTERACTION

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Adjacent cells can communicate via gap junctions (GJs) that are formed of connexin (Cx) hexamers which allow exchange of small molecules. Gap junction intercellular communication (GJIC) plays important roles in health and disease. Cx43 is the most highly and widely expressed Cx in human tissues. We have shown that in human placental trophoblasts and liver epithelial cells Cx43 GJIC is regulated by phosphorylation of Cx43 by an Ezrin-anchored pool of PKA which leads to opening of the Cx43 channels. Interaction between Cx43 and Ezrin is a protein-protein interaction (PPI) where we have defined the regions of interaction by peptide arrays and mutagenesis. Targeting this PPI with small molecular compounds to displace the PKA-Ezrin complex closing the channels could have benefit in conditions where GJIC can be damaging like in cancer and heart disease. Using the AlphaScreen assay we screened libraries of ~45000 small molecular compounds searching for compounds that can disrupt the Cx43-Ezrin PPI. Promising hits were further characterized and tested in a cell-based high-throughput assay for gap junctions that we recently developed as well as in the gap-FRAP assay. A few hit compounds showed the ability to significantly reduce GJIC in two cell-based assays. Such compounds may be a useful tool to prevent cancer cell invasion and EMT or to modulate the immune suppression by regulatory T cells towards effector T cells mediated through Cx43 and in that way alleviate inhibition of anti-tumor immunity.
Epithelial to Mesenchymal Transition plays a critical role in cancer metastasis. Connexins contribute to cancer metastasis through intercellular interaction between subpopulation of cells and their microenvironment. Pannexins share structural and functional features with connexins. Three pannexins have been described in human: PANX1, PANX2 and PANX3. These pore forming hemi-channels are involved in intracellular calcium, extracellular ATP release, and ROS production. PANX 1 and PANX 2; but not PANX3, are expressed in MDA-MB231. PANX1 and PANX2 expressions are significantly influenced by Cx43 expression in vitro as well as in vivo. We demonstrated that blocking PANX 1 hemi-channel with probenecid, not only affected intercellular communication, but also induced cell cycle arrest and enhanced the epithelial marker E-cadherin ad other markers that include Cx26 an PANX2, leading to a change in cellular morphology. Additionally, it decreased N-cadherin expression and the hypoxic marker HIF-1α, thus generating a less invasive form of these cells; only treatment surviving cells displayed a more likely invasive form of cancer. Furthermore, the MMP9 protein expression levels were decreased upon PANX1 blockage in MDA-MB231 cells; but not MMP2. Finally, PANX1 and PANX2 levels are significantly up-regulated in triple negative patients as well as in HER2+ and HER2- breast cancer patients. Pannexins serve as promising prognostic biomarkers and may constitute a potential therapeutic target in metastatic breast cancer. These findings demonstrate the enhancement of anti-hypoxic effect and attenuation of cancer invasion induced by inhibiting pannexin hemi-channels, suggesting a therapeutic advantage of combining this treatment with other anti-metastatic and chemotherapeutic drugs.
PS1.59

ANTI-ANGIOGENESIS THERAPY INDUCE AN INFLAMMATORY STATE IN MDA-MB-231 BREAST CANCER CELL LINE IN VITRO AND IN VIVO

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VEGF-A stimulates angiogenesis in cancer. Avastin (Av), the recombinant antibody targeting VEGF, improves progression-free but not overall survival in metastatic breast cancer. Studies in a diabetic model showed that avastin treatment increased inflammation by pre-activation of RAGE signaling. Furthermore, inflammatory factors in the tumor microenvironment induce epithelial-to-mesenchymal transition of non-transformed breast epithelial cells. On the other hand, both VEGF and direct cancer cell-endothelial cell interaction are crucial in extravasation. In this study, we evaluated the expression of inflammatory mediators (RAGE, TNF-α, IL-1β, IL-17 and IL-13) in triple negative breast cancer that lacks estrogen receptor (ER), progesterone receptor (PR) and epidermal growth factor receptor (HER2) rendering it refractory to available targeted therapy. We also investigated the effect of Av on inflammatory mediators in metastatic breast cancer (MDA-MB-231) cells and evaluated the levels of IL-1β, RAGE, and NF-κB pathway in a metastatic model of breast cancer in vitro and in a xenograft murine model injected with MDA-MB-231 cancer cells treated with Av at the secondary site (lung tissues). Results showed that tumor tissues showed an elevated expression of inflammatory mediators as compared to their normal counterparts. However, TNBC showed significant low levels of these mediators as compared to normal breast tissue. Remarkably, data showed that Av treatment increases expression of inflammatory mediators including RAGE, IL-1β and TNF-α as well as other metastatic factors including MMP2 and MMP9 at transcriptional and protein levels after treatment in vitro and in vivo. Interestingly, upregulation of Cx43 in combination with Av treatment alleviated the effect of avastin on inflammation. Therefore, Ineffectiveness of avastin treatment may be due to avastin-induced inflammatory microenvironment. Overexpression of Cx43 enhances sensitivity to Av treatment. We postulate that therapy with avastin is more significant in tumors that express Cx43 and may become more effective if coupled with Cx43 upregulation in breast cancer.
MOLECULAR MECHANISM OF GAP JUNCTION INTERNALIZATION

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Gap junctions turn over with a short half live of only a few hours, however the reason for this short and for membrane channels untypical turnover kinetics is not known. One possibility is that channels, once accrued to a gap junction plaque permanently close, become non-functional and thus need to be replaced by newly synthesized functional channels. Similarly, to allow cell migration, cells will need to physically uncouple from their neighbors, and this uncoupling requires the coordinated, rapid removal of all cell-cell junction types, including gap junctions. Regulated turnover is important for gap junction function, as abnormal turnover characteristics can lead to pathological conditions. We have characterized connexin 43 (Cx43) gap junction turnover on a molecular basis and characterized a sequence of posttranslational modifications that occur on Cx43 in gap junctions (ZO-1 binding and release, phosphorylation/de-phosphorylation on several sites that occur earlier and later, and K63-poly-ubiquitination) that occur in sequence to transition functional channels into non-functional channels that then can interact with clathrin/clathrin-adaptors to internalize and recycle gap junctions. Results have been published or are currently under review. Our findings will be summarized in this presentation.
DIFFERENTIAL ROLES FOR CONNEXIN 43 IN WOUND CLOSURE EVENTS IN FIBROBLASTS AND KERATINOCYTES

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During wound healing, fibroblasts and keratinocytes play an important role in cell proliferation, migration, re-epithelisation, wound contraction and remodelling of the extracellular matrix (ECM). Connexin43 (Cx43) is differentially remodelled during ‘normal’ and ‘chronic’ wound healing events and inhibiting Cx43 expression or function is known to have therapeutic potential but the mechanisms remain unresolved.

We investigated the effect of Gap27 on hemichannel activity, ECM deposition and cell migration in fibroblasts sourced from human juvenile foreskin (JFF), neonatal fibroblasts (HNF) and adult skin fibroblasts (ADF). In addition, the impact of Gap27 and Cx43SiRNA on JFF, ADF and adult keratinocyte (AK) cell migration rates under normoxic and hypoxic conditions (1% oxygen, 5% carbon dioxide and 94 % nitrogen) were explored. mRNA was extracted at set time points and MMP-9 expression determined by qPCR.

Acute calcium deprivation stimulated hemichannel activity and ATP release was attenuated by100mM - 100nM Gap27 in JFF and HNF cells. Exposure of JFF to Gap27 (100mM-100nM) and Cx43SiRNA enhanced scrape wound closure rates by 50% compared to non-treated cells. In HNF and ADF 100mM-100nM Gap27 had minimal influence on cell migration on standard plastic, collagen or fibronectin. Further, Cx43SiRNA only marginally improved migration of ADF cells and had no effect on ADF migration under hypoxia. In AK, Gap27 and Cx43SiRNA enhanced cell migration under normoxic and hypoxic conditions. In HNF and ADF, Gap27 had no effect on MMP-9 expression. Cx43SiRNA showed a decrease of MMP-9 under normoxia and an increase under hypoxia. In AK, MMP-9 was increased after exposure to Gap27 under normoxic and hypoxic conditions but no change with Cx43SiRNA occurred.

In conclusion, in juvenile foreskin derived fibroblasts and adult keratinocytes connexin signalling events play a key role in co-ordinating wound closure events. By contrast in adult dermal fibroblast the role of Cx43 is less important in cell migration.
INHIBITION OF CONNEXIN HEMICHANNLES BY AMINOGLYCOSIDES USING A NOVEL E. COLI-BASED ASSAY FOR RAPID SCREENING OF HEMICHANNEL FUNCTION

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Connexins form the gap-junctional channels that mediate intercellular communication and also form plasma membrane hemichannels (HCs). Connexin HCs are involved in the pathophysiology of several disorders including deafness, stroke and cardiac infarct, making HCs an attractive therapeutic target. Most available pharmacological inhibitors are either non-selective or toxic, which hampers their translational application. Aminoglycosides (AGs) are broad-spectrum antibiotics that have also been identified as inhibitors of connexin HCs. However, their potential as HC inhibitors has not been explored in detail. Here, we tested commercial AGs using a simple bacteria-based screening assay that we have developed and optimized recently. This assay is robust and easily scalable to high-throughput multi-well platforms. For the assay, we used the E. coli strain LB2003 as host-system. LB2003 cells cannot grow in low-[K+] medium due to the deletion of three key K+ uptake transport systems, but growth can be restored by supplementing the medium with [K+], or expressing recombinant K+-selective channels or connexin HCs. Several AGs inhibited Cx26 and Cx43 HC function in a dose-dependent manner, with IC50s in the low-µM to high-nM. We also showed that the HC inhibition by AGs is independent of their antibacterial effect. Since AGs are well known from many decades of use in humans, they have a high translational potential for use in disorders associated with increased HC activity.

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CONNEXIN-43 (CX43) REGULATES MITOCHONDRIAL TRANSFER FROM HEMATOPOIETIC STEM AND PROGENITOR CELLS TO BONE MARROW (BM) STROMAL CELLS VIA AMPK AND ATP SIGNALING

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Slow BM regeneration following chemotherapy causes morbi-mortality in cancer patients. Murine BM Cx43 facilitates intercellular communication between hematopoietic stem and progenitor cells (HSPC) and BM stromal cells (BMSC). Cx43 activity mediates BM HSPC survival and efficient blood formation by transfer of damaging excess reactive oxygen species (ROS) levels to BMSC after chemotherapy, preventing lethal hematology failure. Only primitive HSPC functionally express Cx43. We hypothesized that ROS transfer is mediated via mitochondria transfer. Chimeric mice created by transplanting mitochondrial-Dendra2 labeled (mito-Dendra2) HSPC to wild type (WT) mice showed about 88% of host BMSC containing donor hematopoiesis-derived mitochondria. This transfer is bidirectional, as determined in reversely transplanted chimeric mice where only 26% of the non-transgenic, donor-derived HSPCs acquired recipient BMSC mitochondria. Mitochondrial transfer is cell contact dependent and mediated by HSPC Cx43. In vivo Cx43 KO HSPC are not protected from chemotherapy and in vitro co-culture of mito-Dendra2 HSPC from Cx43 deficient (KO) mice with WT or Cx43KO BMSC impaired mitochondrial transfer and the concomitant increase of ROS levels in BMSC. Mitochondrial transfer and ROS levels were rescued by Cx43 re-expression but not by mutants that impair hemichannel docking or form functional channels with C-terminus truncation. Importantly, inhibition of the metabolic regulator AMP-activated protein kinase (AMPK) in hematopoietic cells dramatically increased mitochondrial transfer from HSPC to BMSC leading to increased ROS content of BMSC. ATP reduces AMPK activity in HSPC, in a correlation with Cx43 availability. Upon Cx43-mediated high mitochondrial transfer there are elevated ATP levels in HSPC and reduced in BMSC. These results suggest a potential transfer of ATP by Cx43 from BMSC to HSPC in order to reduce AMPK activity and further increase mitochondrial transfer back to BMSC. Our study discovered a Cx43 dependent dynamic metabolic state between BM HSPC and their stromal microenvironment, which is facilitated by mitochondrial transfer.
SPIRONELOACTONE CAN ACT ON PANNEXIN1 CHANNELS IN SMOOTH MUSCLE CELLS TO REGULATE VASOCONSTRICTION AND BLOOD PRESSURE

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Using an unbiased screen for potential inhibitors of pannexin1 (Panx1) channels, we identified spironolactone, a potent anti-hypertensive, as being able to block Panx1 channels. This was especially interesting considering spironolactone has been considered a mineralcorticoid receptor (MR) antagonist. We have previously demonstrated that Panx1 channels on smooth muscle cells (SMCs) can regulate α-adrenergic vasoconstriction and blood pressure, therefore, we hypothesized that spironolactone would also inhibit α-adrenergic vasoconstriction. Indeed, we found that spironolactone inhibited α-adrenergic vasoconstriction from resistance arteries isolated from hypertensive humans. This effect was also seen in C57Bl6 mice, mice lacking SMC MR, or mice lacking Panx1 in endothelial cells. However, mice with SMC specific deletion of Panx1 (SMC-Panx1 deficient) did not show any change in α-adrenergic vasoconstriction when treated with spironolactone. TO-PRO dye uptake on intact arteries further confirmed these functional results. Using radiotelemetry, we found that C57Bl6 mice and SMC MR deficient mice injected with spironolactone had a significant drop in blood pressure acutely. In line with the vasoreactivity experiments, SMC-Panx1 deficient mice did not have any change in blood pressure. Injection of finerenone, an MR antagonist that does not contain the steroid structure, did not reduce blood pressure acutely, whereas trovafloxacin, which has previously been demonstrated to inhibit Panx1 channel function, acutely decreases blood pressure in mice that have SMC Panx1. Together these data suggest that Panx1 is a novel target of spironolactone and inhibition of Panx1 by spironolactone may contribute to this drug’s anti-hypertensive effects. Funding: HL088554, HL120840, HL007284, HL131399.
THE H2 DOMAIN IN THE CONNEXIN43 CARBOXYL TERMINUS REGULATES CARDIOPROTECTION FROM ISCHEMIA REPERFUSION INJURY

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Cardiac ischemia-reperfusion injury occurs in patients with acute coronary syndromes undergoing percutaneous intervention to re-establish blood flow in blocked or narrowed arteries. Presently, there are no therapies approved to prevent the loss of heart muscle, scar tissue formation and contractile dysfunction that result from ischemia-reperfusion injury. We have previously reported that αCT1, a Connexin 43 (Cx43) mimetic peptide currently in Phase III testing in humans (GAIT1; NCT02667327) as a therapy for chronic skin wounds (PMID:27856288; 25072595), improves contractile function of mouse hearts following injury in association with increases in PKCε-mediated phosphorylation of Cx43 at serine 368 (S368) (PMID: 21273554). Here, we report that induction of S368 phosphorylation and cardioprotection from ischemia-reperfusion injury by αCT1 involves direct protein-protein interaction of αCT1 with a short alpha-helical sequence within the Cx43 carboxyl terminus (CT) termed the H2 domain. The interacting protein domains involved in Cx43 S368 phosphorylation were identified and validated by a combination of techniques including tandem mass spectroscopy, surface plasmon resonance, immunoprecipitation and thermal shift assays. These studies indicated that αCT1 induced S368 phosphorylation (pS368) via a novel mechanism involving interaction between a pair of positively charged lysines (K344, K345) in the Cx43 CT H2 domain and a cluster of negatively charged amino acids in αCT1. It is further shown that the known ability of αCT1 to interact with Zonula Occludens-1 (ZO-1), has no direct role in either the induction of S368 phosphorylation or protection from ischemia-reperfusion injury. Finally, we identify a small, novel variant of αCT1 (αCT11 – RPRPDDLEI) that induces S368 phosphorylation and robustly preserves ventricular contractile function and cardiac muscle when administered after ischemic injury. Results indicated selective targeting of the secondary structure and phospho-status of Cx43 CT by small peptidergic drugs may provide novel and potent pharmacological approach to preservation of cardiac muscle following ischemia reperfusion injury.
HOMOLOGUES OF GAP JUNCTION PROTEINS IN THE PROTOZOA Trypanosoma cruzi

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Introduction: Innexins are proteins expressed in invertebrates and present properties remarkably similar to those of pannexin found in vertebrates. However, the presence of these proteins in unicellular organisms has not been described. The aim of our study was to investigate whether Trypanosoma cruzi have innexin-formed hemichannels.

Methods: Multiple nucleotide alignments of T. cruzi, C. elegans, and D. melanogaster were carried out (ClustalW, EMBL-EBI). Cloning was performed using specific primers designed from C. elegans innexin sequences and genomic DNA of T. cruzi as template. The hemichannel functional state was evaluated by dye uptake (YOPRO-1: 375 Da, 2+) measurements in epimastigotes, and the sensitive to Probenecid (a pannexin hemichannel blocker) was assayed pharmacological characterization.

Results: In T. cruzi we identified one gene encoding a potential protein homolog to innexin. The gene identity was 44.77% and 37.67% with INX10 of C. elegans and INX2 of D. melanogaster. The membrane topology of protein showed a 4TMDs with intracellular C and N-terminal domain (Protter). Exposure to divalent cation-free solution increased (~50%) YOPRO-1 uptake in T. cruzi, effect that was blocked by 400 µM Probenecid. Fluorescein isothiocyanate-dextran (70,000 Da) did not enter into the cells, indicating that dye uptake was not the result of cell membrane damage.

Conclusions: These results strongly suggests the presence of innexins-like channels in T. cruzi. Due to the absence of innexin proteins in vertebrates, their identification in T. cruzi could lead to a new molecular target for the therapy of Chagas’ disease.

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CONNEXIN37-DEPENDENT MECHANISM ALTERS THE DEVELOPMENT OF RENIN-DEPENDENT HYPERTENSION

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Gap junction channels made of Connexin37 (Cx37) are expressed between 1) the endothelial cells of blood vessels, 2) the renin-secreting cells of the kidneys and 3) the smooth muscle cells (SMCs) of vessels from hypertensive mice. Cx37-deficient mice (Cx37-/-) are normotensive but feature a reduced basal nitric oxide (NO) release. We have shown that Cx37-/- mice display an increased media thickness and higher levels of Cx43 between SMCs. In this study, we investigated the role of Cx37 in the vascular signaling and in the control of the renin-angiotensin system. Wild-type (WT) and Cx37/-/- mice were subjected to the two-kidney, one clip (2K1C) procedure (renin-dependent hypertensive model) or L-N-nitro-arginine-methyl-ester (L-NAME) treatment. Compared to hypertensive WT 2K1C mice, Cx37/-/- mice did not increase their blood pressure (BP) in response to the 2K1C procedure, whereas they were as hypertensive as WT mice after L-NAME treatment. Four weeks after the 2K1C procedure, the Cx37/-/- left clipped kidney weighed less than the kidneys of untreated Cx37/-/- mice, whereas the contralateral kidney and the heart were heavier than controls, confirming the efficiency of the surgery. Despite the absence of increased BP, the Cx37/-/- 2K1C mice featured increased renin levels in clipped kidneys and in the plasma. To confirm these results, ALZET osmotic mini-pumps loaded with angiotensin II (AngII) were inserted during fourteen days in WT and Cx37/-/- mice. WT treated mice display a 50% increase in BP (149.7mmHg) whereas Cx37/-/- feature a significantly lower BP increase (33%, 133.2mmHg). Altogether, these data provide evidence that Cx37 is mandatory for a proper AngII signaling.
CONNEXIN37 REDUCES THE DEVELOPMENT OF INTIMAL HYPERPLASIA IN A MOUSE MODEL OF CAROTID ARTERY LIGATION

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Intimal hyperplasia (IH) is an abnormal response to vessel injury characterized by the dedifferentiation, migration, and proliferation of vascular smooth muscle cells (VSMC) from the media to form a neointima layer over the site of injury. Wild type (WT) mice were subjected to carotid artery ligation (CAL), a surgical model of IH development. The vessel injury triggered the linear formation of a neointima layer. Cx43 levels were upregulated in the media and neointima layers of ligated carotids whereas Connexin37 (Cx37) levels were selectively increased in the media layer but not in the neointima layer. We examined the development of IH in Cx37-deficient mice (Cx37⁻/⁻) subjected to CAL. The formation of the neointima layer was decreased 14 days after the carotid ligature in Cx37⁻/⁻ compared to WT mice. However, it was markedly increased 28 days after the carotid ligature. The presence of a large neointima layer was correlated with a two-fold increase in cell proliferation in the media and neointima regions between 14 and 28 days in Cx37⁻/⁻ mice compared to WT mice. In vitro, the de novo expression of Cx37 in primary VSMC from human veins reduced cell proliferation and triggered the degradation of Cx43. In contrast, Cx43 levels were increased in the media of Cx37⁻/⁻ mice compared to WT and primary VSMC from Cx37⁻/⁻ mice proliferated faster than their WT counterpart. Our data support a protective role of Cx37 against VSMC proliferation and IH, which counteracts the pro-proliferative effect of Cx43.
**CONNEXIN37 DELETION ALTERS ANGIOGENESIS IN THE DEVELOPING MOUSE RETINA**

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Connexins (Cxs) form intercellular gap junction channels, allowing the communication between neighboring cells through free exchange of molecules up to 1 kDa, including ions, small metabolites, secondary messengers and miRNAs. Connexin37 (Cx37) is expressed between vascular endothelial cells (EC) and participates to the coordination of the vasomotor tone in large arteries. Using the neovascularization model of the mouse neonatal retina in Cx37 deficient mice (Cx37⁻/⁻), we show that Cx37 contributes to developmental angiogenesis. The extension of the superficial vascular network is decreased at post-natal days 4 (P4) but not at P6 in Cx37⁻/⁻ retina, compared to wild type (WT) mice. However, at P6, the absence of Cx37 leads to an increase in the vascular density and the number of sprouts at the angiogenic front. Interestingly, those events occur mainly in the venous network compared to the arterial network. The vessel density is unaltered in adult Cx37⁻/⁻ retina. However, the number of veins and arteries is decreased in Cx37⁻/⁻ retina compared to WT retina. To understand how the Cx37 may influence the arteriovenous differentiation, a process mainly governed by Notch signaling, WT and Cx37⁻/⁻ mice were treated with an inhibitor of the Notch pathway (DAPT). DAPT-treated WT mice had an increased blood vessel density similar to what is observed in control Cx37⁻/⁻ mice. In contrast, DAPT-treated Cx37⁻/⁻ mice displayed no major change compared to untreated Cx37⁻/⁻ mice suggesting that Cx37 may interfere with the Notch pathway. The data provide evidence that Cx37 plays a role in angiogenesis possibly by altering of the Notch signaling.
EXPRESSION OF PROTEINS OF CELL COMMUNICATION IN THE RUMEN OF FETUSES, NEWBORN AND ADULT BOVINES

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The bovine stomach is a four chambered organ (rumen, reticulum, omasum and abomasum). Differently from the true stomach (abomasum), at birth the rumen stratified epithelium is not fully differentiated and functional, which will only occur after weaning. Connexins are involved in several metabolic functions of the epithelial cells, which are important to prepare the rumen to the weaning process. The study of Cxs expression and behavior in the rumen epithelium may offer an interesting model to understand their contribution to epithelial tissue specialization. Samples from the ruminal mucosa of fetuses (110- and 150 day-old), newborn calves and adult bovines were collected (dorsal and ventral portions) and the expression of Cx26, 32, 40 and 43 were studied by immunohistochemistry. The Cx26 and Cx32 were found in the cytoplasm (juxtanuclear) of the basal epithelial cells of fetuses and newborn calves and at the cell membrane in higher epithelial layers; the expression in adults of both connexins was very low. In adults, most of the Cx32 was found in the cytoplasm. Cx40 was observed in all epithelial layers of all groups, both at the membranes and in the cytoplasm. CX43 was found in all groups, with an intense expression in adults and a low expression in other groups. Cx26 and Cx32 expression pattern in fetuses and newborns suggest that they are more important to the formation and development phases of the ruminal epithelium architecture. Cx40, highly expressed in all groups, probably has a function more related with the integrity and homeostasis of the rumen epithelium in all its phases of development. Cx40 and Cx43, the last one expressed more intensely after weaning, must be involved in helping and keeping the final functional differentiation of the stratified squamous epithelium of the rumen and the final process of keratinization of the upper layers.
MODULATING THE EFFECTS OF ROTIGAPTIDE ON CONDUCTION BY ALTERING EXTRACELLULAR IONIC COMPOSITION

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Background: Previously, we demonstrated that elevating perfusate sodium (Na⁺) and calcium (Ca²⁺) can promote normal conduction velocity (CV) in the heart despite reduced gap junction (GJ) coupling. However, it is unknown whether the same interventions can increase conduction with compounds that enhance GJ coupling.

Methods and Results: Langendorff-perfused guinea pig hearts were pretreated with 80nM Rotigaptide or vehicle for 15 minutes and optically mapped with normal Tyrode’s (NT) solution containing 146mM [Na⁺] and 1.25mM [Ca²⁺], elevated Na⁺ (155mM [Na⁺], 1.25mM [Ca²⁺]), or elevated Na⁺/Ca²⁺ (155mM [Na⁺], 2.0mM [Ca²⁺]). At baseline, perfusate composition did not change CV. However, Rotigaptide significantly increased CV for NT (by 5±1%), but not in the other perfusates. During 30 minutes of no-flow ischemia without Rotigaptide, elevated [Na⁺] and elevated [Na⁺]/[Ca²⁺] delayed the time to ischemia-induced conduction block to 12 minutes, from 8 minutes with NT. Importantly, elevated [Na⁺]/[Ca²⁺] significantly attenuated CV slowing during ischemia. Western blots demonstrated that perfusate effects were independent of changes in total or phosphorylated connexin43 at serine368. Interestingly, for NT, Rotigaptide prolonged the time to conduction block from 8 to 12 minutes of ischemia. Subsequent reperfusion led to frequent ventricular fibrillation (VF) in all perfusates. With NT, there was a trend for Rotigaptide to reduce VF duration from 15+ minutes (vehicle) to 12 minutes. In contrast, Rotigaptide did not modulate reperfusion arrhythmias in the perfusates with elevated [Ca²⁺] and/or [Na⁺].

Conclusions: Elevating perfusate Na⁺ and/or Ca²⁺ can promote normal conduction when GJ coupling is reduced, but this effect is not enhanced by treatment with Rotigaptide. Importantly, previous reports of enhanced conduction with Rotigaptide during ischemia/reperfusion may be dependent on the choice of perfusate, suggesting that future studies investigating the loss of GJ coupling during ischemia should consider the effects of this class of compounds in the context of underlying ionic fluid composition.
Peucedani Radix (PR) is commonly used to eliminate sputum, relieve cough, reduce bronchus contraction, protect cardiac and hepatic disease, control inflammation, and suppress aggregation of platelets in Korea, China and Japan. The purpose of this paper is to investigate the anticancer effect and its underlying mechanism of PR in A549 human non-small lung cancer cells. PR decreased the proliferation of A549 cells in a dose dependent manner (IC50= 1.12 µm/ml). PR treatment specifically changed the morphology of lung cancer cells, which were related to apoptosis and autophagy. Western blot analysis indicated that PR up-regulated the levels of Death receptors, FasL and activated caspase-3, -8, -9 and subsequent cleavage of poly(ADP-ribos)polymerase and increased LC3B. Furthermore, the cell cycle analysis indicated that PR caused cell cycle arrest of A549 cells at G2/M phase upregulating cyclin B1, p53 and p21. Taken together, these data suggested that PR can be expected to be a novel agent for lung cancer treatment (NRF-2016R1C1B2016529).
THE EFFECT OF CHRONIC HYPOXIA ON PULMONARY VASCULAR REACTIVITY IN CONNEXIN 43 HETEROZYGOUS MICE

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Pulmonary hypertension (PH) is a chronic progressive disease associated with abnormal vasoconstriction and vascular remodelling. Altered connexin mediated signalling is implicated in abnormal vasoconstriction and smooth muscle cell proliferation in PH. These vascular conditions are primarily mediated by serotonin (5-HT) and endothelin-1 (ET-1), and evidence showed 5-HT passes through myoendothelial gap junctions and induced smooth muscle cell differentiation. As hypoxic conditions lead to the development of PH, we assessed the effects of hypoxia on pulmonary vascular reactivity in connexin 43 heterozygous (Cx43+/−) mice. The effect of hypoxia on Cx43 expression in lungs and pulmonary arteries from WT and Cx43+/− mice was also explored.

Male wild type (WT) and Cx43+/− mice (C57BL6, 7 to 9 months old) were exposed to chronic hypoxia (550mbar, ~10% O2) for two weeks. Mice were culled using 60mg/kg pentobarbitone and heart and lung tissue dissected out. Intralobar pulmonary arteries (IPAs) were mounted on wire myographs and cumulative concentration response curves (CCRCs) constructed to ET-1 and 5-HT. qRT-PCR was conducted on main and branch pulmonary arteries to assess Cx43 gene expression. Cx43 protein expression was visualised in lung sections using confocal microscopy.

ET-1 was more efficacious (P<0.05) in producing maximal contractile response in IPAs from hypoxic Cx43+/− mice compared to hypoxic WT mice. In addition IPAs from hypoxic Cx43+/− mice were more sensitive to ET-1 compared to IPAs from hypoxic WT mice (P<0.001). Under normoxic conditions, the potency of ET-1 in IPAs of Cx43+/− mice was significantly (P<0.05) greater than in WT mice, however the maximum response to ET-1 was unchanged. No difference in pulmonary vascular reactivity to 5-HT between WT and Cx43+/− mice was observed. Hypoxia mediated a reduction in gene and protein expression of Cx43 in both WT and Cx43+/− mice.

In conclusion reduced connexin 43 signalling may be associated with increased pulmonary vascular reactivity.
INTRAMOLECULAR LOOP/TAIL INTERACTIONS INVOLVE THE SH3 BINDING DOMAIN, CONTROLLING Cx43-HEMICHANNEL ACTIVITY

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Interactions between C-terminal tail (CT) and cytoplasmic loop (CL) critically control the activity of Cx43 hemichannels. We previously identified the last 9 amino acids of the CT as a critical determinant for binding the 2nd part of the CL and as an important region for Cx43-hemichannel function. Here, we show that deletion of the last 18 amino acids in the CT only partially lowers the binding to the L2 region, indicating that a second L2-binding region is present in the CL. We propose that the SH3-binding domain present in the CT is a target of L2, since the CTDSH3D18 fails to bind biotin-L2 in surface plasmon resonance (SPR) experiments. Moreover, membrane-permeable SH3 peptide (TAT-SH3) removed the high [Ca2+]i brake on Cx43-hemichannel activity measured via Ca2+ waves and ATP release in HeLa cells ectopically expressing Cx43 as well as in primary bovine corneal endothelial cells. These results were confirmed at the single channel level using whole-cell patch clamp experiments and demonstrated that SH3 peptide acts to promote [Ca2+]i-triggered hemichannel opening. Moreover, TAT-SH3 restored the activity of CT-truncated Cx43 hemichannels (Cx43M242) in response to 2 µM A23187. We also identified the critical role of SH3-binding domain in controlling Cx43-based hemichannels in the full-length Cx43 using 5 mM EGTA or 2 µM A23187-induced ATP-release experiments. We found that ATP release mediated by Cx43DCT18 and Cx43DSH3-based hemichannels was impaired but not completely abolished. However, Cx43DSH3DCT18-based hemichannels completely failed to mediate ATP release in response to EGTA or A23187. Finally, we show that in contrast to activity of Cx43M242, the ATP-release properties of Cx43Dgap19M242-based hemichannels in response to 2 µM A23187 could not be restored by TAT-SH3. This shows that both the CT18 region and the SH3-binding domain in the CT of Cx43 are important for Cx43-hemichannel activity.
PHOSPHORYLATION STATE OF TYROSINE 332 REGULATES CX37 GAP JUNCTION CHANNEL FUNCTION AND ITS GROWTH SUPPRESSIVE PROPERTIES

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Increased number of collaterals in the pial circulation and improved recovery of hindlimb perfusion following surgically induced ischemia in Cx37 knockout compared to wildtype mice suggests vascular growth processes (vasculogenesis, arteriogenesis, angiogenesis) are regulated by Cx37. Although available data implicate Cx37 in the regulation of vascular growth and remodeling, its mechanisms of action remain unclear. Cx37 is a phosphoprotein with several predicted high probability (>90%) phosphorylation sites targeted by growth factor activated kinases located in its intracellular carboxyl-terminus (CT). A CT truncated form of Cx37 fails to limit rat insulinoma (Rin) cell proliferation, whereas full length Cx37 profoundly slows proliferation, an effect that also requires gap junction channel (GJCh) functionality. These and other data suggest growth factor activated signaling cascades lead to serine phosphorylation, which is necessary for Cx37-mediated cell cycle progression. However, Cx37 tyrosine phosphorylation has not been explored for its impact on proliferation or channel function. A likely critical residue is tyrosine 332, as it is the only tyrosine in Cx37 with >90% predicted probability of phosphorylation. To study phosphorytrosine-dependent regulation of Cx37-mediated growth suppression and channel function, point mutations were made at Y332 to mimic dephosphorylated (phenylalanine) or phosphorylated (aspartate) states and expressed in Rin cells. Expression of Cx37-Y332F in Rin cells limits proliferation comparably to Cx37-WT expression. In contrast, expression of Cx37-Y332D eliminates the anti-proliferative properties of Cx37. Cx37-Y332F forms functional GJChs, but there is no evidence of coupling between cells expressing Cx37-Y332D, further supporting previous observations that GJCh function is necessary for Cx37-mediated growth suppression. Despite the lack of functional GJChs, cells expressing Cx37-Y332D have active hemichannels, which further indicates that hemichannel function is not sufficient for Cx37-mediated suppression of cell proliferation. Together, these data suggest that Cx37-pY332 regulates cell proliferative phenotype and GJCh functionality.
The cellular process of epithelial-mesenchymal transition (EMT) is central to progression of cancer metastases, and can be induced by transforming growth factor-β (TGF-β). A hallmark of EMT is breakdown in intercellular communication including dissolution of connexin 43 (Cx43) gap junctions. Internal translation of the Cx43 (GJA1) transcript generates N-terminally truncated isoforms, with a predominating 20 kDa isoform (GJA1-20k) capable of regulating gap junction formation. Given that PI3K/AKT/mTOR is activated by TGF-β, a pathway that suppresses internal translation of GJA1, we hypothesized that this contributes to losses in intercellular gap junction formation during TGF-β-induced EMT.

Inducing EMT in human and mouse epithelial cells, we find suppression of GJA1-20k expression despite increased levels of full-length Cx43, indicating a shift in translation initiation concomitant with losses in gap junction formation. Immunofluorescence, gene expression, and biochemical assays reveal sequestration of Cx43 in intracellular compartments, together with induction of established EMT marker expression. Suppression of GJA1-20k expression is also sufficient to mimic decreased stability of full length Cx43 seen with TGF-β stimulation. In order to elucidate effects on EMT progression and gap junction formation, we constitutively expressed GJA1-20k using lentiviral transduction. Maintenance of GJA1-20k expression rescues Cx43 gap junctions in TGF-β treated cells, confirming that suppression of internal translation is a necessary event in gap junction disassembly during EMT. To better understand the mechanism by which GJA1-20k regulates gap junctions, we investigated the Cx43 hexamer/monomer ratio in TGF-β treated cells. Stable expression of GJA1-20k alters this ratio and suggests a role for GJA1-20k in promoting hexamer formation. These data highlight alternate translation initiation as a regulator of the proteome and cellular junctional status.
POSSIBLE ROLES FOR ATP RELEASE FROM RBC EXCLUDE THE cAMP-MEDIATED PANX1 PATHWAY

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Red blood cells (RBCs) have previously been observed to release adenosine triphosphate (ATP) in a Pannexin 1 (Panx1)-dependent manner in response to hypoxia and shear stress. This extracellular ATP is believed to signal via purinergic receptors on the vascular endothelium to initiate vasodilation and increase blood flow in response to elevated cellular oxygen demand. The initiation of either pathway (hypoxia or shear stress) in RBCs is predicted to converge on an intracellular cyclic adenosine monophosphate (cAMP)-dependent RBC mechanism; however little direct evidence for this conclusion exists. We hypothesized that stimulation of intracellular cAMP in RBCs would cause Panx1-dependent ATP release. To test this hypothesis, we used genetic mouse models to confirm that Panx1 is responsible for ATP release from RBCs. Using Western blot, immunofluorescence and multiple Panx1 antibodies, we confirmed the presence of Panx1 on RBC plasma membranes. Next, we stimulated RBCs with novel and previously-reported activators of ATP release, detecting an increase in intracellular ATP in the absence of cAMP-stimulated ATP release. Similar results were detected following stimulation of RBCs with hypotonic potassium gluconate solution or the active cAMP analog 8Br-cAMP, where ATP release strongly correlated with levels of hemolysis. Moreover, we found no difference in background ATP levels or controlled ATP release between WT and Panx1⁻/- mice. Lastly, from these results we pioneered novel methodology for assessing both ATP release and hemolysis from RBCs, which necessitate measurements of hemoglobin absorbance peaks at an isosbestic wavelength and minimization of background extracellular ATP. We conclude that the previously reported Panx1-dependent ATP release from RBCs is not regulated via cAMP.
ENHANCEMENT OF GAP JUNCTIONAL ACTIVITY BY iPS CELL-DERIVED NEURAL STEM CELLS IN STEM CELL-BASED SUICIDE GENE THERAPY

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Background: Stem cell-based suicide gene therapy is a feasible treatment strategy for malignant brain tumors. The mechanism includes several steps; (1) intratumorally implanted effector cells transduced with herpes simplex virus-thymidine kinase (HSVtk) gene migrate to the tumor site and form gap junctions; (2) systematically administrated ganciclovir (GCV), an antiviral agent, is converted to phosphorylated GCV by HSVtk, and transferred to the tumor cells via gap junction; and (3) GCV-triphosphate cause tumor cell death thorough DNA synthesis inhibition. HSVtk-transduced neural stem cells (NSCtk) are useful as effector cells because of their tumor tropic property and “bystander effect”. However the role of gap junctional activity in the bystander effect is not elucidated. In this study, we approach this theme with assessments of gap junctional function and amount of connexine 43 (Cx43).

Methods: Induced pluripotent stem cell-derived NSCs (iPS-NSCs) and mouse glioma cells (GL261) were used. The parachute assay with donor cells (iPS-NSCs or GL261) and recipient cells (GL261) were observed by flow cytometry and fluorescent microscope. Expression of Cx43 was assessed by immunocytochemistry and western blot in culture of GL261 alone and co-culture of iPS-NSC and GL261 for 3 days. Effector cells transduced with HSVtk gene (iPS-NSCtk and GL261tk) were co-cultured with GL261 cells in 5µg/ml of GCV. Cell viability was assessed on day 7 by MTT assay.

Results: Dye transfer from iPS-NSC to GL261 was stronger than that from GL261 to GL261. Expression of Cx43 was enhanced when iPS-NSC and GL261 were co-cultured compared with culture of GL261 alone. Finally, the bystander effect was stronger in iPS-NSCtk/GL261 co-culture (tk/tumor cell ratio < 1:32) than GL261tk/GL261 co-culture (tk/tumor cell ratio < 1:8).

Conclusion: Our results suggest that iPS-NSC forms functionally enhanced gap junction with GL261 through increased expression of Cx43. This may contribute to potent bystander effect in stem cell-based suicide gene therapy.
CONSTITUTIVE TURNOVER OF CONNEXIN 43 AND MODULATION OF GAP JUNCTION INTERCELLULAR COMMUNICATION INVOLVE BOTH AUTOPHAGY AND THE ENDOCYTIC PATHWAY

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The available experimental evidence suggests that connexin 43 (Cx43) can follow different pathways en route to lysosomes. In one pathway, annular gap junctions are sequestered by autophagosomes, which subsequently fuse with lysosomes in a process known as macroautophagy (autophagy hereafter). In a second pathway, Cx43 may undergo sorting to lysosomes along the endocytic pathway. However, the relative importance of these two pathways in the degradation of Cx43 under basal conditions has remained elusive. Moreover, how the modulation of Cx43 trafficking via either of these pathways affects gap junction intercellular communication is incompletely understood. Here, we have studied the mechanisms involved in the constitutive degradation of Cx43 by using HeLa cells stably transfected with Cx43 and C33A cells, which express Cx43 endogenously, as model systems. The degradation pathway followed by Cx43 was analyzed by confocal microscopy, super-resolution microscopy, and immunoelectron microscopy. In accordance with previous studies, the constitutive degradation of Cx43 was found to involve autophagy. We also found that a subpool of Cx43 was sorted along the endosomal pathway. To functionally investigate the role of these two pathways in Cx43 degradation, cells were depleted of Atg5, which is important for the maturation of autophagosomes, or Tsg101, which is required for the sorting of ubiquitinated cargo along the endocytic pathway. Depletion of either Atg5 or Tsg101 resulted in increased Cx43 protein levels, enlarged gap junctions, and enhanced gap junctional communication. Similar effects on Cx43 and gap junctional communication were observed in response to depletion of the small GTPase Rab5, which is essential for the biogenesis of the endolysosomal system and may also have a role in autophagy. Collectively, our data suggest that autophagy and the endosomal pathway act in parallel in mediating Cx43 degradation and in the modulation of gap junctional communication under basal conditions.
A ROLE FOR CX43 HEMICHANNELS IN SEPSIS-INDUCED RENAL VASCULAR PERMEABILITY

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To date, sepsis is the leading cause of death in intensive care units. Within the field of septic shock, both the multiplicity of simultaneously occurring events, and the difficulty in translating basic research to the clinic, complicate the understanding and treatment. Extended vascular hyperpermeability is a common feature of many diseases, including septic shock, which is typically connected to changes in expression patterns of different cell junction proteins. We aimed to explore the less-understood contribution of connexin (Cx)-mediated signaling in a mouse model of sterile sepsis: TNF-induced Systemic Inflammatory Response Syndrome (SIRS). Using TAT-Gap19 as a specific blocker of Cx43 hemichannels (HCs), which does not inhibit gap junctions, we found that mice, injected with a lethal dose of TNF, were protected against mortality and hypothermia. Conversely, TAT-CT9 that promotes Cx43 HC opening worsened mortality and hypothermia. We also observed increased vascular permeability during TNF-induced SIRS, most prominently in the kidney; TAT-Gap19 and Tie2 Cre-mediated Cx43 KO (endothelial cells and others) protected against this permeability disturbance. Furthermore, we also identified a role for RIPK3 in vascular permeability, which is known to signal to necroptosis via MLKL as well as to phosphorylate CaMKII, which has well-recognized phosphorylation sites on Cx43, and is thus able to regulate Cx43 channels. Together, our results suggest a novel role for Cx43 HCs in contributing to vascular permeability and shock. Further work needs to be done to see whether Cx targeted treatments can be of use in the field of septic shock.
Situated between the blood circulation and the brain, the blood-brain barrier (BBB) protects the brain from circulating toxins while securing a specialized environment for neuro-glial signaling. Unique features of BBB-endothelial cells (ECs) include highly restrictive tight junctions that prevent paracellular diffusion, and low prevalence of non-specific transcytosis. Previous work demonstrated that the EC cytoplasmic Ca2+ concentration ([Ca2+]i) is an important determinant of BBB function and that connexin hemichannels (CxHCs) contribute to [Ca2+]i dynamics and BBB alterations. BBB compromise, as observed in inflammatory conditions, involves paracellular leakage and/or increased, non-selective transcytotic events, the latter being the subject of this study. CxHCs may contribute to transcytosis by providing a direct diffusion pathway for small MW molecules (<1kDa), or they may exert control over [Ca2+]i that is critical in the transcellular pathway. We used lipopolysaccharide (LPS) to induce systemic inflammation and to trigger a BBB permeability increase in mice. Identification of vesicles in BBB-ECs was done by immunostaining for early endosome antigen-1 and TSG101. An increase in vesicular transport was seen in BBB-EC of LPS treated mice and this increase was prevented by in vivo intravenous injection of the Ca2+ chelator BAPTA-AM and the CxHC blocking peptide Gap27. Our results suggest that transcytosis may play a role in increased BBB permeability in inflammatory conditions and that Ca2+ signaling and CxHCs are crucial in this transcellular transport.
INHIBITION OF STRETCH-ACTIVATED PANNEXIN 1 CHANNELS BY A PKA-DEPENDENT PATHWAY

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Pannexin1 channels allow the passage of monovalent ions and small signaling molecules between intra and extracellular compartments. These channels can be opened by increased cytoplasmic calcium ion concentration, positive membrane potentials, high extracellular potassium ion concentration and mechanical stretch. In contrast, mechanisms that promote pannexin1 channel closure are poorly understood. Since pannexin1 is known to be a phosphoprotein, one possibility might be via changes in phosphorylation state. Therefore, we studied the possible role of protein kinases in closing pannexin1 channels activated by mechanical stretch.

The activity of pannexin1 channels was evaluated by using the dye uptake assay (DAPI as a permeability tracer) in parental and pannexin1 transfected HeLa (HeLa-Panx1) cells. Channels were activated by mechanical stretch induced by drops of extracellular saline solution, dropped from 10 cm high. Mechanical stretch–induced DAPI uptake was detected only in HeLa-Panx1 cells, and adenosine or cAMP analogues (8-CPT-cAMP and Db-cAMP) closed the channels. PKI (PKA inhibitor) pretreatment prevented channel closure, whereas phloretin, SB203580, Akt inhibitor VIII or KN62 (PKC, p38 MAPK, Akt or CamKII inhibitor, respectively) did not. Since adenosine may be transduced by A2A or A2B receptors, which upon activation increase cAMP concentration that, in turn, activates PKA, we performed a bioinformatic analysis using the NetPhosK 3.1 server, and found five putative PKA-phosphorylation residues in pannexin1: T21, S205, T302, S328 and S417. Site-directed mutagenesis of S205 did not prevent the Db-cAMP-induced closure of pannexin1 channels. Generating pannexin1 mutated in other putative PKA-phosphorylation sites is an ongoing endeavor. Future evaluation of phosphorylation states of pannexin1 might be necessary to assess whether it can be a substrate for PKA.

Our results suggest a negative modulation of pannexin1 channels by a PKA-dependent pathway, and indicate that paracrine communication via pannexin1 channels could be modulated by several Gαs-coupled receptors (e.g., nucleoside, hormone and neurotransmitter receptors).
N-TERMINALLY ELONGATED SPLIINX2 AND SPLIINX3 REDUCE BACULOVIRUS-TRIGGERED APOPTOSIS VIA HEMICHANNEL CLOSURE

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The hemichannel and gap junction channel are major portals for the release of factors responsible for the effects of apoptotic cells on the spread of apoptosis to neighboring cells and apoptotic corpse clearance, typically by phagocytes. The N-terminal cytoplasmic domain in the connexins, gap junction proteins in vertebrate, has been implicated in regulating channel closure. However, little is known about how the hemichannel close responds to apoptotic signaling transduction leading to the reduction of neighboring cellular apoptosis in an invertebrate. An insect Bac-to-Bac expression system, pFastBacTM HT A, allows us to construct an N-terminally elongated SpliInx2 (Nte-Inx2) and SpliInx3 (Nte-Inx3). Here, we demonstrated that recombinant baculovirus Bac-Nte-Inx2 (reBac-Nte-Inx2) and Bac-Nte-Inx3 (reBac-Nte-Inx3) closed the endogenous hemichannel on the Sf9 cell surface. Importantly, primary baculovirus infections significantly caused early apoptosis, and this apoptosis was reduced by hemichannel-closed Sf9 cells at 24 hrs post-infection (PI). Although N-terminal elongated residue led to the increase of the phosphorylated sites in both Nte-Inx2 and Nte-Inx3 and an additional transmembrane domain in Nte-Inx3, both the proteins localized on the cell surface, suggesting Nte-Inx proteins could mediate hemichannel closure. Further supporting evidence showed that hemichannel closure was dependent on N-Inx3 expressed by baculovirus polyhedrin promoter, which began to express at 18-24 hrs PI. These results identify an unconventional function of N-terminal elongated innexins that could act as a plug to manipulate hemichannel closure and provide a mechanism connecting the effect of hemichannel closure directly to apoptotic signaling transduction from intracellular to extracellular compartment.
We created a 2D model of cardiac tissue consisting of discrete cells connected through modulatable gap junctions. The model was developed by combining Noble equations, which describe cardiac excitability (CE), and a 36-state model (36SM) of gap junction (GJ) channel gating, which evaluates junctional conductance ($g_j$) changes at a given transjunctional voltage ($V_j$). The 36SM accounts for the presence of the fast and the slow gate in each of two hemichannels. A combined model (CE-36SM) allowed us to study an interaction between the spread of cardiac excitation and GJ channel voltage gating. Modeling results using CE-36SM showed that during propagation of cardiac excitation the delay between action potentials (APs) in neighboring cells generates high amplitude (~100 mV) $V_j$ spikes. Each $V_j$ spike can cause a small decrease of $g_j$, and such process has a tendency to accumulate at frequencies of excitation, comparable to those during tachycardia. Interestingly, $g_j$ decrease was more significant in transverse than in longitudinal direction of cardiac syncytium, exhibiting well expressed electrical anisotropy. This phenomenon is caused by different delays between APs and, consequently, different shape of $V_j$ spikes in longitudinally and transversely oriented gap junctions. Thus, voltage gating of GJ channels could change a natural electrical anisotropy of cardiac tissue. Modeling results in larger clusters of cardiomyocytes revealed that GJ channel gating increases arrhythmogenicity of cardiac tissue. For example, our data show that dynamic modulation of $g_j$ can facilitate reentry formation, which results to spiral waves of excitation in cardiac tissue. Moreover, under certain conditions voltage gating of GJ channels can cause the drift of spiral wave core or increase the probability of spiral wave multiplication, which leads to a fibrillation-like process.

In memory of the late Feliksas Bukauskus
RESTRAINT OF SKIN FIBROBLAST MOTILITY, MIGRATION AND CELL SURFACE ACTIN DYNAMICS BY PANNEXINS/PURINERGIC SIGNALING

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Fibroblast migration is a critical step that takes place during the inflammatory and proliferative phases of skin wound healing. Intercellular communication, either through connexins or pannexins (Panxs) has been proposed to be critical for effective wound healing. We found that Panx1 and Panx3 are expressed in human and mouse skin fibroblasts, and that the absence or inhibition of pannexins significantly increase single cell motility and accelerate fibroblast migration during wound healing tested in vitro. In addition, the inhibition of pannexins induces cell surface actin redistributions, which is required for cell migration. On the other hand, our results suggest a functional association between pannexins and the purinergic receptor P2X7 that slowing down fibroblasts migration. Finally, using an in vivo assay for cell migration into artificial skin scaffolds, we found that the lack or inhibition of Panx1 increases migration of skin cells into the scaffolds. Taken together our results indicate that pannexins and P2X7 receptors have a important role in skin fibroblast migration by controlling actin dynamics and cell motility, which may be critic for the proper timing of cell arrival required for skin healing.

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RhoA AND ACTIN CYTOSKELETON CONTROL THE FUNCTIONAL BALANCE BETWEEN GAP JUNCTION CHANNELS AND HEMICHANNELS

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The trafficking of Cxs to the plasma membrane is Cxs-isoform dependent since some Cxs assemble in the Endoplasmatic reticulum (ER)/Golgi apparatus (GA) network and traffic to the plasma membrane, following the canonical exocytic/secretory pathway, and others exhibit direct trafficking to the plasma membrane from the ER. Several studies support the role of microtubules and actin microfilaments for trafficking of Cxs and GJCs plaque formation. However, it is unknown whether actin-Cxs association differentially regulates the traffic and functional state of GJCs and HCs. We have found that depolymerization of the actin microfilaments with Cytochalasin B (Cyto-B), reduce the size of GJCs plaques at appositional membranes formed by Cx43 or Cx26 and increase the transport of Cx43 or Cx26 HCs to non-appositional plasma membrane. Functional studies show that actin depolymerization reduces the functional state of Cx43 GJCs but in contrast significantly increases HCs activity. Interestingly; functional state of GJCs and HCs composed by Cx26 were not affected by Cyto-B. In addition, the effect of the small GTPase RhoA protein, which is an important modulator of the actin cytoskeleton rearrangements, was also evaluated by overexpression of constitutive active form, or by pharmacologic inhibition, or expression of dominant negative and siRNAs to reduce its activity. We found that the inhibition of RhoA activity, in HeLa cells expressing Cx43 or Cx26, produces a reduction in the amount of stress fibers and the GJCs plaque size. However, in cells expressing Cx43, but not Cx26, inhibition of RhoA activity and expression produce reduction in GJCs coupling and an increased of dye uptake compared to the control condition resembling the effect observed with Cyto-B treatment. Our results show a novel functional association in which actin’s dynamics control the trafficking and functional state of GJCs and HCs, by a mechanism that could involve RhoA activity that differentially regulates Cx26 and Cx43 channels. Work supported by Fondecyt-1130855 and 1171240 (to ADM) and the Chilean Millennium Institute, Centro Interdisciplinario de Neurociencia de Valparaíso (P09-022-F)
MOLECULAR DETERMINANTS UNDERLYING THE GAIN OF FUNCTION ELICITED BY THE
CX26 SYNDROMIC DEAFNESS MUTATION G12R

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This has been shown that mutations in connexin26 (Cx26) related to keratitis ichthyosis deafness
(KID) syndrome promote gain of function hemichannels. We investigated the biophysical properties
of the amino terminal mutant Cx26G12R (G12R) that generates gain of function hemichannels. On
G12R hemichannels expressed in Xenopus laevis oocytes we found large currents that not reach a
steady state at depolarizing voltages. The analysis of the relaxation time constants and calculations
of the apparent affinity for Ca2+ strongly suggest that the mutation impairs the slow gating in G12R
hemichannels. Interestingly, single channel recordings in G12R hemichannels reveal absence of
transition to the subconductance state, indicating altered fast gating. Moreover, although the single
channel conductance was similar to wild type Cx26 hemichannels, the open probability of the fully
open state in G12R hemichannels is largely augmented. In studying the underlying mechanism,
molecular dynamic simulations indicate the arginine insertion makes the N-terminus helix displaced
to the intracellular side, allowing interactions with the TM2/IL border residues, especially Arg99.
Disrupting this interaction totally reverts the effect induced by the mutation. All together these
results reveal the molecular mechanism of gain of function elicited by syndromic G12R mutation,
and strongly suggest that both, fast and slow gates works coupled rather than separates entities.
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EHD1 REGULATES THE INTERNALIZATION OF CX43 - A NEW PLAYER OF ISCHEMIA-INDUCED REMODELING OF GAP JUNCTIONS IN THE HEART?

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Electrical conduction in the heart is mainly governed by an efficient gap junction-mediated intercellular communication (GJIC) between cardiomyocytes. Expectedly, several cardiomyopathies, including myocardial ischemia, are associated with impaired channel activity, increased degradation and lateralization of connexin43 (Cx43), the main ventricular GJ protein. Results from our group demonstrated that Cx43 is ubiquitinated and undergoes autophagic degradation in cardiomyocytes during ischemia and ischemia/reperfusion (I/R). However, little is known about the players and signals underlying the subcellular redistribution of Cx43 in this setting. Recently, we unveiled the Cx43 interactome in ischemic rat hearts, which showed that ischemia positively regulates interaction of Cx43 with Eps15 homology domain-containing protein 1 (EHD1). Since EHD1 has been associated with internalization and recycling of several membrane proteins, the main objective of the present study was to investigate the role of EHD1 in ischemia-induced remodeling of GJ.

Our results show that co-localization of EHD1 with Cx43 increases in adult human primary cardiomyocytes subjected to ischemia in vitro, and in ischemic ex vivo rat hearts, using the Langendorff apparatus. Additionally, we establish that phosphorylation and ubiquitination of Cx43 enhance the association between Cx43 and EHD1. To further characterize the molecular mechanisms underlying EHD1-mediated intracellular trafficking of Cx43, we genetically manipulate the levels of EHD1. Our results demonstrate that internalization of Cx43 is promoted after overexpression of wild-type EHD1 in HEK-293 cells. On the other hand, both the knockdown of EHD1 and the overexpression of a mutant form of the protein results in the stabilization of Cx43 at the plasma membrane.

Altogether, we have identified EHD1 as a new player involved in the modulation of intracellular trafficking of Cx43, which may ultimately contribute to GJ remodeling during myocardial ischemia.
NOVEL ROLES FOR SUMOylated AND EXOSOMAL CONNEXIN43 IN MELANOMA: SEARCHING FOR NEW THERAPEUTIC TARGETS

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Loss of gap junction intercellular communication (GJIC) and/or downregulation of connexins (Cxs) have been reported both in cell lines as well as in tissues of many tumor types including melanoma. Cxs have been described as tumor suppressors only in the earlier steps of cancerogenesis. Indeed, Cxs are tumour drivers during tumor cell invasion and metastasis but their role is controversial. In this study we have investigated different roles of Cx43 in proliferation and metastasis capacity in human melanoma. BRAF- and NRAS-mutant melanoma cell lines showed low Cx43 levels associated with cytoplasmic distribution and low incidence of dye coupling (GJIC). Interestingly, Cx43 was found mainly SUMOylated. Pharmacological proteasome inhibition combined with inhibitors of SUMOylation restored the unmodified form of Cx43 and significantly reduced proliferation and colonies grown. Cell proliferation tested by iCelligence and Click-it assays showed a correlation between Cx43 protein levels and proliferation rates between different melanoma cell lines. Ectopic Cx43 gene expression using plasmid vectors incremented total Cx43 levels, restored Cx43 native band detected by western-blot, plasma membrane localization detected by immunofluorescence, and raised GJIC. Cx43 significantly increased cell adhesion but reduced cell proliferation, migration and melanoma cell colonies grown. To further investigate the role of Cx43 in metastatic potential we have studied the effect of tumor-derived exosomes. Exosomes containing Cx43, secreted from melanoma cells overexpressing Cx43, decreased cell proliferation and blocked colonies grown in different melanoma cell lines. In summary, these results indicate that SUMOylation of Cx43 in melanoma affects cell proliferation, migration and grown. Exosomal Cx43 may play a role in the crosstalk between the primary tumor and host cells to promote metastatic niche formation. These findings provide new role of Cx43 as conditional tumor suppressor and invasion capacity providing melanoma features with prognostic and therapeutic potential.
THE CARBOXYL TAIL OF CONNEXIN43 INFLUENCES B CELL RECEPTOR SIGNALING-INDUCED CYTOSKELETAL REARRANGEMENTS IN B-LYMPHOCYTES

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Cellular processes involving cytoskeletal reorganization such as adhesion, membrane spreading, immune synapse formation, motility and cell migration are important for normal B–Lymphocyte (B cell) development and for immune responses. Signaling by the B cell antigen receptor (BCR), as well as integrins and chemokine receptors, result in small GTPase-dependent actin cytoskeleton remodeling. We have previously shown that the Cx43 carboxyl tail (CT) is important for these processes in B cells, in particular the region of the CT between amino acids 246-307. In addition, we have shown that the CT alone, without the rest of the Cx43 protein is sufficient to support these types of cytoskeletal changes in B cells. The CT region contains a number of tyrosine (Y) and serine (S) residues that are potential protein interaction sites. Using a mutational approach, and the cell-spreading assay as a simple read-out to assess cytoskeletal rearrangements, the effects of individual Cx43 point mutations or combinations of mutations have been assessed. Our analysis of the serine mutants, S255A, S262A, S279A and S282A showed that they had differential effects on B cell spreading. We propose that this reflects unique roles for these serine residues in leading to actin remodeling. Future studies will include assessing the effects of these mutations on cell motility and directed migration, as well as experiments to identify the proteins that interact with the Cx43 CT. The challenge will be in determining how these interactions could influence the dynamics of the actin network. This information will reveal critical interactions that are nucleated by Cx43 and link signaling receptors to molecular mechanisms that control cellular cytoskeletal architecture that influences B cell development and immune responses. This could lead to identifying potential targets for the development of therapeutics to treat immune diseases. (Supported by grants from the CIHR).
ROLE OF CONNEXINS IN HYPOXIC-INDUCED PROLIFERATION AND MIGRATION IN RAT PULMONARY ARTERY FIBROBLASTS

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Rationale: Pulmonary arterial hypertension (PAH) is a disease associated with increased pulmonary arterial pressure and is mediated by both constriction and remodelling of the pulmonary arteries. One cause of PH is chronic hypoxia which increases cellular proliferation contributing to vascular remodelling. Pulmonary artery fibroblasts (PAFs) play a key role as they proliferate rapidly in response to hypoxia and can increase pulmonary artery smooth muscle cell proliferation in co-culture. It is likely that p38 MAPK plays an important role in hypoxic pulmonary hypertension since it is activated in models of acute and chronic hypoxia. Dysfunctional connexin-mediated signalling is thought to play a role in the pathophysiology of PAH. The aim of this study was to investigate the effects of gap junction inhibitor Gap 27 on rat PAF (rPAF) proliferation, migration and phosphorylated p38 expression.

Methods: rPAFs were exposed to hypoxic conditions (5%O\textsubscript{2}) or normoxic conditions for 24 hours in the presence and absence of Gap 27 (300µM). Cells were counted using the Countess II (Life Technologies) to assess proliferation. Scratch assays were conducted in order to assess migration. Protein was harvested for Western blots.

Results: Hypoxia mediated an increase in proliferation of rPAFs which was inhibited in the presence of Gap 27 (P<0.001). Hypoxia also significantly increased cell migration, an effect which was attenuated in the presence of Gap 27 (P<0.001). Gap 27 inhibited hypoxic-induced increases in expression of phosphorylated p38 in rPAFs (P<0.05).

Conclusions: Our results show connexins play a role in hypoxic-induced proliferation and migration of rPAFs via activation of the p38 MAPK pathway.
Mutations in the gap junction protein connexin47 (Cx47) are associated with primary and secondary lymphedema. These mutations result in amino acid changes in various domains of the CX47 protein; i.e. H19P (N-terminus), S48L (first extracellular loop), R125Q and G149S (intracellular loop), R260C (second extracellular loop) and P316L (C-terminus). The functional consequences of these mutations on CX47 channels are largely unknown. Moreover, the role of Cx47 in lymphatic pathophysiology is unidentified. Here, we studied the in vivo function of lymphatic Cx47 using Cx47-deficient mice. Cx47 was found in lymphatic endothelial cells by quantitative PCR. Cx47-deficiency did not affect lymphatic contractility (contractile amplitude or frequency) or lymphatic morphology (vessel diameter or number of valves). Interstitial fluid drainage or dendritic cell migration through lymphatic vessels was also not affected by Cx47-deficiency. Cx47 did not affect the uptake of long-chain fatty acids from the gut but rather affected serum lipid handling with prolonged elevated triglyceride levels after oral lipid tolerance tests. When crossed with Apolipoprotein E-deficient (Apoe/-) mice, LDL-cholesterol was decreased in young Cx47/-/Apoe/- adults as compared to Apoe/- mice, which was inverted later in life. Finally, advanced atherosclerotic plaques in thoracic-abdominal aortas of 15 months-old mice tended to be larger in Cx47/-/Apoe/- mice. These plaques contained fewer macrophages but similar amounts of T lymphocytes, collagen and lipids than plaques of Apoe/- mice. In conclusion, Cx47 is expressed in lymphatic endothelium and seems modestly implicated in multiple aspects of lymphatic pathophysiology.
After injury to the skin, the normal adult wound healing process can result in cutaneous scars that are not only unpleasant to look at, but also stiff, painful, and liable to cause further injury at the wound site, sometimes severely affecting the patient’s quality of life. A peptide mimic of the carboxyl terminus of Connexin 43 (Cx43), αCT1 reduces Cx43 hemichannel density and activity in the membrane. We recently reported a Phase II study on 92 patients where αCT1 treatment resulted in improvements in cutaneous scar appearance by 9 months. To explore the anatomical basis of this change we examined patient scar biopsies sampled 1 month post-wounding from an earlier Phase I clinical trial. It was determined that deeper sectors of treated scar progenitor tissue in the dermis exhibited changes in collagen density, maturity and organization compared to placebo scars. Behavioral differences have also been identified in fibroblasts, wherein treated cells exhibited an αCT1 dose-dependent increase in migration speed and decrease in directional persistence. Preliminary findings suggest these changes in cell behavior are associated with defects in tail retraction of migrating cells. When αCT1-induced changes in fibroblast motility were programmed into a computational model, the simulated collagen matrix generated resembled that of αCT1-treated human scar biopsies. Preliminary analysis of splinted-wound rat skin at multiple time points exhibited trends that appeared to be consistent with this deep collagen difference at 4 and 6 weeks, and also displayed a decreased density of collagen fibers in the treated samples. In summary, we present in vivo scar tissue collagen organization and density data from rats and humans, in vitro fibroblast movement data, and an in silico analysis, exploring the mechanism and potential of αCT1 as a scar-reducing drug.
DIRECT SYNTHESIS OF TRUNCATED CONNEXINS: MECHANISM, REGULATION, AND FUNCTION IN HEALTH AND DISEASE

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We and others have demonstrated that direct protein synthesis of N-terminally truncated forms of Cx43 can occur. We showed this is mediated through a unique cap-dependent mechanism. We now provide further evidence towards the mechanism of translation initiation, its regulation, possible functions, and prevalence of internal translation in the connexin gene family.

We show phosphorylation of the cap-binding initiation factor eIF4E, in conjunction with several other trans-acting factors, regulates internal translation efficiency. Mutational analysis strongly suggests leaky ribosomal scanning is a key mechanism, and that the 5’UTR enhances this. Deletion analysis suggests that no internal IRES sequence is utilized and that the major 20-kDa form may be synthesized through a highly unusual non-AUG translation initiation mechanism. Expression of the 20-kDa isoform appears linked to the cell cycle, can be regulated by multiple signalling pathways including mTOR and Mnk1/2, and is subject to phosphorylation that depends on the presence of full-length Cx43. The physiological role of the C-terminal fragments remain poorly defined, but retroviral overexpression studies suggest major isoform-specific differences in terms of function and localization (to the cytoplasm, mitochondria, or nucleus). Other ongoing studies suggest translation of truncated connexin forms occur throughout the gap junction gene family, highlighting an important layer of complexity to be considered towards understanding the role of connexins and their associations in health and disease.

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DISTINCT INHIBITION AND MOLECULAR DETERMINANTS OF FLUORESCENT DYE PERMEABILITY VERSUS ION CONDUCTANCE IN ASTROCYTIC (HEMI) CHANNELS

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Astrocytic large-pore channels, such as connexin (Cx) hemichannels and pannexins (Px), serve, as yet, unresolved physiological roles as their individual permeability profiles remain disputed. The overlapping inhibitor profiles of these channels, and their different isoforms, complicate determination of the exact (patho)physiological contribution of each channel in complex cell systems. The astrocytic Cx hemichannels, Cx30 and Cx43, are both gated by extracellular Ca²⁺, the removal of which promote fluorescent dye uptake in Xenopus oocytes overexpressing these connexins. Only in Cx30-expressing oocytes is this fluorescent dye uptake paralleled by ion conductance. To determine the molecular determinants giving rise to the ability of Cx43 hemichannels to discriminate between atomic ions and fluorescent dye, we employed site-directed mutagenesis of predicted pore-lining residues in Cx43. In our ongoing studies, we seek to identify the pore-lining residues in Cx43 responsible for the prevention of hemichannel-mediated conductance. Furthermore, we characterized the individual inhibitory profile of commonly used inhibitors of the astrocytic hemichannels and Px1, and their distinct effect on dye uptake versus ion conductance. Lanthanum inhibited both transfer modes in the tested connexin hemichannels, whereas Px1 was largely insensitive to this inhibitor. Flufenamic acid showed isoform-specific inhibition of the connexin hemichannel-mediated conductance with little effect on their fluorescent dye uptake and on Px1 activity. Probenecid and carbenoxolone both efficiently inhibited Px1-mediated conductance, with little effect on its dye permeability, while only carbenoxolone exerted a slight inhibitory effect on Cx hemichannel-mediated conductance. The obtained inhibitor characterization promote caution when drawing conclusions based on commonly used (hemi)channel inhibitors and suggest that the inhibitory profiles of the astrocytic (hemi)channels depend on the nature of the permeant; inhibition of fluorescent dye uptake does not necessarily reflect inhibition of current and vice-versa.
CONNEXIN43 DOMAIN-SPECIFIC MUTATIONS PRESENT UNIQUE GAIN AND LOSS OF FUNCTION PHENOTYPES IN ODDD

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Oculodentodigital dysplasia (ODDD) is associated with over 78 mutations in the GJA1 gene encoding connexin43 (Cx43). In patients, mutations have been identified across all structural domains of Cx43 and are associated with a pleiotropic variety of disease phenotypes across tissues; however, functional outcomes of Cx43 domain-specific mutations remain difficult to predict as to whether they will be gain- or loss- of function mutations. We continue to expand our library of Cx43 gene mutations to include the first segment of the Cx43 cytoplasmic loop (CL) and 2nd extracellular loop (EL2). In early studies, EL2 Cx43 mutants demonstrated impaired plasma membrane localization in GJ-deficient Hela cells, where CL mutants did not. Ongoing investigations will continue to characterize domain-specific mutants to identify unique gain- or loss- of function Cx43 properties which may extend to changes in the Cx43 interactome. Astonishingly, cardiopathologies are rarely observed in ODDD patients, raising questions as to how the heart, a Cx43-abundant organ, is spared from disease. Recent findings revealed that proper GJ communication and Cx43 localization to cardiac intercalated discs (ICD) is regulated by a novel cardiac-enriched protein transmembrane 65 (Tmem65) (Sharma et al., 2015). In ODDD, we are investigating the notion that Tmem65 may continue to localize reduced levels of functional Cx43 to the ICD to preserve cardiovascular function. In the Cx43G60S/+ murine model of ODDD, ventricular tissue demonstrated similar Cx43 and Tmem65 mRNA expression between groups (littermate wildtype, n=6; mutant, n=4). Interestingly, protein expression of Tmem65 was similar between groups, where Cx43 expression was reduced in mutant ventricular tissue. Our studies will continue to characterize the interaction of Cx43, as well as other connexins present in the heart, with Tmem65 at the ICD. Overall, our findings will elucidate the mechanisms contributing to the pleiotropic domain- and tissue-specific functions of Cx43 in ODDD. Supported by the CIHR.
INTRINSIC ONCOGENIC FUNCTION OF INTRACELLULAR CONNEXIN PROTEINS IN HEAD AND NECK SQUAMOUS CELL CARCINOMA CELLS

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It has long been known that gap junction is down-regulated in many tumours. One of the downregulation mechanisms is translocation of connexin proteins from cell membrane into cytoplasm, nucleus, or Golgi apparatus. Interestingly, as tumours progress and reinforce their malignant phenotype, the amount of aberrantly-localised connexin increases in various malignant tumours including oesophageal squamous cell carcinoma, thus suggesting that such an aberrantly-localised connexin should be oncogenic although gap junctional connexin is tumour-suppressive.

To define the dual roles of connexin in head and neck squamous cell carcinoma (HNSCC), we introduced wild-type connexin26 (wtCx26) or Golgi-targeting mutant Cx26 (mtCx26) gene into human SAS-1 and FaDu lingual cancer cell lines, both of which had lost the expression of connexin during carcinogenesis. The wtCx26 protein was trafficked to the cell membrane and formed gap junction, which successfully exerted cell-cell communication. On the other hand, the mtCx26 protein was retained in the Golgi apparatus on the way to the cell membrane. While the forced expression of wtCx26 suppressed both cell proliferation in vitro and tumorigenicity in mice in vivo, mtCx26 significantly enhanced both cell proliferation and tumorigenicity compared with the mock control clones, indicating that an excessive accumulation of connexin protein in the Golgi apparatus should be involved in cancer progression and that restoration of proper subcellular sorting of connexin might be a therapeutic strategy to control HNSCC.
Psoriasis, a chronic inflammatory disease affecting 2-3% of the population, is characterised by epidermal hyperplasia, a sustained pro-inflammatory immune response and primarily thought to be a T-cell driven disease (inside-out). A shift in the microflora and elevated levels of the opportunistic skin pathogen Staphylococcus aureus occur on psoriatic plaques suggesting external factors play a role in disease progression (outside-in). Transcriptomic studies reported an 18 fold upregulation of Connexin26 (Cx26) in psoriatic tissue. We hypothesise that the shift in skin microflora coupled with the dysregulation of connexin expression are important drivers of the condition.

Biopsies from normal and psoriatic patients were collected. RNA was isolated and subject to real time PCR analysis to assess changes in gene expression between normal and psoriatic samples. Tissue was processed for histological (wax embedded) and immunohistochemical (frozen section) analysis (n=7). Human keratinocytes were exposed to peptidoglycan (PGN) isolated from S. aureus for 6 hours in the presence or absence of Cx channel blockers and IL-6 levels monitored by ELISA assays.

Histological analysis revealed classical hyperproliferative epidermal tissue within psoriatic plaques. Cx26 protein levels were upto 40x higher than normal and expressed in hyperproliferative psoriatic epidermal layers. By contrast Cx43 remained restricted to basal layers with a similar profile to unaffected and normal tissue. Real time PCR analysis further confirmed the upregulation of Cx26 gene expression (upto 100x greater than normal epidermis). Cx30, IL-6, TLR2 and Ki67 gene expression levels were also enhanced in psoriatic tissue while no change in Panx1 gene expression was observed. In a keratinocyte model cell line PGN isolated from S. aureus induced Cx26 expression, hemichannel signalling and IL-6 expression. Exposure to Cx channel blockers attenuated the PGN induced IL-6 response. Together the data suggest that controlling Cx26 expression and function may have therapeutic benefit for psoriasis and other inflammatory skin disorders.
PS2.22

MAGNESIUM-DEPENDENT PLASTICITY IN CONNEXIN 36 GAP JUNCTION CHANNELS

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Electrical synapses formed of connexin36 (Cx36) gap junction (GJ) channels are present throughout the mammalian brain and play an important role in neuronal synchronization. Cx36-mediated junctional conductance (gj) is modulated by changes in the concentration of intracellular free magnesium ([Mg²⁺]i); low or high [Mg²⁺]i increases or decreases gj, respectively. Cx36 GJs are permeable to Mg²⁺ ions and their effect on gj is fully reversible. Cx36-containing electrical synapses between neurons of the thalamic reticular nucleus (TRN) in mouse brain slices are similarly affected by changes in [Mg²⁺]i. Our data suggests that Mg²⁺ modulates Cx36 GJs by interacting with a Mg²⁺-binding site in the channel lumen. Chimeras were generated by exchanging corresponding domains from Cx36 to Cx43. Among sixteen generated chimeras, eight formed junctional plaques (JPs) and from those, only four exhibited functional GJ channels. Homotypic chimeric GJs in which the first extracellular loop (E1) of Cx36 was transferred to Cx43 (CH2 and CH3) showed enhanced sensitivity to Mg²⁺, while transferring NT/M1 of Cx43 into Cx36 (CH1) did not affect the Mg²⁺-sensitivity. Single channel conductance of CH3 and CH4 remained typical for Cx43 at high or low Mg²⁺. Hence, E1 of Cx36 contains a Mg²⁺-sensitive domain that controls the strength of electrical transmission. Mutations targeted to E1 of Cx36 revealed that aspartate in position 47 (D47) is determinant for high sensitivity to Mg²⁺ of Cx36. Furthermore, a Cx43G46D mutant gained strong Mg²⁺-sensitivity while a CH3D47G lost its high Mg²⁺-sensitivity, without changes in their single channel conductance. In summary, a number of chimeras and mutations demonstrate that D47 located in E1 of Cx36 determines the Mg²⁺-dependent plasticity in Cx36-containing electrical synapses. We suggest that this Mg²⁺-dependent plasticity could underlie changes in neuronal synchronization under conditions in which ATP levels, and consequently [Mg²⁺]i, are modified.
Previous in silico studies, developed in our laboratory, suggest that gating of human Cx26 hemichannels can be modulated by an intracellular water pocket (IC pocket). The presence of a water pocket has also been reported in Cx32 hemichannels. Both Cx26 and Cx32 belong to the β subfamily of connexins. To elucidate whether members of other connexin subfamilies also contain a water pocket, we chose human Cx50 (hCx50), a member of the α subfamily of connexins. Cx50 is expressed in the eye lens, and several mutations in its gene have been associated with congenital cataracts in humans. To test for the presence of the IC pocket in hCx50 and its possible functional role, we performed all-atom molecular dynamics simulations on wild type hCx50 hemichannels and on hemichannels formed by hCx50 containing mutations of some of the amino acid residues lining the IC pocket.

Our analyses are focused on the structure and dynamics of water molecules inside the IC pocket and ionic currents across the channel pore. We calculated occupancy, survival probability, and dipole vector orientation of water molecules, and made structural comparisons. In addition, we performed functional mode and essential dynamics analyses to identify relevant collective atomic motions of the hemichannel. Our results suggest that hCx50 hemichannels contain an IC pocket. Moreover, water occupancy and dynamics imply the presence of highly structured waters that make electrostatic interactions with particular amino acid residues lining the IC pocket. Thus, the existence of an IC pocket is a general feature of hemichannels formed by different connexins. Ongoing studies will evaluate its importance for hCx50 function.

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GAP JUNCTIONS ENHANCE THE ANTIPROLIFERATIVE EFFECT BY TRANSFER OF microRNA IN GLIOMA CELLS

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Objective: This study aimed to investigate the permeability of gap junction composed of connexin 43 (Cx43) for microRNAs (miRNAs) and the impact of gap junction-mediated transfer of miRNAs in glioma U87 cells.

Methods: Co-culture assay demonstrated the transmission of miRNAs through gap junction channel into adjacent cells. U87 cells were labeled with green fluorescein protein (GFP) as receivers and cells were transfected miRNAs as donors. Receiver cells and donor cells were mixed together in a ratio of 1:1. After 12h co-culture, cells were separated using a BD influx flow cytometer based on the GFP labeled. Quantitative real-time polymerase chain reaction (qRT-PCR) was applied detect to the expressions of miRNAs and Cx43 mRNA. Western blotting was performed to detect the protein expressions of Cx43 and GFP in U87 cells. CCK-8 assay is used to detect cell growth.

Results: Co-culture assays demonstrated miR-34a could transfer between U87 cells. The role of the contact independent could also transfer of miR-34a. Gap junctions inhibitor (CBX and 18-α-GA) showed lower miR-34a expression than co-culture group, whereas gap junctions enhancer (RA and Galanglin) enhanced miR-34a expression. Knockdown of Cx43 could significantly decrease the transferring of miR-34a between U87 cells. The length of twenty two or twenty three nucleotides miRNAs (miR-200b, miR-504, miR-517c and miR-135b) were similar to the expression of miR-34a between U87 cells. Additionally, we demonstrated that gap junctions mediate the effect of antiproliferation mediated by miR-34a in U87 cells. The functional inhibition of gap junctions using either siRNA or inhibition eliminated the miR-34a mediated antiproliferation, whereas the enhancement of gap junctions treatment augmented this miR34a-mediated antiproliferation.

Conclusion: Our study demonstrates that gap junction composed of Cx43-mediated transfer miRNAs in length of twenty two or twenty three nucleotides and gap junction-mediated transfer of miR-34a enhance the antiproliferative effect in glioma U87 cells.
ASSESSING THE ROLE OF CHARGES OVER IONIC TRANSPORT IN GAP JUNCTION CHANNELS

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The conductance of ion channels can be modulated by a transmembrane potential difference, due to alterations on ion-mobility and also by changes in the pore structure. Despite the vast knowledge regarding the influence of voltage on transport properties of ion channels, little attention has been paid to describe, with atomic detail, the modulation of ionic transport in gap-junction channels (GJCs). Hence, molecular dynamics simulations were performed to explore the conductance of simple dual-membrane systems accounting for the very basic features of GJCs. In doing so, we studied the influence of different charge distributions in the channel pore on these idealized systems under external electric fields, paying attention to the behavior of the electrostatic potential, ion density, ion currents, and equilibrium properties. Our results demonstrate that the incorporation of a charge distribution akin GJCs decreased anionic currents, favoring the transport of cationic species. Moreover, a thermodynamic characterization of ionic transport in these systems demonstrate the existence of a kinetic barrier that hinders anionic currents, reinforcing the role played by the internal arrangement of charges in GJCs. Overall, our results provide insights at the atomic scale on the effects of charge distributions over ionic transport, constituting a step forward into a better understanding of GJCs.

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SPATIOTEMPORAL REGULATION OF CONNEXIN43 GAP JUNCTION COMMUNICATION IN THE HEART

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In the heart, β-adrenergic receptor (β-AR) stimulation activates protein kinase A (PKA) and increases electrical conduction velocity (dromotropy). This process is mediated by connexin 43 (Cx43) that forms gap junction (GJ) channels and thus ensures a proper propagation of the electrical impulse, essential for sequential and coordinated contractions of cardiomyocytes. Regardless the causes, a chronic activation of the β-AR signaling cascade leads to heart failure and contributes to an aberrant Cx43 expression. This affects electrical conduction, induces arrhythmias and uncoordinated contractions. We have previously shown that in placental cells, PKA anchored to ezrin allows rapid and direct phosphorylation of Cx43, which enhances GJ communication. Considering the key role of Cx43 in the heart, we decided to investigate the putative presence of a PKA-ezrin-Cx43 complex in cardiac cells and to explore its functional role in cardiac physiology and pathophysiology. By immunoblotting, we found the expression of ezrin, Cx43 and PKA in purified neonatal rat ventricular cardiomyocytes (NRVCs). Immunolocalization studies showed that ezrin is expressed at the plasma membrane of NRVCs and adult ventricular cardiomyocytes. Furthermore, proximity ligation assays and co-immunoprecipitations revealed that ezrin forms a molecular complex that directs PKA in the vicinity of Cx43. Finally, gap FRAP (Fluorescence Recovery After Photobleaching) experiments in which PKA was displaced from A-kinase anchoring proteins (AKAPs) by HT31, presented a reduction of GJ communication in NRVCs upon chronic β-AR stimulation. These preliminary data suggest a role of PKA-ezrin-Cx43 complex in the regulation of GJ communication in the heart.
We have previously shown that inducing Cx37 expression in iRin cells profoundly suppresses their proliferation. This anti-proliferative effect requires interaction of the Cx37 carboxyl terminus (CT) with a Cx37 pore-forming domain able to form functional gap junction channels; the ability to form functional hemichannels (HCh) is not sufficient. Closer examination of iRin37 proliferative behavior following induced Cx37 expression reveals an early period of cell death, followed by a period of cell cycle arrest and ultimately proliferation. Here we address the possibility that differences in HCh function might determine these phenotypes. Cx37-WT forms HCh that function in the presence of 1mM \([\text{Ca}^{2+}]_o\). The closed state of the channel predominates, but WT-HChs open to multiple conductance states that are evident at membrane potential (Vm) of +25mV. The open probability of Cx37-WT channels is sensitive to \([\text{Ca}^{2+}]_o\), with 5mM generally sufficient to reversibly close the channels. We have identified mutations of Cx37 that specifically induce iRin cells to display each of the growth phenotypes induced by Cx37-WT (death, cell cycle arrest or proliferation). Despite the different phenotypic consequences of mutant expression, all of the assessed mutants form HChs that function in 1mM \([\text{Ca}^{2+}]_o\) (Vm= +25mV). It appears that HCh formed by mutants that induce apoptotic cell death or support proliferation may have a higher open probability of larger conductance HChs than mutants inducing cell cycle arrest. We have assessed the permeation characteristics of HChs formed by WT and a truncation mutant (proliferative phenotype) using KCl, tetraethylammonium chloride and potassium glutamate. As expected, WT HChs are overall cation selective (3.7:1); truncated Cx37 is also cation selective, but the preference for cations is reduced (2.4:1). The data suggest HCh permselective and open probability characteristics could contribute to Cx37-induced growth phenotype, but the regulatory sites in the CT and critical phenotypic-permeants remain unclear.
IDENTIFICATION OF THE E3 UBIQUITIN LIGASE ITCH AS A NOVEL REGULATOR OF CONNEXIN 43 DEGRADATION AND GAP JUNCTION INTERCELLULAR COMMUNICATION

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Increasing evidence suggests that gap junction intercellular communication can be regulated at the level of connexin turnover. Similar to other connexin isoforms, connexin 43 (Cx43) has a high turnover rate under basal conditions, typically displaying a half-life of 1.5-5 hours. Moreover, many oncogenes and tumor promoters, such as the protein kinase C activator TPA (12-O-tetradecanoylphorbol 13-acetate), are potent inducers of Cx43 degradation. However, the molecular basis underlying the regulation of Cx43 degradation remains poorly understood. The NEDD4 (neural precursor cell-expressed developmentally downregulated gene 4) family of E3 ubiquitin ligases consists of nine members, of which NEDD4 is the founding member. NEDD4 was the first E3 ubiquitin ligase found to interact with Cx43. Subsequently, two other members of the NEDD4 family, WWP1 and SMURF2, were shown to regulate Cx43 degradation. Here, we identify a fourth member of the NEDD4 family, termed ITCH, as a novel regulator of Cx43 degradation and gap junction intercellular communication. Furthermore, using HeLa cells stably transfected with Cx43 as a model system, we provide evidence that ITCH acts in concert with NEDD4 and SMURF2 to mediate Cx43 turnover. Simultaneous depletion of NEDD4, SMURF2, and ITCH by siRNA was found to result in a significantly lower Cx43 ubiquitination level and reduced Cx43 degradation under basal conditions. This was associated with increased size of gap junctions and enhanced gap junction intercellular communication. The triple knock-down of these three E3 ubiquitin ligases was also found to strongly counteract the TPA-induced degradation of Cx43. Collectively, these data indicate that ITCH acts together with NEDD4 and SMURF2 to mediate the basal and TPA-induced turnover of Cx43. To our knowledge, the data represent the first evidence that ubiquitination and turnover of Cx43, as well as the modulation of gap junction intercellular communication, is controlled by the concurrent activity of multiple E3 ubiquitin ligases.
LIPID PEROXIDES-INDUCED MODULATION OF CX46 HEMICHANNELS

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Hemichannels (HCs) are ion channels composed of six connexin (Cxs). They participate in several physiological and pathological processes. Therefore, the study of the molecular mechanisms that control HC activity appears to be essential for understanding their role of HCs in these processes. Carbon monoxide (CO) is a gaseous transmitter that modulates several cellular processes, some of them through modulation of ion channel activity. Recently, it has been demonstrated the application of CO donors to the bath solution inhibited Cx46 HC currents in Xenopus Laevis oocytes. Interestingly, the effect of CO was sensitive to reducing agents (e.g., GSH). CO can activate guanylate cyclase and induces direct carbonylation of proline, threonine, lysine and arginine. However, through a lipid peroxide dependent process, it can also induce cysteine (Cys) carbonylation. In this study was test the hypothesis that lipid peroxides can induce changes in Cx46 HCs. In this study was used Xenopus oocytes transfected with Cx46, and whole cell voltage clamp experiments were performed. Was found that the lipid peroxides 4-Hydroxy-2-nonenal (4-HNE) and 4-Oxo-2-NE inhibits Cx46 HC currents by 60 and 40 % respectively. However, other arachidonic acids products such as 12-HpETE and 15-HpETE which are produced by the action of enzymes produced the activation of Cx46 HCs by 67 and 123% respectively. Additionally, we observed that CO was able to induce lipid peroxides production in Xenopus Laevis and Vitamin C –a lipid peroxide inhibitor– importantly reduced (by 50%) the effect of CO upon Cx46 HCs. All these data suggest that CO induce (at least in part) the inhibition of Cx46 HCs through the production of Lipid peroxides, which in turn could induce the secondary carbonylation of this Cx.
USP8-MEDIATED DEUBIQUITINATION REGULATES THE LIFE-CYCLE OF CX43

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Connexin43 (Cx43) is a channel-forming transmembrane protein that allows the exchange of substances between the cytoplasm of adjacent cells, through gap junctions (GJ), or with the extracellular milieu, via undocked channels called hemichannels (HC). Although various mechanisms have been implicated in the regulation of intercellular communication (IC), including the number of functional Cx-containing channels at the plasma membrane, the molecular players and signals involved in the regulation of the intracellular trafficking of Cx43 remain largely unknown. We have previously shown that Cx43 ubiquitination mediated by the balanced action of the E3 ligase Nedd4 and the deubiquitinating enzyme (DUB) AMSH regulates the internalization and degradation of GJ. However, it is likely that other DUBs play important roles in modulating the levels of ubiquitination of Cx43, with consequences at the level of IC. Based on a DUB screening analysis, we identified USP8, as a new DUB acting upon Cx43. In this study, we show that USP8 interacts with Cx43 and mediates its deubiquitination and intracellular trafficking. Using siRNA depletion, we demonstrate that decrease in USP8-mediated deubiquitination of Cx43, leads to a reduction in degradation rate and increase in total levels of Cx43. Moreover we demonstrate that USP8 depletion leads to an accumulation of Cx43 both intracellularly and at the plasma membrane. Importantly, functional studies demonstrate that augmented levels of Cx43 at the plasma membrane contribute to increase HC activity. Overall, these data provide strong evidence that USP8-mediated deubiquitination of Cx43 constitutes an additional level of regulation of Cx43 intracellular trafficking. Furthermore, we suggest that impairment of USP8 activity can account to cellular dysfunction due to a enhanced activity of Cx43-containing HC.
INCREASED CONNEXIN43 EXPRESSION CORRELATES WITH THE LOSS OF BLOOD VESSEL INTEGRITY IN DIABETIC RETINOPATHY

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Background: Diabetic retinopathy is a chronic disease that develops due to hyperglycaemia and inflammation-induced vascular disruptions in the retina. This study investigated the interplay between hyperglycemia and inflammation using endothelial cells, mouse eyes and human donor tissues.

Methods: Primary human retinal microvascular endothelial cells (hRMECs) were exposed to high glucose (25 mM) or pro-inflammatory cytokines IL-1β and TNF-α (10 ng/mL each) or both. The lactate dehydrogenase (LDH) assay was used as a measure of cell death and Connexin43 expression was determined using immunohistochemistry. Cx43, glial fibrillary acidic protein (GFAP), and plasmalemma vesicular associated protein (PLVAP) were labelled in eye sections of wild-type (C57BL/6), Akita (diabetic) and Akimba (diabetic retinopathy) mice. The expression of Cx43 and GFAP was also studied in retinas from human donors with confirmed diabetic retinopathy and compared to normal eyes.

Results: Co-application of HG and pro-inflammatory cytokines increased LDH release in hRMECs relative to HG or pro-inflammatory cytokines alone. There was increased expression of Cx43 and GFAP in the ganglion cell layer in Akimba mice compared to wild-type, but not in Akita mice. On PLVAP-positive vessels, Cx43 was higher in Akimba mice but unchanged in Akita compared to wild-type. Cx43 expression was elevated in human donor retinas with confirmed diabetic retinopathy compared to normals, as seen in Akimba mice and correlating with the in vitro results.

Conclusions: The pathology of diabetic retinopathy seems to require the concerted action of hyperglycemia and inflammation and appears to be associated with increased Cx43 expression in areas of astrocytosis and neovascularization. These findings support a causal role of Cx43 channels in disease progression. Targeting Cx43 channels may be an effective strategy for the treatment of diabetic retinopathy.
Extra Villous Trophoblast (EVT) migration/invasion occurs in a hypoxic environment (2% of oxygen), as placental circulation has yet to be established. This condition would be interpreted as a cellular injury by many cell types, but EVT needs low levels of oxygen to thrive. Research in other tissues (i.e., lens and tumoral cells), has linked Connexin 46 (Cx46) with resistance to hypoxia, enabling the cell to survive longer periods under hypoxic conditions. Expression of Cx46 in Jar cell line (derived from choriocarcinoma) has been previously described, showing a decrease of Cx46 levels under hypoxia. This conflicting evidence has led to believe that regulation of Cx46 under low levels of oxygen may be tissue specific. However, expression of Cx46 in normal placenta derived cell lines has not been reported yet.

Objectives: this study aims to evaluate the expression of Cx46 in EVT cells and to study whether Cx46 plays a role in EVT survival under hypoxic conditions.

Methods: HTR8/SVneo cells were exposed to hypoxia (1% O2) for 24 or 48 h and compared with cells grown in 21% O2 environment. Western blot, indirect immunofluorescence and RT-PCR were used to determine protein and transcript expression, while dye uptake assays were performed to stablish hemichannel functionality.

Results: our results show that contrary to what has been described in Jar cells, HTR-8/Svneo constitutively express Cx46, and exposure to hypoxia increases its expression up to 4 times compared to control condition (21% O2).

Conclusions: These results suggest that hypoxia regulates Cx46 expression in HTR8/SVneo cells. As such, Cx46 could play an important role in hypoxia survival of EVT cells. Further experiments are required to stablish the influence of Cx46 over functional outcomes such as cell viability and migration/invasion capacity. If so, Cx46 would be an interesting target for pre-eclampsia studies.
ACTIVE ACETYLCHOLINE RECEPTORS PREVENT THE ATROPHY OF DENERVATED SKELETAL MUSCLES

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The denervation of skeletal muscles induces severe muscle atrophy, which leads to loss of muscle strength. Skeletal myofibers without neuronal supply manifest cellular alterations, prior to muscle atrophy, such as increased plasma membrane permeability affecting the electrochemical gradient, decreased in resting membrane potential and protein unbalance (Cea et al. PNAS 2013, Cisterna BBA 2016). However, up to now conditions or factors that prevent the unfolding of these changes remain unknown. In the present work we found that active acetylcholine receptors prevent the phenotype changes induced by denervation. Using in vitro assays, non-hydrolyzable acetylcholine analogs were found to represses the expression of connexin43 and connexin45 hemichannels. Our results show how the ACh released by motoneurons exerts a hitherto unknown function, independent of myofibers contraction. We anticipate our assay to be a starting point for more sophisticated studies intended to understand and use the protective effects of acetylcholine on denervated skeletal myofibers. For example, to identify the molecular mechanism by which the acetylcholine receptor inhibits the expression of connexin hemichannels. Therefore, acetylcholine receptors and connexin hemichannels are potential molecular targets for therapeutic intervention in a variety of pathological conditions with reduced synaptic neuromuscular transmission.
CONNEXIN 62 HEMICHANNELS AND GAP JUNCTIONS REGULATE PLATELET FUNCTION VIA ACTIVATION OF PKA IN A cAMP INDEPENDENT MANNER

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Introduction: Previously, we reported the presence of Cx37 and Cx40 in human platelets and that their selective inhibition, or deletion in transgenic-mice, attenuates platelet functions. Notable levels of Cx62 transcripts were also reported in megakaryocytes. The objective of this study is to determine the role of Cx62 in human platelets.

Methods: Immunoblotting and immunocytochemistry were performed to detect Cx62 in human/mouse platelets. A selective inhibitor (62Gap27) targeting Cx62 was designed. Platelet aggregation and dense granule secretion was analysed in washed platelets. Flow cytometry was used to study the degree of fibrinogen binding and α-granule secretion. Calcium mobilisation was performed by spectrofluorimetry in FURA-2AM loaded platelets and outside-in signalling was analysed by measuring clot retraction. Effects of 62Gap27 on in vitro (human blood) and in vivo (mice) thrombus formation were also examined.

Results: Western blotting and immunocytochemistry confirmed the expression of Cx62 in human/mouse platelets. CRP-XL or thrombin-stimulated platelet aggregation and dense granule secretion was inhibited by 62Gap27 treatment. No effect of scrambled peptide treatment was observed. 62Gap27 reduced CRP-XL or thrombin-stimulated fibrinogen binding and P-selectin exposure, indicating fundamental role of Cx62 in regulating inside-out signalling and secretion. Consistent with this, treatment with 62Gap27 also reduced calcium mobilisation and clot retraction. Thrombus formation under in vitro and in vivo setting was inhibited substantially by 62Gap27 suggesting the likely role of Cx62 in influencing haemostasis and thrombosis. An increase of PKA activation, VASP S157 phosphorylation, in a cAMP independent manner was also observed.

Conclusion: Cx62 is present in human/mouse platelets and exposure to the newly designed mimetic peptide 62Gap27 dampens platelet functions. Work is ongoing to identify the mechanisms/signalling through which Cx62 function in platelets and to determine the nature of the molecules trafficking through Cx62 hemichannels (isolated platelets) or gap junctions (within thrombus).
NOVEL FUNCTION OF CX30.3 IN MOUSE EMBRYONIC STEM CELLS

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Based on the dynamic expression patterns, 19 connexin (Cx) isoforms in mouse embryonic stem cells could be classified into pluripotent state specific, differentiating stage specific, and non-specific Cxs. We focused on Cx30.3 as typical of the first category. Cx30.3 was pluripotent state-specific and upregulated by leukemia inhibitory factor (LIF), a specific cytokine that maintains the pluripotent state of ES cell, via a Jak signaling pathway. Cx30.3 protein was localized to both the cell membrane and cytosol. The dynamic movement of Cx30.3 in the cell membrane was suggested by the imaging analysis by means of overexpressed Cx30.3-EGFP fusion protein. The cytosolic portion was postulated to be a ready-to-use Cx pool. The Cx30.3 expression level in ES cell colonies dramatically decreased immediately after their separation into single cells. It was suggested that mRNA for Cx30.3 and Cx30.3 protein might be decomposed more rapidly than mRNA for Cx43 and Cx43 protein, respectively. These indicate possible involvement of Cx30.3 in the rapid formation and/or decomposition of gap junctions; implying a functional relay between Cx30.3 and other systems such as adhesion proteins. Cx30.3 transfected ES cells revealed statistically significant larger cell population compared with wild type. This indicates that Cx30.3 affected the cell cycle control system in undifferentiated ES cells.
INSTABILITY OF CX43 GAP JUNCTION PLAQUES INCREASES BLOOD BRAIN BARRIER PERMEABILITY

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It has previously been shown that Cx30/Cx43 null mice display higher blood-brain-barrier (BBB) permeability following brief carotid perfusion with elevated hydrostatic pressure (Ezan et al, JCBFM 2012) and that infarct volume was increased in a stroke model involving mice with truncated Cx43 (Cx43K258stop mice) (Kozoriz et al. J Neuropathol Exp Neurol. 2010). Our group recently showed that differently from two astrocyte connexins (Cx26 and Cx30) which readily diffuse within the plaque structures, Cx43 remains persistently immobile after bleaching and that the cytoplasmic C terminus of Cx43 is required for Cx43 plaque stability. To further understand how astrocyte gap junctions control organization of this endfoot complex and thus establish and maintain BBB integrity, we used elevated hydrostatic pressure during perfusion to cause acute BBB damage and compared BBB integrity between the genotypes using HRP staining. In addition, we used immunocytochemistry and confocal and super-resolution microscopy to determine degree of co-localization of Cx43 and Cx30 at the interface between astrocytes and brain vasculature and determined the number and size of Cx43 and AQP4 particles. Our confocal data showed increased co-localization of Cx43 and Cx30 in astrocyte domains near vasculature of Cx43\textsuperscript{258/-} mice, and SIM analyses revealed that although the total number of Cx43 and AQP4 particles present in Cx43+/brain section did not differ from those obtained from Cx43\textsuperscript{258/-}, Cx43 and of AQP4 particles were significantly smaller in Cx43\textsuperscript{258/-} than in Cx43+/. The number of micro-hemorrhages per mouse and the density of micro-hemorrhages was significantly higher in Cx43\textsuperscript{258/-} brains compared to those from Cx43+/ mice. These results showing altered astrocyte nexus components (AQ4 and Cx30) in truncation mutants and increased BBB permeability support the hypothesis that localization and mobility of gap junction proteins and their binding partners determines organization of astrocyte endfeet which in turn impacts BBB integrity. Support: NS092466.
Gap junction channels (GJC) are built between neighbouring cells by docking of two connexons (hemichannels), which are made up of six connexins (Cx). It is assumed that hemichannels can be homomeric or heteromeric, depending on whether they are composed of one or more connexin types. Accordingly, the formation of gap junctions with variable connexin combinations has been postulated as a mode of fine tuning of the gap junction channels and their regulation in tissue.

eGFP labelled monomeric hCx26 and hCx46, two homodimers (hCx46-hCx46, hCx26-hCx26) and two heterodimers (hCx26-hCx46, hCx46-hCx26) were expressed in communication deficient HeLa cells. Confocal laser scanning microscopy showed that the tandems had the capability to form gap junction plaques, which, however, had a reduced plaque area compared to the monomeric hCx26 or hCx46. Using the whole-cell patch-clamp configuration with pipette solution containing Lucifer Yellow or AMCA (7-Amino-4-methyl-3-coumarinylacetic acid), we found that the homodimeric and heterodimeric constructs formed metabolically coupled gap junction channels like the monomeric constructs. Expressing the constructs in Xenopus laevis oocytes, two-electrode voltage-clamp (TEV) technique revealed that the tandems, as well as the monomeric constructs formed hemichannels, which were opened by depolarizing voltage steps and inhibited by classical hemichannel blockers, such as externally applied Ca²⁺ or La³⁺. Furthermore, we analysed the single channel activity of the different variants with the inside-out patch-clamp configuration. The two heterodimers hCx46-hCx26 and hCx26-hCx46 showed a higher single channel fluctuation rate, as well as more open states than the monomers and the homodimers. The study shows, that the concatemerization of connexins can be used to analyse the physiological consequences of the formation of heteromeric connexons, as well as the heteromeric-heterotypic gap junction channels.
INDUCTION OF CELL DEATH AND GAIN-OF-FUNCTION PROPERTIES OF CONNEXIN26 MUTANTS PREDICT DISEASE SEVERITY

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Connexin26 (Cx26) is a gap junction protein that oligomerizes in the cell to form hexameric transmembrane channels called connexons. Cell surface connexons dock between adjacent cells to allow for gap junctional intercellular communication. Numerous autosomal dominant mutations in the Cx26-encoding GJB2 gene lead to many skin disorders and sensorineural hearing loss. Although some insights have been gained into the pathogenesis of these diseases, it is not fully understood how distinct GJB2 mutations result in hearing loss alone or in skin pathologies with comorbid hearing loss. Here, we investigated five autosomal dominant Cx26 mutants (N14K, D50N, N54K, M163V, and S183F) linked to various syndromic or nonsyndromic diseases to uncover the molecular mechanisms underpinning these disease links. We demonstrated that when gap junction–deficient HeLa cells expressed the N14K and D50N mutants, they undergo cell death. The N54K mutant was retained primarily within intracellular compartments and displayed dominant or transdominant properties on wild-type Cx26 and coexpressed Cx30 and Cx43. The S183F mutant formed some gap junction plaques, but was largely retained within the cell and exhibited only a mild transdominant reduction in gap junction communication when co-expressed with Cx30. The M163V mutant, which causes only hearing loss, exhibited impaired gap junction function and showed no transdominant interactions. These findings suggest that Cx26 mutants that promote cell death or exert transdominant effects on other connexins in keratinocytes will lead to skin diseases and hearing loss, whereas mutants having reduced channel function but exhibiting no aberrant effects on coexpressed connexins cause only hearing loss. Moreover, cell death–inducing GJB2 mutations lead to more severe syndromic disease. Supported by the CIHR to DWL.
PS2.60

PANNEXIN1 SUSTAINS ELECTROPHYSIOLOGICAL RESPONSIVENESS OF RETINAL GANGLION CELLS

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Pannexin1 forms ATP-permeable membrane channels is expressed at high levels in ganglion cells (RGCs) of the retina and involved in purinergic signaling. However, its normal physiological function in the CNS is not defined. In this study, we used patch clamp recordings and pattern electroretinogram (PERG) to examine a role of Panx1 in generation of normal membrane currents in retinal neurons and visual function, respectively.

Membrane currents, characteristic for the Panx1 channel activity, were selectively evoked in RGCs. A targeted ablation of the Panx1 gene in RGCs resulted in an impaired PERG. Under pathological conditions, induced by elevated intraocular pressure, Panx1 played a distinct role and facilitated selective RGC loss, which was suppressed by either gene ablation or pharmacological channel blockade. We discovered robust activation of the inflammasome in the mouse retina with IOP-induced ischemia and/or glaucoma. Acute increase in the levels of inflammasome caspase-1 and -11, as well as secretion of mature IL-1b were significantly decreased in the retinas of Casp1-null, Panx1-null and WT probenecid-treated mice relative to IR-exposed control retinas. These results imply that high level Panx1 expression in RGCs is essential for the visual function in the inner retina. The high activity of Panx1, however, makes these cells highly sensitive to mechanical and ischemic stresses, such as those imposed by elevated intraocular pressure. These findings suggest that controlling inflammasome via manipulation of Panx1 activity can protect retinal neurons in glaucoma therapies.
Pannexins (Panx1, Panx2, Panx3) are a family of channel-forming glycoproteins at the plasma membrane that are generally thought to mediate the release of molecules and ions from the cell to the extracellular environment. However, since their discovery, the breadth of subcellular localization for both endogenous and ectopically expressed Panxs has varied widely, and is often attributed to the Panx subtype expressed or the cell type being investigated. In order to understand the scope of Panx functions, it has become critically important to clearly resolve where Panxs reside in both non-polar and polarized cells. To this end, we are investigating endogenously and ectopically expressed human and rodent Panx1 and Panx3 in live and fixed reference cells that include the use of next-generation fluorescent protein tags. In non-polar BICR-M1Rk rat mammary tumor cells, Panx1-GFP was highly mobile and evenly distributed within all plasma membrane domains, including filapodia and lamellipodia. In MDCK cells, both Panx1-moxGFP and Panx3-moxGFP were found at the cell surface but frequently co-localized with the endoplasmic reticulum marker PDI, though it is unclear whether this cellular distribution was due to the presence of the fluorescent tag or the host cell type. When these same MDCK cells were grown in 3D, the resulting lumen-containing cyst-like cellular structures revealed that endogenous Panx1 may be regulated by the cellular microenvironment. Studies investigating whether Panx1/3 reside in the apical and/or basolateral domains of 3D organoids as well as in vivo are currently underway. Preliminary results suggest that Panx1/3 acquire multiple subcellular residencies, retain dynamic mobility within cellular membranes, and their expression levels may be dynamically regulated by signals received from the cellular microenvironment. Supported by the Canadian Institutes of Health Research.
NEW 4-STATE MODEL OF GAP JUNCTION CHANNEL VOLTAGE GATING TO DESCRIBE FAST KINETICS OF JUNCTIONAL CONDUCTANCE IN EXCITABLE CELL TISSUES

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Gap junctions formed of connexin (Cx) protein ensure the spread of electrical signal in cardiac tissue. Although it is well established that gap junctional conductance (gj) depends on transjunctional voltage (Vj), in most of models, which describes networks of excitable cells, it is assumed that gap junctions exhibit a constant and ohmic conductance. On the other hand, gap junctional conductance is usually described by a steady state gj-Vj relationship using the Boltzmann function. Such approach is not applicable in cardiac tissue models, because it requires a few seconds to reach a steady state gj value at a given Vj in electrophysiological experiments, while the typical Vj spikes which develop during cardiac action potentials are much shorter (~0.1-0.3 s). In this study, we present a new 4-state model which could adequately describe the fast kinetics of junctional conductance in response to rapid transjunctional voltage changes. In our model, each hemichannel exhibits two states - open (o) and closed (c) - therefore overall number of states is 4 (oo, oc, co and cc). The rates of hemichannel gating transitions are estimated under an assumption that free energy differences between system states linearly depend on transjunctional voltage across each hemichannel. The feasibility of the model is verified by electrophysiological experiments in cells transfected with Cx43 and Cx45, which are abundantly expressed in cardiac tissues. We also illustrate the influence of voltage gating in simulated spread of cardiac excitation between cells connected through Cx43 and Cx45 gap junctions. Our modeling results demonstrate that Vj spikes, which develop during the spread of cardiac action potentials are capable to significantly reduce gj, especially in a more Vj sensitive Cx45 gap junction.
Gap junctional intercellular communication (GJIC) is essential for regulation of main physiological processes in male reproductive system, including testicular development and homeostasis, cell proliferation and differentiation, regulation of hormone release, initiation and maintenance of spermatogenesis. Thus, untimely dysregulation of GJIC during critical stages can result in male reproductive dysfunctions such as impaired spermatogenesis, increased germ cell apoptosis and anarchic proliferation, loss of blood-testis barrier integrity, hyperplasia of androgen producing Leydig cells, and decreased sperm motility. This talk will focus on GJIC as an important but overlooked molecular target for environmental toxicants in male reproductive system and will discuss the molecular signalling events occurring in response to toxicants and how these pathways are integrated with GJIC. Findings from experiments with somatic testicular cells exposed to environmentally relevant toxicants such as pesticides and polycyclic aromatic hydrocarbons aromatic hydrocarbons will be presented in the context of cell regulatory mechanisms of gap junctions with an emphasis on linking these molecular signalling events with cellular responses such as steroidogenesis, apoptosis and proliferation. Acknowledgment: This research is supported by Czech Science Foundation Project No. GA16-10775Y.
STRUCTURAL STUDIES OF THE NEDD4 WW DOMAINS AND THEIR SELECTIVITY FOR THE CX43 CARBOXYL-TERMINUS

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Nedd4 was the first ubiquitin protein ligase identified to interact with Cx43 and its suppressed expression results in accumulation of gap junction plaques at the plasma membrane. Nedd4-mediated ubiquitination of Cx43 is required to recruit Eps15 and target Cx43 to the endocytic pathway. While the Cx43 residues that undergo ubiquitination are still unknown, in this study we address other unresolved questions pertaining to the molecular mechanisms mediating the direct interaction between Nedd4 (WW1-3 domains) and Cx43 (carboxyl terminus, CT). All three WW domains display a similar three antiparallel β-strand structure and interact with the same Cx43CT₂₈₃PPₓY₂₈₆ sequence. While Y286 is essential for the interaction, MAPK phosphorylation of the preceding serine residues (pS279 and pS282) increases the binding affinity by two-fold for the WW domains (WW2>WW3>>WW1). The structure of the WW2/Cx43CT₂₇₆-₂₈₉(pS279,pS282) complex reveals that coordination of pS282 with the end of β strand 3 enables pS279 to interact with the back face of β strand 3 (Y286 is on the front face) and loop 2, forming a horseshoe-shaped arrangement. The close sequence identity of WW2 with WW1 and WW3 residues that interact with the Cx43CT PPₓY motif and pS279/pS282 strongly suggest that the significantly lower binding affinity of WW1 is the result of a more rigid structure. This study presents the first structure illustrating how phosphorylation of the Cx43CT domain helps mediate the interaction with a molecular partner involved in gap junction regulation.
ASTROCYTE CONNEXIN43 STABILIZES ENDOTHELIAL TIGHT JUNCTIONS AND CONTROL TIGHTNESS OF THE BARRIER

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BBB integrity is reportedly challenged in Cx43/Cx30 double knockout (KO) mouse brain (Ezan et al. JCMFM. 2012); such impact of connexins on BBB function was attributed to reduced AQP4 and β-dystroglycan at the astrocyte endfeet. We hypothesized that astrocyte Nexus besides contributing to the structure and composition of endfoot markers also stabilize endothelial cell tight junctions and thus decrease BBB permeability. To that end, we co-cultured immortalized wildtype (iWT) and Cx43KO (iCx43KO) astrocytes with mouse brain endothelial (bEnd.3) cells. Fluorescence recovery after photobleach (FRAP) of sfGFP-ZO-1 expressing bEnd3 cells in absence of astrocytes indicated relatively rapid recovery of ZO-1 (0.33±0.07µm\textsuperscript{2}/sec) but no recovery when co-cultured with astrocytes; thus, astrocytes stabilize ZO-1. Trans-endothelial resistance measurements revealed slightly higher barrier resistance in astrocyte co-cultures compared to monocultures (22.7±4.3 vs 31.1±1.8 ohm.cm\textsuperscript{2}, N=3). Permeability to 10 kDa fluorescein dextran of monocultures was significantly lower than co-cultures with astrocytes (1.41±0.26e-006cm/s vs 5.16±1.34e-007cm/s, N=10), indicating that astrocytes promote endothelial barrier tightness. Cx43 contribution to this process was revealed by comparing permeability of bEnd.3 co-cultured with iWT and with iCx43KO astrocytes; permeability of bEnd.3/iCx43KO astrocyte co-cultures was 1.46±0.16 (N=10) fold higher than in bEnd.3/iWT astrocyte co-cultures. Transient barrier disruption in this in vitro BBB model with brief pulsed ultrasound increased BBB permeability within few minutes which was restored 3 hours after the insonation. Five min after insonation, permeability of co-cultures with iCx43KO astrocytes was 1.75±0.29 (N=6) fold higher than with iWT astrocytes. Immunocytochemistry indicated that in both type of co-cultures, endothelial ZO-1 was remodeled after insonation and partially recovered after 3 hrs. Compared to non-sonated iWT astrocyte co-cultures, the amount of ZO-1 present at bEnd.3 cell-cell contacts was substantially lower. Together, these results indicate that astrocyte Cx43 contributes to BBB tightness by promoting and stabilizing tight junctions in endothelial cells. Support: NS092466.
CONNEXIN43 AND RUNX2 INTERACT TO AFFECT CORTICAL BONE GEOMETRY, SKELETAL DEVELOPMENT, AND OSTEOBLAST AND OSTEOCLAST FUNCTION

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The coupling of osteoblasts and osteocytes by connexin43 (Cx43) gap junctions permits the sharing of second messengers that coordinate bone cell function and cortical bone acquisition. However, details of how Cx43 converts shared second messengers into signals that converge onto essential osteogenic processes are incomplete. Here, we use in vitro and in vivo methods to show that Cx43 and Runx2 functionally interact to regulate osteoblast gene expression and proliferation, ultimately affecting cortical bone properties. Using compound hemizygous mice for the Gja1 (Cx43) and Runx2 genes, we observed a skeletal phenotype not visible in wild-type or singly hemizygous animals. Cortical bone analysis by microCT revealed that 8-week-old male, compound Gja1+/-Runx2+/- mice have a marked increase in cross-sectional area, endosteal and periosteal bone perimeter, and an increase in porosity compared to controls. These compound Gja1+/-Runx2+/- mice closely approximate the cortical bone phenotypes seen in osteoblast-specific Gja1-conditional knockout models. Furthermore, microCT analysis of skulls revealed an altered interparietal bone geometry in compound hemizygotes. Consistent with this finding, Alizarin red/Alcian blue staining of 2 day-old Gja1+/-Runx2+/- neonates showed a hypomorphic interparietal bone, an exacerbation of the open fontanelles, and a further reduction in the hypoplastic clavicles compared to Runx2+/- neonates. Gene expression of several osteoblast genes, including osteocalcin, osterix, periostin, and Hsp47, were markedly reduced in tibial RNA extracts from compound hemizygous mice. Osteoblasts from compound hemizygous mice displayed an increase in proliferative capacity. Further, the reduced osteocalcin expression and hyperproliferative nature of osteoblasts from Cx43 deficient mice was rescued by the overexpression of Runx2. In summary, these findings provide evidence that Cx43 and Runx2 functionally intersect in vivo to regulate osteoblast differentiation and cortical bone properties, and the convergence of Cx43 and Runx2 contributes to aspects of the skeletal phenotype of Cx43 conditional knockout mice.
THE CONNEXIN 43 CARBOXYL-TERMINUS AFFECTS TIGHT JUNCTION FUNCTION VIA ZO-1 TARGETING

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Cx43 is the most widely expressed and best-studied connexin. There is evidence that Cx43 may alter cell-to-cell contact arrangements and cytoskeletal dynamics, as well as participate in transcriptional regulation. Cx43 shares a common adaptor molecule and signaling hub, Zonula Occludens 1 (ZO-1), with adherens junctions and tight junctions, linking multiple families of junctional transmembrane proteins to the actin cytoskeleton. Using both classical and automated in-vitro methods of measuring resistance/impedance across epithelial monolayers, recent studies have demonstrated that αCT1, a Cx43 carboxyl-terminus (CT) mimetic peptide, stabilized tight junctional complexes. In a classical Transepithelial Resistance method, αCT1 prevented VEGF-mediated breakdown of tight junction-based barrier function and stabilized RPE tight junctions in a model of Age Related Macular Degeneration (Obert, Strauss et al. 2017 PMID 28132078). Another well-known model of barrier function disruption with Ca²⁺ chelator EGTA resulted in consistent findings, as assessed by real-time impedance monitoring of cell monolayers by electronic cell substrate impedance sensing (ECIS). In this model, αCT1-treated cells produced a greater percentage of barrier function recovery compared to EGTA-alone treated cells. Moreover, using αCT1 variant peptides designed to abrogate ZO-1 binding, our preliminary data indicates that barrier function recovery with αCT1 requires ZO-1 interaction. Importantly, the ECIS experiments were undertaken in Cx43-deficient MDCK cells, indicating that any effect on barrier function and/or tight junctions was independent of Cx43. Taken together, αCT1, a Cx43 CT mimetic, stabilized tight junctions in two independent models (ARPE-10 and MDCK cell monolayers) triggered by two methods (VEGF and calcium chelation) in a manner that appears to be independent of Cx43 function.
CARDIAC CONNEXIN-43 IN RATS WITH ALTERED THYROID STATUS AND OMEGA-3 FATTY ACIDS OR RED PALM OIL SUPPLEMENTATION

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Thyroid hormones are powerful modulators of heart function. We have previously shown that hyperthyroidism down-regulates myocardial Cx43 as well as PKCε and it was associated with increased susceptibility of the heart to ventricular fibrillation (VF). This study was designed to explore impact of both hyper-and hypo-thyroidism on cardiac Cx43 in rats without and with cardio-protective edible oils intake. Experiments were performed on adult male euthyroid, hyperthyroid (treated with T3/T4), hypothyroid (treated with methimazole) rats supplemented or not with red palm oil (RPO, 100 µl/100g/day) and Omacor (omega-3, 20 mg/100 g/day) for 6 weeks. Left ventricle tissue was used for detection of Cx43 and PKCε protein expression and for matrix metalloproteinases-2 (MMP-2) activity. VF threshold was examined using isolated heart preparation. Total expression of Cx43 and its phosphorylated forms were significantly increased in hypothyroid rats compared to euthyroid controls. In contrast, the total levels of Cx43 and its functional phosphorylated forms were decreased in hyperthyroid rats. In parallel, the expression of PKCε that phosphorylates Cx43 was increased in hypothyroid but decreased in hyperthyroid rat hearts. Activity of MMP-2, implicated in extracellular matrix remodeling, was significantly decreased in hypothyroid rats but unchanged in hyperthyroid rat hearts. VF threshold was significantly lower in hyperthyroid rat hearts, while in hypothyroid group, even the highest threshold (50mA) did’t induce VF. RPO and Omega-3 intake resulted in significant increase of phosphorylated forms of Cx43 as well as PKCε expression in hyperthyroid rats. It was associated with decreased vulnerability to VF. In conclusion, there is an inverse relationship between expression of cardiac Cx43 and the levels of circulating thyroid hormones. It appears that increased propensity of hyperthyroid while decreased of hypothyroid individuals to malignant arrhythmias may be in part attributed to down-and up-regulation of Cx43. Edible oils intake partly ameliorated adverse changes caused by excess of thyroid hormones.

Key words: Thyroid hormones, Rat heart, Connexin 43, PKCε, MMP-2, Edible oils intake

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NONJUNCTIONAL CX32 MEDIATES ANTI-APOPTOTIC AND PRO-TUMOR EFFECTS VIA EPIDERMAL GROWTH FACTOR RECEPTOR IN HUMAN CERVICAL CANCER CELLS

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The role of connexin proteins (Cx), which form gap junctions (GJ), in progression and chemotherapeutic sensitivity of cervical cancer (CaCx), is unclear. Using cervix specimens (313 CaCx, 78 controls) and CaCx cell lines, we explored relationships among Cx expression, prognostic variables and mechanisms that may link them. In CaCx specimens, Cx32 was upregulated and cytoplasmically localized, and three other Cx downregulated, relative to controls. Cx32 expression correlated with advanced FIGO staging, differentiation and increased tumor size. In CaCx cell lines, Cx32 expression suppressed streptonigrin/cisplatin-induced apoptosis in the absence of functional GJ. In CaCx specimens and cell lines, expression of Cx32 upregulated epidermal growth factor receptor (EGFR) expression. Inhibition of EGFR signaling abrogated the anti-apoptotic effect of Cx32 expression. In conclusion, upregulated Cx32 in CaCx cells produces anti-apoptotic, pro-tumorigenic effects in vivo and vitro. Abnormal Cx32 expression/localization in CaCx appears to be both a mechanism and biomarker of chemotherapeutic resistance.
DIFFERENTIAL LOCALIZATION OF MURINE CaMKII ISOFORMS INDICATES CaMKII-Beta AS A SPECIFIC ELEMENT OF RETINAL GAP JUNCTIONS MADE OF CONNEXIN36

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All amacrine cells are essential interneurons of the primary rod pathway and transmit rod-driven signals onto cone bipolar cells in order to enable vision under scotopic conditions. Gap junctions made of connexin36 (Cx36) mediate electrical coupling between these cells and were shown to underlie a remarkable degree of plasticity as they are modulated by different signaling cascades. In particular Calcium/calmodulin-dependent protein kinase II (CaMKII) has been characterized as an important regulator of Cx36, capable of potentiating electrical coupling. However, most observations focused on the physiological effects of the enzyme rather than its different isoforms. In order to obtain a more detailed understanding of the subunit composition of CaMKII at retinal gap junctions, we analyzed the cellular expression and distribution of all four CaMKII isoforms at electrical synapses using confocal microscopy. These experiments revealed a differential distribution for all CaMKII isoforms: Strong CaMKII-α expression was detected in starburst amacrine cells which represent retinal interneurons known to lack electrical coupling. CaMKII-β labeling was absent in these cells but strongly evident in AII amacrine cells, which contain the majority of Cx36-immunoreactive puncta in the inner retina. CaMKII-γ was diffusely distributed in the entire retina, whereas CaMKII-δ was prominently localized in the synaptic terminals of rod bipolar cells. Furthermore, we performed double labeling experiments with Cx36 and each CaMKII isoform. Among all four CaMKII isoforms only CaMKII-β colocalized with Cx36. This association appeared to be connexin-specific since we did not observe an apparent colocalization between Cx45 and any of the other tested CaMKII isoforms. Taken together, our study identifies CaMKII-β as a Cx36-specific regulator in the mouse retina.
DOWN-REGULATION OF CONNEXIN45 IN HUMAN AORTIC SMOOTH MUSCLE CELLS ATTENUATES CONTRACTILE ABILITY

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Background Connexin (Cx) isoforms expressed in vascular smooth muscle cells (SMCs) have been reported not only to form gap junction channels but also possess other biological function. Previous studies showed that connexin isoforms expressed in SMCs are Cx37, Cx40, Cx43 and Cx45, of which the role of Cx45 remains unclear, even after comprehensive investigation of Cx45 knockdown animals.

Methods and Results Connexin isoform expression level was determined in human aortic smooth muscle cells (HASMCs) using real-time PCR and Cx43 was most abundant followed by Cx45, Cx40 and Cx37 (respectively 1/100, 2/10000, and 1/10000 of Cx43). Cx45 expression was reduced by 3 different sequences of short interfering RNA (siRNA) specific to human Cx45 followed by phenotypic characterization of the cells. The results showed that at a level down to 30% expression of Cx45, gap junctional intercellular communication (GJIC) evaluated using fluorescence recovery after photobleaching (FRAP) was minimally affected. In contrast, HASMC proliferation was reduced by Cx45 siRNA, similar to cells treated with Cx43 siRNA. When HASMCs were shifted from synthetic to contractile phenotype, cell contraction ability, analyzed using collagen gel assay, and alpha smooth muscle actin (α-SMA) expression were reduced by Cx45 siRNA (contraction, 30% decrement; α-SMA, 50% decrement), but minimally affected by Cx43 siRNA. Intracellular levels of cyclic adenosine monophosphate (cAMP), a second messenger molecule involving cell relaxation pathway, was found increased after Cx45 down-regulation. However, cyclic guanosine monophosphate (cGMP) remained stationary. Down-regulation of α-SMA by siRNA did not significantly affect the contractility.

Conclusion Although Cx45 was not critical for GJIC in HASMCs, down-regulation of Cx45 lead to reduced contractility of the cells via cAMP regulation, suggesting that Cx45 may play a key role in maintenance of vascular tone.

Keywords: SMC, connexin45, contractility, cAMP
PS2.84

E3 UBIQUITIN LIGASE NEDD4 INDUCES ENDOCYTOSIS AND LYSOSOMAL SORTING OF CONNEEXIN 43 TO PROMOTE LOSS OF GAP JUNCTIONS

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The E3 ubiquitin ligase NEDD4 (neural precursor cell-expressed developmentally downregulated gene 4) has previously been demonstrated to interact with the C-terminus of connexin 43 (Cx43) and to facilitate its autophagy-mediated degradation in response to serum starvation. The objective of the present study was to obtain a better understanding of the role of NEDD4 in the regulation of Cx43 degradation and gap junction levels under basal conditions and in response to protein kinase C (PKC) activation. Moreover, since NEDD4 is a proto-oncogene and is frequently overexpressed in human carcinomas, we aimed to investigate how overexpression of NEDD4 in carcinoma cells affects Cx43 protein and gap junction levels. NEDD4 contains a C-terminal HECT (homologous to E6-AP carboxyl terminus) domain, which catalyzes the covalent attachment of ubiquitin to substrate proteins. Within the HECT is a catalytic cysteine residue, which acts as a site for thiol ester formation with ubiquitin prior to the transfer of ubiquitin to a lysine residue in the substrate protein. Our data demonstrate that NEDD4 controls the Cx43 protein level and gap junction size in HeLa cells under basal conditions. NEDD4 was also found to be involved in mediating the PKC-induced endocytosis and degradation of Cx43. Evidence is further provided that ectopic overexpression of NEDD4 results in loss of gap junctions in both HeLa and C33A cervical cancer cells. In contrast, overexpression of NEDD4 containing an inactivating mutation in the catalytic cysteine of the HECT domain did not affect gap junction levels. Collectively, the data provide new insights into the molecular basis underlying the regulation of gap junction levels and represent the first evidence that an oncogenic E3 ubiquitin ligase promotes loss of gap junctions and Cx43 degradation in human carcinoma cells.
REGULATION OF CX32 BY EPHRIN RECEPTORS AND TC-PTP

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All stages of the connexin life cycle, which include assembly, degradation, regulation of electrical and metabolic coupling, as well as regulation of interactions with other proteins, involve phosphorylation. Cx32, the major gap junction protein in liver and brain, also found in a number of other tissues, contains 12 intracellular Ser residues and can be in vitro phosphorylated by CAMKII, PKA, and PKC. Additionally, Cx32 contains three intracellular Tyr residues (NT, Y7; CT, Y211 and Y243) and has been shown to be phosphorylated on Tyr through activation of the EGFR, however the specific residue and its functional implications were not identified. Based upon the limited available information of Cx32 regulation by tyrosine kinases, we used an in vitro kinase-screening assay, which identified Ephrin family members as novel tyrosine kinases that phosphorylate Cx32. EphB1 phosphorylates the Cx32CT residue Y243. Contrary to Cx43, phosphorylation of Cx32 by EphB1 increases the number of gap junctions at the plasma membrane in HeLa cells, which led to enhanced cell-cell communication. We also demonstrate that the TC-PTP dephosphorylates Cx32CT residue pY243, both in vitro and in HeLa cells. The importance of this study is that it directly links phosphorylation of a specific Cx32 tyrosine residue to intercellular communication and provides support that characterization of the same kinase for different connexin isoforms is important, as the functional significance cannot be inferred from another isoform.
DISTINCT PERMEABILITY PROPERTIES OF LENSES’ GAP JUNCTION CHANNELS TO SECOND MESSENGERS cAMP, IP3 AND Ca2+

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A striking feature of connexin channels is their diverse selectivity in intercellular passage of larger solutes like second messengers, which may have specific roles in growth control and differentiation of different cell types.

We compared the permeability of cAMP, IP3, and Ca2+ through gap junction channels of lens connexins. Intercellular solute transfer was investigated in HeLa cells expressing Cx43, Cx46, or Cx50 and measuring cell-to-cell flux while simultaneously monitoring junctional conductance. For cAMP detection, recipient cells were transfected with the cyclic nucleotide-modulated channel (SpIH), which acted as a reporter. cAMP was introduced via patch pipette into the cell not expressing SpIH within a pair. In the case of cAMP diffusion to a neighboring SpIH transfected cell, SpIH-derived currents increased over time. For IP3 and Ca2+ detection, cells were loaded with Fluo-8, the Ca2+-binding dye. IP3 and/or Ca2+ were delivered via patch pipette to one cell of a pair or to a monolayer while fluorescence intensity changes were recorded.

Homotypic Cx43 channels were permeable to cAMP, IP3, and Ca2+ over a wide range of conductances. Conversely, homotypic Cx50 channels were impermeable to IP3 and cAMP, while exhibiting high permeation to Ca2+. Negatively charged species (cAMP and IP3) showed a similar permeability order: Cx43>>Cx50. Positively charged Ca2+ permeability was comparable: Cx43≈Cx50. In addition, preliminary data shows that Cx46 channels exhibited moderate cAMP permeability: Cx43>Cx46>>Cx50.

These results confirm that connexin channels can discriminate between solutes based on size and/or charge, suggesting that channel selectivity is a key factor in cell signaling. Furthermore, second messenger permeability of Cx43 results in a rapid delivery of cAMP, IP3, and Ca2+ between cells in sufficient quantities to trigger relevant cellular responses. Alternatively, reduced Cx50 permeability to cAMP and IP3 may play a role in regulating cell division, differentiation, and homeostasis of the lens.

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CONNEXIN43 OVEREXPRESSSION MIMICS OSTEOARTHRITIC PHENOTYPE IN HUMAN ADULT ARTICULAR CHONDROCYTES

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Osteoarthritis (OA) is the most common degenerative rheumatic disease, being its ethiology still poorly understood. The disease is primarily characterised by articular cartilage degradation following by joint degeneration. In the early stages, osteoarthritic chondrocytes (OACs) undergo phenotypic changes increasing cell proliferation, cluster formation and production of matrix-remodelling enzymes. OACs contain high levels of Cx43, that together with increased gap junction intercellular communication (GJIC) and changes in Cx43 interaction network have been associated with development and progression of OA. The aim of this study was to investigate the role of Cx43 in the dedifferentiation processes that suffer OACs. The human chondrocyte T/C-28a2 cell line was transfected with a plasmid containing the human Cx43 sequence. Cx43 overexpression and cellular localization was confirmed by western blot, qPCR, flow cytometry and immunofluorescence. The overexpression of Cx43 led to a significant increase in the expression of the inflammatory mediators COX-2 and IL-1 and matrix degrading enzymes such as MMP-3. Collagen type 2 levels were significantly diminished in Cx43 transfected chondrocytes in comparison with the control cells. Cell proliferation measured by iCelligence and Click-iT assays and GJIC tested by lucifer yellow transfer increased significantly when Cx43 is upregulated. Flow cytometry analysis revealed an increase in the levels of stemness-like cell surface markers related with an immature state when Cx43 is overexpressed. Our results suggest that Cx43 upregulation is involved in the phenotypic changes and dedifferentiation processes detected in OACs responsible for degradation of cartilage and its predisposition to develop OA. In the light of these results, it is important to take into account Cx43 levels and function in the scope of designing more effective targeted therapies for prevention and treatment of OA.
SPECIFICITY OF THE CONNEXIN W3/4 LOCUS FOR FUNCTIONAL GAP JUNCTION FORMATION

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The N-terminal (NT) domain of the connexins forms an essential transjunctional voltage (Vj) sensor and pore-forming domain that when truncated, tagged, or mutated often leads to formation of a nonfunctional channel. The NT domain is relatively conserved among the connexins though the α and δ-group connexins possess a G2 residue not found in the β- and γ-group connexins. Deletion of the connxin40 G2 residue (Cx40G2Δ) affected the Vj gating, increased the single channel conductance (γj), and decreased the relative K+/Cl- permeability (PK/PCI) ratio of the Cx40 gap junction channel. The conserved α/β-group connexin D2/3 and W3/4 loci are postulated to anchor the NT domain within the pore via hydrophilic and hydrophobic interactions with adjacent connexin T5 and M34 residues. Cx40D3N and D3R mutations produced limited function with progressive reductions in Vj gating and noisy low γj gap junction channels that reduced the γj of wild-type Cx40 channels from 150 pS to < 50 pS when coexpressed. Surprisingly, hydrophobic Cx40 W4F and W4Y substitution mutations were not compatible with function despite their ability to form gap junction plaques. These data are consistent with minor and major contributions of the G2 and D3 residues to the Cx40 channel pore structure, but not with the postulated hydrophobic W4 intermolecular interactions. Our results indicate an absolute requirement for an amphipathic W3/4 residue that is conserved among all α/β/δ/γ-group connexins. We alternatively hypothesize that the connexin D2/3-W3/4 locus interacts with the highly conserved FIFR M1 motif to stabilize the NT domain within the pore.
EXPLORING THE EFFECTS OF VOLUME VARIATIONS IN THE IC POCKET OF HUMAN CX26 HC: A MOLECULAR SIMULATIONS STUDY

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On a previous work we showed that human Cx26 (hCx26) monomers have an intracellular cavity between TM2 and NTH: the IC pocket. This pocket is filled with water molecules presenting particular dynamic properties with respect to the bulk water. By a combination of both molecular dynamics (MD) and in vitro experiments, we proposed that the IC pocket could modulate the positioning of NTH, an important segment for the fast gating mechanism of hCx26 HC. To test this hypothesis we modulated the inner volume of the IC pocket to first remove all water molecules. We then simulated a vacuum bubble in the center of the pocket exerting a repulsive force on the protein atoms. The magnitude of this repulsive bubble was manipulated to change the inner volume of the IC pocket. In doing so, we evaluated its effects on channel structure and function.

Our results suggest that the mobility of NTH changes remarkably with changes in the IC pocket volume. In the absence of water molecules, the NTH move freely with a high RMSF in comparison with unrestricted simulations (each IC pocket holds between 5 and 15 water molecules). When increasing volume of the pocket, the NTH displaces toward the center of the pore changing the inner radius and affecting ionic currents.

These results point to a relevant function of the IC pocket in maintaining a correct position of NTH. Therefore, changes in the IC pocket volume could eventually module channel function. Of note, our structural analyses show that changes in the position of the NTH also modify the position of the PH, providing further evidence for a gating coupling between the NTH and PH.
bFGF-INDUCED MODULATION OF CELL COUPLING AND CONNEXIN TURNOVER IN ANTERIOR PITUITARY FOLLICULO-STEMMATE CELLS

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The basic fibroblast growth factor (bFGF) is a mitogenic and differentiating cytokine affecting the anterior pituitary endocrine cell proliferation and secretion. The folliculo-stellate (FS) cells are the major source of bFGF in the anterior pituitary. bFGF increases its own expression through a direct action on FS cells. The FS cells send cytoplasmic processes organized into a reticular network around endocrine cells. Within the anterior pituitary, intercellular communication is mainly mediated through FS cell gap junctions. Here, we tested whether if bFGF regulates FS cell coupling and if it influences Cx43, Cx46 and Cx50 gap junction protein expression in the FS cell line TtT/GF. A short-term (10-30 min) bFGF treatment induced a transient cell uncoupling whereas a long-term (1-4 hr) treatment momentarily increased cell coupling. A short-term exposure to bFGF increased PKC-α-mediated Cx43 phosphorylation without modifying Cx43 levels. As well, short-term incubation with bFGF transiently increased full-length Cx46 levels and the increase was due to a decrease in 14 kDa and 25 kDa Cx46 breakdown products. By contrast, Cx50 levels were not modified by a short-term incubation with bFGF. A long-term bFGF incubation temporarily increased Cx43 and Cx50 levels without affecting Cx46 expression. Together the data show that bFGF modulates TtT/GF cell coupling. A short-term bFGF exposure reduces cell-to-cell communication as a mean to “desynchronize” FS cells whereas a long-term exposure enhances cell-to-cell communication and facilitates the coordination of FS cells action in the gland. The opposite effects of short-term and long term exposure to bFGF on TtT/GF cell coupling may in part result from the differential action of the growth factor on the Cx43, Cx46 and Cx50 turnover. Supported by Natural Sciences and Engineering Council of Canada.
A CRUCIAL ROLE OF THE ELECTRICAL SYNAPSES IN HIPPOCAMPAL SIGNALS TRANSMISSION

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As we know, the hippocampus plays a crucial role in memory information controlling, so the neuronal connections among the hippocampal neurons provides a window for us to reveal the mechanism of memory information controlling, but how the electrical and chemical connections among the neuronal ensembles in hippocampus affect the memory information control is still unclear. We propose a mathematical connection model that will provide a new view for the signals transmission among the neuronal ensembles in hippocampus. So how the controlling signalling pathways are executed via the chemical or electrical ways will be discussed in details in our model with the feedback from the structural information of the related channels.

Key Words:
Hippocampus, Neuronal Ensemble, Memory, Electrical Synapse, Chemical Synapse
SIMVASTATIN-INDUCED UP-REGULATION OF GAP JUNCTIONS COMPOSED OF CONNEXIN 43 SENSITIZE LEYDIG TUMOR CELLS TO ETOPOSIDE: AN INVOLVEMENT OF PKC PATHWAY

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Some of lipophilic statins have been reported to enhance toxicities induced by antineoplastic agents but the underling mechanism is unclear. The authors investigated the involvement of Cx43-mediated gap junction intercellular communication (GJIC) in the effect of simvastatin on the cellular toxicity induced by etoposide in this study. The results showed that a major component of the cytotoxicity of therapeutic levels of etoposide is mediated by gap junctions composed of connxin 43(Cx43) and simvastatin at the dosage which does not induce cytotoxicity enhances etoposide toxicity by increasing gap junction coupling. The augmentative effect of simvastatin on GJIC was related to the inhibition of PKC-mediated Cx43 phosphorylation at ser368 and subsequent enhancement of Cx43 membrane location induced by the agent. The present study suggests the possibility that upregulation of gap junctions may be utilized to increase the efficacy of anticancer chemotherapies.
Pannexins (Panx) are ubiquitously expressed transmembrane channel proteins, with Panx1 being the best-characterized member of the protein family. Panx1 is implicated in sensory processing, as a result, panx1 knockout (KO) animal models have become the primary tool to investigate the role(s) of Panx1 in sensory systems. Extending previous work from our group on primary olfaction, we have now compared gene expression patterns of pannexins in the vomeronasal organ (VNO), an auxiliary olfactory sense organ with a role in reproduction and social behavior. Using qPCR and immunohistochemistry, we confirmed the loss of Panx1, found equal Panx2 expression, and a significant upregulation of Panx3 in mice with a global ablation of Panx1. In KO mice, Panx3 showed upregulated expression in nerve fibers of the non-sensory epithelial layer in juvenile mice and in the sensory layer of adults, which overlaps with Panx1 expression in WT populations and most likely reflects the functional reorganization of the VNO when animals become sexually mature. Consistent with the hypothesis that Panx3 can compensate for the loss of Panx1 as an ATP release channel, the evoked ATP-release from ex vivo preparations of the VNO was indistinguishable between WT and KO animals. This result led us to compare Panx1 and Panx3 channels, which showed both similar and distinct dye uptake and ATP release properties in vitro. Outcomes of this study strongly suggest that Panx3 may functionally compensate for Panx1 during chemosensory processing in the VNO. These results extend previous reports on the upregulation of Panx3 in arterial walls and the skin of Panx1 KO mice and have general implications regarding compensatory regulation between pannexins.
REGULATION OF PANNEXIN 1 AND PANNEXIN 3 LEVELS DURING SKELETAL MUSCLE DEVELOPMENT, REGENERATION, AND DUCHENNE’S DYSTROPHY

Tammy Le Pham²,³, Aymeric Ravel-Chapuis²,³, Tara E. Crawford Parks³,⁴, John A. Lunde³,⁴, Xiao Xiang²,³, Stéphanie Langlois¹,², Silvia Penuela⁵, Bernard J. Jasmin³,⁴, Kyle N. Cowan¹,²,³

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Pannexin 1 (Panx1) and Pannexin 3 (Panx3) are single membrane channels recently implicated in myogenic commitment, as well as skeletal muscle myoblast proliferation and differentiation in vitro. However, their expression patterns during skeletal muscle development and regeneration had yet to be investigated. Here we show that Panx1 levels increase during skeletal muscle development becoming highly expressed in adult tissue. A switch in Panx3 expression pattern was observed as its ~70 kDa immunoreactive species was mainly expressed in embryonal/neonatal muscles while its ~40 kDa species was the main form expressed in adult skeletal muscle. In adult mice, Panx1 and Panx3 were differently expressed in fast-twitch and slow-twitch muscles. We also report that Panx1/PANX1 and Panx3/PANX3 are co-expressed in mouse and human satellite cells, which play crucial roles in skeletal muscle regeneration. Interestingly, Panx1 and Panx3 levels were modulated in muscle degeneration/regeneration experiments, similar to that seen during skeletal muscle development. As Duchenne’s muscular dystrophy is characterized by skeletal muscle degeneration and impaired regeneration, we next used mild and severe mouse models of this disease and found a significant down-regulation of Panx1 and Panx3 (~40 kDa) levels in dystrophic skeletal muscles, while the ~70 kDa immunoreactive species of Panx3 was up-regulated. Together, our findings are the first demonstration that Panx1 and Panx3 are differently expressed amongst skeletal muscle types with their levels being highly modulated during skeletal muscle development, regeneration, and dystrophy. This suggests that Panx1 and Panx3 channels may play important and distinct roles in skeletal muscle health and disease.
PANNEXIN 1 IS DOWN-REGULATED IN Rhabdomyosarcomas and Its Reintroduction Inhibits Tumor Growth: A Potential Novel Therapeutic Target

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Rhabdomyosarcoma (RMS) is an aggressive skeletal muscle-derived paediatric malignancy for which novel therapeutic strategies are needed. RMS cells have lost the ability to terminally differentiate thus proliferating indefinitely. Promoting their differentiation to mature skeletal muscle tissue is thought to be a promising therapeutic approach. We have recently identified Pannexin 1 (PANX1) channels as novel regulators of skeletal muscle differentiation. Indeed, PANX1 levels were very low or below detectable limits in undifferentiated human primary skeletal muscle myoblasts, but became highly expressed during their differentiation, promoting this process. We thus hypothesized that PANX1 is down-regulated in RMS and that restoration of PANX1 levels may induce differentiation of RMS cells thereby alleviating their malignant properties. Briefly, our results demonstrate that PANX1 transcript and protein expression are very low in embryonal (eRMS) and alveolar RMS (aRMS) tumor specimens and patient-derived cell lines similar to that seen in fetal skeletal muscle tissue and undifferentiated myoblasts. Once PANX1 was introduced in Rh18 (eRMS) and Rh30 (aRMS) patient-derived cell lines, PANX1 was not sufficient to overcome the inability of RMS to reach terminal differentiation, but significantly decreased cell proliferation and migration. Moreover, expression of PANX1 in Rh18 and Rh30 cells abolished spheroid formation and growth by inducing apoptosis. When PANX1 was expressed after the spheroids had formed, it caused complete RMS spheroid regression. Furthermore, our pre-clinical orthotopic xenograft studies demonstrated that PANX1 expression significantly suppresses the growth of human eRMS and aRMS tumors in vivo. Importantly, our preliminary studies suggest that expression of PANX1 in established xenografts induced their regression. Taken together, our findings unravel tumor suppressive functions of PANX1 in human cancer cells in vitro and in vivo and suggest that PANX1 may constitute a novel therapeutic target for RMS.
PS2.108

THE ROLE OF CONNEXIN ISOFORMS IN SMOOTH MUSCLE PROGENITOR CELLS

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Background Smooth muscle progenitor cells (SPCs) participate in neovascularization and contribute to tissue repair following ischemic injury. Our previous studies showed that reduction of connexin43 (Cx43) impaired the repairing activity of endothelial progenitor cells (EPCs). However, the role of connexins in the biological function of SPCs remains unclear.

Methods and Results SPCs isolated from healthy donor peripheral blood expressed smooth muscle cell markers, such as SMMHC, caponin and desmin. Real-time PCR assay showed that connexin37 (Cx37), Cx43 and Cx45, but not Cx40, existed in the SPCs and Cx43 expressed 10 folds more than Cx45 and 10,000 folds than Cx37. Switch of SPCs to contractile phenotype was associated with increased expression of both Cx43 and Cx45. The cellular activities of SPCs treated with short interference RNA (siRNA) specific to Cx43 or Cx45 were compared with untreated cells. The results showed that Cx43 siRNA reduced the gap-junctional communication function (GJIC), migration and proliferation of SPCs, to an extent more than Cx45 siRNA did. In SPCs of contractile phenotype, Cx45 siRNA reduced alpha smooth muscle actin (α-SMA) expression and contraction function (evaluated using collagen gel assay). Cx43 siRNA did not affect the contraction.

Conclusion Gap junctions made of Cx37, Cx43 and Cx45 existed in human SPCs and Cx43 was the predominant isoform. Reduced expression of Cx43 impaired SPCs migration and proliferation, suggesting that Cx43 is essential to the reparative function of SPCs. Reduced expression of Cx45 lead to reduced contractility and α-SMA expression of SPCs, suggesting that Cx45 played a role in regulation of SPC contractile function.

Keywords: SPC, connexin43, connexin45, siRNA
ABNORMALLY UPREGULATION OF CONNEXIN 32 CAUSED BY INFLAMMATION PROMOTES THE PROGRESSION OF CERVICAL CANCER

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Generally, the function of gap junction and the expression of connexins were reduced or disappeared in the development of tumor. However, our data previously indicated that connexin32 was abnormally upregulated and accumulated in the cytoplasm compared with their matched adjacent non-tumor tissues in human cervical cancer tissues. But the function and the underlying mechanism of the upregulation of connexin32 remain unclear. Now, in vitro study revealed that the expression of connexin32 was obviously upregulated and transferred to cytoplasm after exposure to LPS, TNF-α, IL-6, IL-1β in cervical tumor cell, meanwhile the connexin43 was downregulated. Furthermore, inhibition or knockdown of Nf-κB, the upregulation of connexin32 caused by inflammation cytokines was reversed, suggesting that Nf-κB signaling pathway was involved in the regulation of connexin32. Functionally, knockdown of connexin32 led to a significant reduction in the proliferation of cervical cancer cells via induction of cell cycle arrest at the G1 stage, with or without gap junction intercellular communication. These results would lead us to further explore whether connexin32 could be considered as a predictor of poor prognosis, and whether simultaneous use of inflammation inhibitor could be developed as an adjuvant therapeutic strategy.
SRC PHOSPHORYLATION OF CX43 RESIDUE Y313 IS INVOLVED IN CHANNEL CLOSURE

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Phosphorylation regulates many aspects of Cx43 function from assembly into functional channels to coupling at the plaque. So far, Src is the only Tyr kinase known to both phosphorylate Cx43 (Y247 and Y265) and affect gap junction intracellular communication (GJIC). However, the Cx43CT contains additional Tyr residues and mass spectrometry (MS) data identified Y313 as a potential phosphorylation target (PhosphoSitePlus). Based upon the study of Lin et al. (2001) J. Cell Biol., which still observed Tyr phosphorylation by Src when using a Cx43 Y247F/Y265F mutant, we addressed the possibility of Y313 phosphorylation by Src. In vitro Src phosphorylation of Cx43CT followed by MS revealed Src phosphorylates Y313. This observation was confirmed by repeating the in vitro phosphorylation using different combinations of Cx43CT Y to F mutants and a general anti-pTyr antibody. Next, we generated a phospho-specific antibody to help characterize the importance of Src phosphorylation at Y313. Antibody specificity was verified by preincubation of the inoculating phosphorylated peptide prior to Western blot and immunostaining. We established an in cyto experimental system by stably expressing Cx43 WT and mutants (Y247F, Y265F, Y313F, Y247F/Y265F, Y247F/Y313F, Y265/Y313F, or Y247F/Y265/Y313F) in Cx-deficient HeLa cells. Cx43 WT and mutants, in the absence of v-Src, localized to the plasma membrane and formed GJs. When v-Src was over-expressed, Cx43 WT localized intracellularly, while all Cx43 mutants were still able to form GJ plaques, albeit variable in number and length. Furthermore, Cx43 Y265F inhibited the ability of v-Src to phosphorylate Y247 and Y313 as well as phosphorylation at all three sites was necessary to inhibit Luciferase Yellow transfer, indicating that Y313 phosphorylation is involved in channel closure. Finally, we observed in diseased human tissues (heart, lung, and breast), for which Src is activated, an increase of Cx43 Y313 phosphorylation.
**UNDERSTANDING GAP JUNCTION CHANNELS THROUGH MOLECULAR DYNAMICS SIMULATIONS**

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Despite generically sharing the same role in cell communications, channels composed by different connexin subunits display a large variety of behaviours in their regulation and permeation properties. The molecular mechanisms behind such important properties are still not completely understood, due to the difficulties in obtaining high resolution structures (so far only two X-ray structures have been published, to our knowledge [1,2]). Achieving a reliable theoretical framework for the study of permeation and gating mechanisms in connexin channels will allow rationalizing the large amount of experimental data and will represent a milestone in the understanding of cell communication and membrane biophysics. In principle, such framework can be obtained by a combination of homology modeling, molecular dynamics simulation and statistical mechanics. In this contribution, we will show that atomistic models provide a reliable tool to study the properties of connexin channels. Indeed, molecular dynamics simulations can be used to understand how conductance depends on the connexin composition of the channel [3], or why point mutations alter its structure or function [4,5]. We will then discuss the limitation of these methods, and show that it is possible to overcome them by integrating the models with quantum mechanics calculation [6] or improving the sampling by using coarse grained force fields.

ENHANCED EFFECT OF CONNEXIN 43 ON SUNITINIB-INDUCED APOPTOSIS VIA Bax INTERACTION IN MALIGNANT MESOTHELIOMA CELLS

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[Background] Connexin (Cx) works as an interactive protein, which would regulate cell functions, in addition to as a component of an intercellular channel called gap junction (GJ). The Cx expression level is often decreased in cancer cells compared to that in healthy ones, and the restoration of its expression has been shown to exert antiproliferative effects. This work aims to evaluate the effect of the restoration of connexin 43 (Cx43) (the most ubiquitous Cx subtype) expression on sunitinib (SU)-induced cytotoxicity in malignant mesothelioma (MM) cells.

[Results & Discussion] Increased Cx43 expression in an MM cell line (H28) improved the ability of SU to inhibit receptor tyrosine kinase (RTK) signaling. Moreover, higher Cx43 expression promoted SU-induced apoptosis. The cell viability test revealed that Cx43 enhanced the cytotoxic effect of SU in a GJ-independent manner. On the other hands, immune-precipitation assay revealed that Cx43 interacted directly with Bax, a pro-apoptotic protein. Then the detailed mechanism was investigated. A treatment of the Cx43-transfected MM cell line with SU for 8 hr increased an expression of the active form of Bax localized at the mitochondria with a decreasing mitochondrial membrane potential, while no expression of the active form was detected in the parental cells. On the other hand, Cx43 mainly located at the plasma membrane but minimally at mitochondria. Therefore, we hypothesized that Cx43 activated Bax at the plasma membrane and then promoted its translocation to the mitochondria. In fact, JNK was activated 2-fold by SU treatment only in Cx43-transfected cells, and the activated JNK is known to promote Bax translocation to mitochondria during a process of apoptosis.

[Conclusion] In conclusion, we found that Cx43 overcame the chemoresistance of MM cells. These findings suggested that Cx43 would promote Bax translocation to mitochondria via JNK activation in the SU-induced apoptosis.
BIological activities of many natural products pass through the gaps

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The health benefit of plant-derived foods is well established since long time and new researches are identifying bioactive natural compounds among thousands of phytochemicals. Throughout life the exposure to specific phytochemicals can affect gene expression via reversible epigenetic mechanisms. This has recently boosted the re-exploration of nutritional, botanical or phyto-pharmaceutical compounds as promising nutraceuticals or cosmeceuticals for their epigenetic effects. Gap junctions have been recognized as therapeutic and chemopreventive target for many diseases and are also emerging as a new potential therapeutic target for the treatment of neurological disorders. The cancer preventive mechanism(s) of many phytochemicals involve(s) the GJIC enhancement including the prevention of the downregulation of GJIC by tumor promoting agents.

The functionality of gap junction-mediated intercellular communications may represent a functional biomarker to assess the health benefits of natural products. Simple assays can be further developed as screening test for a wide range of natural bioactive molecules or lead compounds in specifically chosen cell cultures. When the technological innovation will allow the development of high-throughput analysis systems, the rapid, and hopefully inexpensive, GJIC assessment may serve a broad range of in vitro pharmacological and toxicological needs propaedeutic to the in vivo assays.

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ROLE OF GAP JUNCTION INTERCELLULAR COMMUNICATIONS (GJIC) DURING THE DEVELOPMENT AND LIFE CYCLES OF TWO EARLY BRANCHING METAZOANS

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Gap junctions (GJs) are highly conserved cellular structures in multicellular metazoans, with conserved features in structure, cellular distribution, function, and mechanisms of regulation of their constituent proteins. During embryonic development, GJs play a key role in the regulation of information flow controlling animal body patterning. Developing neurons and other embryonic cell types are often characterized by transient gap-junction networks, which can regulate proliferation, migration, cell death, contact inhibition, as well as synapse formation and disassembly. However, direct evidence of GJ function in invertebrates is still scant.

Here we report on the functionality, GJ gene expression, and role of GJIC throughout life cycle and development of two early branching metazoans, the hydrozoan Clava multicornis and the acoel Symsagittifera roscoffensis. By GJs pharmacological inhibition at different (hydroid and acoel) life stages, and labelling of different (GLW-amide, RF-amide, and 5-HT) neuronal subsets and muscle cells, we gathered information on role of GJs in body patterning, neurogenesis, myogenesis, and larval behaviour. Further, C. multicornis innexins were cloned and their differential expression across pharmacological followed by semi-quantitative PCR analyses.

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<td>O-31</td>
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<td>Boyce, Andrew</td>
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Basing, Sebastian
Batissoco, Ana Carla
Batista-Almeida, Daniela
Bauer, Alison K.
Baum, Rachel
Bayliss, Douglas
Brown, Cherie
Brown, Donald L
Brown, Isola
Brownell, Sarah
Brózman, Ondřej
Bukauskas, Feliksas

Baz-Martínez, Maite
Beach, Rianne
Bechberger, John
Begandt, Daniela
Bell, Cheryl L.
Belli, Carla
Bellido, Teresita
Bennett, Brad
Bennett, Steffany
Benninger, Richard
Berger, Amy

Bultynck, Geert
Buo, Atum
Burboa, Pia
Burt, Janis
Busby, Mélanie
Butcher, Joshua
Bye, Alexander
Byringiro, Innocent

B

Cabrera, Alejandro
Caeiro, José Ramón
Calhoun, Patrick
Calhoun, Patrick J.
Calleja Chuclá, Teresa
Cameron, John
Campbell, RE
Campos, Vania
Cancelas, Jose
Cappuccino, Juan
Carcouët, Agnès
Cárdenas, Ana María
Cardozo, Christopher
Carlen, Peter
Carpintero-Fernández, Paula
Carrer, Andrea
Castro, Claire
Catarino, Steve
Ceren Unal, Yagmur
Chadjichristos, Christos
Chanson, Marc

Chepied, Amandine
Chin, Katrina
Chiu, Yu-hsin
Choi, Kelly
Choi, Yung Hyun
Cifuentes, Fredi
Cisterna, Bruno
Ciubotaru, Catalin
Civitelli, Roberto
Clague, Michael J.
Clarhaut, Jonathan
Clopath, Claudia
Cogliati, Bruno
Concha, Angel
Contreras, Gustavo F
Contreras, Jorge E
Corpataux, Jean-Marc
Cortese-Krott, Miriam
Cortéz, Constanza
Cotter, Maura

Chanson, Marc

C
<table>
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<td>Johnston, Danielle</td>
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<td>Janssen-Bienhold, Ulrike</td>
<td>PS2.80</td>
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<td>Johnstone, Scott</td>
<td>O-45, O-52</td>
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<td>Jara, Oscar</td>
<td>PS1.113</td>
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<td>O-28</td>
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<td>Lagace, Diane</td>
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<td>PS1.75, PS1.77</td>
<td>Lin, Chun-Lin</td>
<td>O-56</td>
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<td>Lamouille, Samy</td>
<td>O-59, PS1.93</td>
<td>Lin, Jian-Sheng</td>
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<td>O-4, O-22, O-51, O-52</td>
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<td>Lang, Susan</td>
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<td>Lin, Ya-Ping</td>
<td>O-41</td>
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<td>Langlois, Stéphanie</td>
<td>PS2.106, PS2.104</td>
<td>Lissoni, Alessio</td>
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<td>Lapidot, Tsvee</td>
<td>O-54</td>
<td>Liu, Ping</td>
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<td>Liu, Yu</td>
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<td>Latorre, Ramon</td>
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<td>Le, Thu</td>
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<td>Lopez, William</td>
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<td>Verónica</td>
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</tr>
</tbody>
</table>
Le Gal, Loïc  PS1.75, PS1.79,  Lorraine, Claire  PS1.63
Lebreton, Jacques  PS1.77, O-3  Lothe, Ragnhild  PS1.99, PS2.84
Lee, Jooae  O-37  Lothe, Ragnhild A.  PS2.38
Lee, Ming Yang  PS2.34  Loureiro, Jesús  PS2.90
Lee, Yi-Nan  O-33  Lovgren, ML  PS1.5
Leithe, Edward  PS2.84  Ludin, Nurin  O-58
Leitinger, Norbert  PS1.69  Lunde, John A.  PS2.104
Leone, Antonella  PS2.122, PS2.120  Luo, Kai-Jun  PS1.107
Leonhardt, Susan  O-25  Luo, Yun  O-24
Leparulo, Alessandro  O-31

M
Maass, Karen  PS2.54  Mendu, Suresh  PS1.69
MacAulay, Nanna  O-36, PS2.14  Menezes, Laura  PS2.54
MacDonald, Alasdair  O-45  Mese, Gulistan  PS2.8
Maciuñas, Kestutis  PS2.64, PS1.109  Mesnil, Marc  O-21, PS1.39
Maes, Michaël  PS1.45  Messier, Claude  PS1.27
Mailloix, Ryan  O-40  Meunier-Balandre, Annie-Claire  PS1.39
Mammano, Fabio  O-34, PS2.116  Mingroni-Netto, Regina C.  O-42
Mannell, Hanna  O-2  Minogue, Peter  PS1.29, PS2.24
Maripillán, Jaime  PS1.111, PS1.113  Minshall, Richard  O-1
Marques, Carla  PS1.19  Mitchell, Cheryl  O-41
Martin, Patricia  O-10, PS2.20,  Mo, Victor  PS2.2
PS1.87, PS2.4,  Mohmoud, Fatima  O-54
PS1.5, PS1.63, O-62
Martínez, Agustín  PS1.111, PS1.113,  Molica, Filippo  O-12, O-47,  O-115
PS1.115
Martins-Marques, Tania  O-7, PS1.117,  Molina, Samuel  PS1.23
PS1.19
Masati, Ester  PS1.49  Moliné, Teresa  O-23
Mathias, Richard  PS1.29  Momboisse, Fanny  PS1.113
Matiukas, Arvydas  PS2.92  Montgomery, Jade  PS2.10
Matsuuchi, Linda  PS2.2  Monvoisin, Arnaud  PS1.31, O-21,  PS1.39
Mayán, María D  PS2.90, PS1.119  Monyer, Hannah  O-17
Mazzolai, Lucia  PS1.75  Morel, Sandrine  O-12
McGowan, Francis  PS1.71  Moreto, Aparecida  PS1.47
McNair, Andrew  PS2.4  Morin, Mario  PS1.27
Meda, Paolo  O-3  Moua, Ong  O-58
Medina, Christopher B.  O-25  Mouthon, Franck  PS1.53
Medina, M  O-50  Moyer, Kurtis  PS2.10
Meens, Merlijn  O-12  Muñoz, María José  O-23
PS1.22

<table>
<thead>
<tr>
<th>Name</th>
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<td>O-37</td>
<td>Polusani, Srikanth</td>
<td>O-56</td>
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<td>Pottier, Géraldine</td>
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<td>Peng, Yuexia</td>
<td>PS2.28, PS2.26, PS2.112</td>
<td>Poulain, Coralie</td>
<td>O-9</td>
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<td>PS1.7, PS2.58</td>
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</tbody>
</table>
Perez-Acle, Tomas | PS2.24, PS2.94, PS2.30
Pérez-Acle, Tomás | PS1.115
Pernelle, Guillaume | PS2.32
Petersen, Ida M.B.S. | O-36
Petrova, Tatiana V. | PS1.37, PS2.6
Pham, Tammy Le | PS2.104
Pham, Tuan | O-16
Phan, Allen | O-44
Proctor, Gordon | O-52
Pronin, Alexey | PS2.60
Puebla, Carlos | PS2.48
Pupo, Amaury | PS1.115
Purginga, Bibiana | PS2.106

Rakoczy, Elizabeth | PS2.44
Ramón y Cajal, Santiago | O-23, PS2.12
Raska, Jan | PS2.66
Rasmussen, Nikoline Lander | PS1.99, PS2.38, PS2.84
Ravel-Chapuis, Aymeric | PS2.104
Ravichandran, Kodi | PS1.69, O-25
Retamal, Mauricio | PS1.3, PS2.46, PS2.40
Ribeiro-Rodrigues, Teresa | O-7, PS1.19, PS2.42
Richardson, William | PS2.10
Rimkute, Lina | PS2.64, PS1.109

Sabile, Jean | O-59
Sabine, Amélie | PS2.6
Sáez, Juan Carlos | PS1.73, PS1.15, PS1.105, PS2.48
Sagdullaev, Botir | PS2.60
Sage, Tanya | PS2.50
Sahli, Khaled | PS2.50
Saito, Mikako | PS2.52
Salat-Canela, Clàudia | PS2.12
Salazar-Silva, Rodrigo | O-42
Sameshima, Tetsuro | PS1.97
Sampson, Jacinda | O-55
Sánchez-Pupo, Rafael E. | O-39
Sansano, Irene | O-23
Sato, Hiromi | O-23
Sayuri Nogueira, Marina | PS1.45

Martín, Francisco | O-16
Petersen, Ida M.B.S. | O-36
Petrova, Tatiana V. | PS1.37, PS2.6
Pham, Tammy Le | PS2.104
Pham, Tuan | O-16
Phan, Allen | O-44
Proctor, Gordon | O-52
Pronin, Alexey | PS2.60
Puebla, Carlos | PS2.48
Pupo, Amaury | PS1.115
Purginga, Bibiana | PS2.106

Rakoczy, Elizabeth | PS2.44
Ramón y Cajal, Santiago | O-23, PS2.12
Raska, Jan | PS2.66
Rasmussen, Nikoline Lander | PS1.99, PS2.38, PS2.84
Ravel-Chapuis, Aymeric | PS2.104
Ravichandran, Kodi | PS1.69, O-25
Retamal, Mauricio | PS1.3, PS2.46, PS2.40
Ribeiro-Rodrigues, Teresa | O-7, PS1.19, PS2.42
Richardson, William | PS2.10
Rimkute, Lina | PS2.64, PS1.109

Sabile, Jean | O-59
Sabine, Amélie | PS2.6
Sáez, Juan Carlos | PS1.73, PS1.15, PS1.105, PS2.48
Sagdullaev, Botir | PS2.60
Sage, Tanya | PS2.50
Sahli, Khaled | PS2.50
Saito, Mikako | PS2.52
Salat-Canela, Clàudia | PS2.12
Salazar-Silva, Rodrigo | O-42
Sameshima, Tetsuro | PS1.97
Sampson, Jacinda | O-55
Sánchez-Pupo, Rafael E. | O-39
Sansano, Irene | O-23
Sato, Hiromi | O-23
Sayuri Nogueira, Marina | PS1.45

Sabile, Jean | O-59
Sabine, Amélie | PS2.6
Sáez, Juan Carlos | PS1.73, PS1.15, PS1.105, PS2.48
Sagdullaev, Botir | PS2.60
Sage, Tanya | PS2.50
Sahli, Khaled | PS2.50
Saito, Mikako | PS2.52
Salat-Canela, Clàudia | PS2.12
Salazar-Silva, Rodrigo | O-42
Sameshima, Tetsuro | PS1.97
Sampson, Jacinda | O-55
Sánchez-Pupo, Rafael E. | O-39
Sansano, Irene | O-23
Sato, Hiromi | O-23
Sayuri Nogueira, Marina | PS1.45

Sabile, Jean | O-59
Sabine, Amélie | PS2.6
Sáez, Juan Carlos | PS1.73, PS1.15, PS1.105, PS2.48
Sagdullaev, Botir | PS2.60
Sage, Tanya | PS2.50
Sahli, Khaled | PS2.50
Saito, Mikako | PS2.52
Salat-Canela, Clàudia | PS2.12
Salazar-Silva, Rodrigo | O-42
Sameshima, Tetsuro | PS1.97
Sampson, Jacinda | O-55
Sánchez-Pupo, Rafael E. | O-39
Sansano, Irene | O-23
Sato, Hiromi | O-23
Sayuri Nogueira, Marina | PS1.45
Scemes, Eliana          O-12, PS2.70, PS2.54
Schadzek, Patrik       PS2.56
Schey, Kevin           PS1.71
Schultz Hansen, Rie    PS1.21
Seguin, Cheryle        O-55
Sevetson, Jessica      O-16
Shao, Qing             O-55, PS2.16, O-30, PS1.7, PS2.62, PS2.58
Shaw, Robin            PS1.17, O-60
Sheng, Zhi             O-59
Sherman, Robyn         PS1.95
Shestopalov, Val       PS2.60, PS1.45
Shimara Pires Ferrão, Juliana PS1.81
Shimizu, Ayaka         O-25
Shu, Shaofang          O-14
Shui, Yuan             O-25
Shum, Michelle         PS2.62
Simonneau, Claire      PS1.31
Spray, David           PS2.68, PS2.114
St.Clair, Josh         O-48, PS2.70, PS2.54, O-15
Stahl, Yannick         PS2.56
Stainer, Alexander     PS2.50
Stains, Joseph         PS2.72
Stautch, Kelly L.      PS2.68
Stout, Randy           PS2.70, PS2.54
St-Pierre, Marie-Eve   PS2.106
Strauss, Randy         PS2.74
Stroemlund, Line       O-36
Waring                 Su, Cheng-Huang PS2.82, PS2.108
Subramaniam, Mohana-Devi PS1.67
Sudhakar, Swathy       PS1.11
Sun, Baiming           Sun, LuZhe O-60
Sun, Peng              Sun, Peng O-45
Su-Taylor, Samantha    PS2.36
Swayne, Leigh Anne     O-8, O-1
Szeiffová Bačová,     PS2.76
Barbara                Sasikuma PS2.50
Parvathy               r,
Tabernero, Arantxa     O-50
Talaverón, Rocío       O-50
Tamm, Lukas            O-25
Tanenbaum, Mira        PS2.110
Tani, Kazutoshi        PS2.28, PS2.26, PS2.78, PS2.100, PS2.112
Tao, Liang             PS2.34, PS1.55
Tasken, Kjetil         PS2.34, PS1.55
Therien, Max           PS1.99, PS2.38, PS2.84
Zachrisson             O-18, O-57
Taylor, Jordan         O-44
Tejada, Mary Grace     PS1.11
Temprana, Jordi        O-23
Terasaki, Mark         O-4
Tessier, Arnaud        O-37
Tetenborg, Stephan     PS2.80
Thevenin, Anastasia    PS1.61
Thierens, Hubert       O-27
Tournier, Nicolas      PS1.53
Toychiev, Abduqodir    PS2.60,
Tribulová, Narcis      PS2.76
Tseng, Ya-Ming         PS2.108
Trenche, Jordan        O-32, PS2.86
Tress, Andrew J.       O-5, O-32, PS2.86
Tress, Andrew J.       O-5, O-32, PS2.86
Trier, Andreas         O-44
Trier, Andrea          O-44
Tseng, Jeffery         PS2.80
Tseng, Ya-Ming         PS2.108
<table>
<thead>
<tr>
<th>Name</th>
<th>Page</th>
<th>Name</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ugur, Deniz</td>
<td>PS2.8</td>
<td>Urbé, Sylvie</td>
<td>PS2.42</td>
</tr>
<tr>
<td>Une, Hayato</td>
<td>O-49</td>
<td>Uzu, Miaki</td>
<td></td>
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<tr>
<td></td>
<td></td>
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</tr>
<tr>
<td>V</td>
<td></td>
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<tr>
<td>V. Van, Haver</td>
<td>PS1.103</td>
<td>Vargas, Siomara</td>
<td>PS1.73</td>
</tr>
<tr>
<td>V.A. Pereira, Isabel</td>
<td>PS1.45</td>
<td>Vázquez, Jacqueline</td>
<td>PS1.111</td>
</tr>
<tr>
<td>Vagner, Josef</td>
<td>O-61</td>
<td>Vázquez-Blanco, María C.</td>
<td>PS1.119</td>
</tr>
<tr>
<td>Valenzuela, Bernardita</td>
<td>PS1.73</td>
<td>Veenstra, Richard</td>
<td>PS2.92</td>
</tr>
<tr>
<td>Valiunas, Virgis</td>
<td>PS2.88</td>
<td>Veeraraghavan,</td>
<td>O-38</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Valiuniene, Laima</td>
<td>PS2.88</td>
<td>Vega, José Luis</td>
<td>PS1.73,</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>PS1.15</td>
</tr>
<tr>
<td>Van Haver, Valérie</td>
<td>O-27</td>
<td>Vera, Joshua</td>
<td>PS2.12</td>
</tr>
<tr>
<td>Vandecasteele, Gregoire</td>
<td>PS2.34</td>
<td>Vidal-Brimi, Laia</td>
<td>PS2.12</td>
</tr>
<tr>
<td>Vandenabeele, Peter</td>
<td>PS1.101</td>
<td>Villanelo, Felipe</td>
<td>PS2.24,</td>
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<tr>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Vandenbroucke, Roosmarijn</td>
<td>PS1.101</td>
<td>Vinken, Mathieu</td>
<td>PS1.45</td>
</tr>
<tr>
<td>Vanhove, Christian</td>
<td>O-27</td>
<td>Vitale, María Leiza</td>
<td>PS2.96</td>
</tr>
<tr>
<td>Varela-Eirin, Marta</td>
<td>PS2.90</td>
<td>Vix, Justine</td>
<td>O-21</td>
</tr>
<tr>
<td>Varela-Vázquez, Adrián</td>
<td>PS1.119</td>
<td>Vodovar, Dominique</td>
<td>PS1.53</td>
</tr>
<tr>
<td>Vargas, Anibal</td>
<td>PS2.48</td>
<td>Veeraraghavan,</td>
<td></td>
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<td>PS2.</td>
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<tr>
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<td>74</td>
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</tr>
<tr>
<td>W</td>
<td></td>
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<tr>
<td>Wagner, Charlotte</td>
<td>PS1.75</td>
<td>Weaver, Rachel</td>
<td>PS1.49</td>
</tr>
<tr>
<td>Wan, Xiaoping</td>
<td>O-38</td>
<td>Wellendorf, Ashley</td>
<td>O-54, PS1.67</td>
</tr>
<tr>
<td>Wang, Bo-Zheng</td>
<td>PS2.82</td>
<td>Welsh, David</td>
<td>PS2.4</td>
</tr>
<tr>
<td>Wang, Chiou-Min</td>
<td>O-56</td>
<td>White, Thomas W</td>
<td>O-33, PS2.88</td>
</tr>
<tr>
<td>Wang, Chmanjen</td>
<td>PS2.98</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wang, Helen Yanran</td>
<td>O-41</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wang, Hong-Zhan</td>
<td>O-33</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wang, Hsueh-Hsiao</td>
<td>PS2.108</td>
<td>Whitehead, Shawn</td>
<td>PS1.21</td>
</tr>
<tr>
<td>Wang, Lingzhi</td>
<td>PS2.100</td>
<td>Whyte-Fagundes, Paige</td>
<td>PS2.102</td>
</tr>
<tr>
<td>Wang, Nan</td>
<td>PS1.89, O-35, O-51</td>
<td>Willebrords, Joost</td>
<td>PS1.45</td>
</tr>
<tr>
<td>Wang, Qin</td>
<td>PS2.28, PS2.26, PS2.78, PS2.100, PS2.112</td>
<td>Willecke, Klaus</td>
<td>O-30, PS1.7</td>
</tr>
<tr>
<td>Wang, Zhao-Wen</td>
<td>O-14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wang, Zhen</td>
<td>PS1.71</td>
<td>Wimberley, Catriona</td>
<td>PS1.53</td>
</tr>
<tr>
<td>Waters, Alex</td>
<td>PS1.5</td>
<td>Wright, Catherine</td>
<td>O-62</td>
</tr>
<tr>
<td>Watkins, Marcus</td>
<td>PS1.41</td>
<td>Wu, Yih-Jer</td>
<td>PS2.82</td>
</tr>
<tr>
<td>Weaver, Rachael</td>
<td>O-1</td>
<td>Wu, Yih-Jer</td>
<td>PS2.108</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Wang, Jasmine</td>
<td>PS1.21</td>
</tr>
</tbody>
</table>
X
Xiang, Xiao
Xiao, Shaohua

Y
Yamaguchi, Hiroo
Yamamoto, Yohei
Yamasaki, Ryo
Yamasaki, Tomohiro
Yao, Jian
Yawer, Affiefa
Ye, Willy
Yeager, Mark
Yeh, Hung-I
Yeung, Ken
Yohannes, Zeremariam
Yue, Benny

Z
Zach, Sydney
Zaidan Dagli, Maria Lúcia
Zamiri, Mozheh
Zamorano, Pedro
Zeitz, Michael
Zhang, Jie
Zhang, Shan-Shan
Zhang, Wei
Zhang, Xian
Zhang, Xiaomin
Zhang, Xiling
Zhang, Zhen
Zhao, Hong-bo
Zhao, Yifan
Zhao, Yinan
Zheng, Li
Zhou, Hu
Zhou, Jade Z.
Zhou, Ruhong
Zhu, Ying
Zoidl, Christiane
Zoidl, Georg
Zoltoski, Rebecca
Zonta, Francesco
Zucker, Shoshanna
Zuo, Zhiyi

PS2.106, PS2.104, PS1.17, O-60
PS2.18, O-49
PS1.97, PS2.66
O-49
PS2.68
PS1.81
PS1.5, PS2.20
PS1.73
O-44, PS2.110, PS1.93
PS2.44, O-46
PS1.17
O-18
PS2.92
PS2.112, PS2.28, PS2.26
O-53
O-53
PS2.92
PS2.105, O-25
PS2.82, PS2.108
PS2.86, O-32, PS2.114
O-29
PS2.78
O-49
PS2.86, O-32, PS2.114
O-40
O-20
PS2.30
O-53
PS2.102
PS2.102
PS1.29
O-31, PS2.116
PS2.118
O-13