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Therapeutic strategies targeting connexins

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Abstract

The connexin family of channel forming proteins are present in every tissue type in the human anatomy. Connexins are best known for forming clustered intercellular channels, structurally known as gap junctions, where they serve to exchange members of the metabolome between adjacent cells. In their single membrane hemichannel form, connexins can act as conduits for the passage of small molecules in autocrine and paracrine signalling. Here we review the roles of connexins in health and disease, focusing on the potential of connexins as therapeutic targets in acquired and inherited diseases as well as wound repair, while highlighting the associated clinical challenges.

Introduction

The discovery of gap junctions and their role in providing direct lines of communication between contacting cells dates back over 50 years, and research into why healthy cells require this mode of cell signalling remains of critical importance [1] . Central to understanding the role of gap junctions in physiology is the need to interrogate the only vertebrate protein family known to form gap junction intercellular channels, the connexins. The breath of the connexin family, numbering 21 in humans [2], and their intermixing allows for an immense spectrum of channel diversity that ultimately dictates their selectivity for thousands of unique small molecules that can pass from one cell to another [3]. Given that connexin expression is different in different tissues this critical form of direct cellular communication all occurs in a tissue selective manner. A more recently discovered gene family, called pannexins (Box 1), were initially proposed to also form intercellular channels [4] but their role appears to be restricted to releasing small molecules to the extracellular milieu, a function shared by connexins in their single membrane hemichannel form [5]. Through findings from numerous *in vitro* and *in vivo* studies, the use of genetically-modified mouse models and extensive epidemiological and genetic studies on patients harboring connexin gene mutations, it has become clear that connexins are intimately linked to healthy development and homeostasis. Conversely, when defective, connexins are causal of diseases including hearing loss, a plethora of skin diseases and neuropathies [6,7].

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Competing Interests:

Given their extensive relationship to disease and wound healing, and in keeping with advance personalized and customized therapeutics, connexins have emerged as viable drug targets. To that end, connexin therapeutics have been the focus of two recent workshops that brought several companies and academic research leaders together in Paris (2016) and Vancouver (2018). While no connexin-specific drugs have yet received approval, the importance of connexins in human physiology and disease, along with their commercial interest, indicate that connexin therapeutics are forthcoming. Although still in its infancy of understanding, the documented relationship between pannexin regulation and disease has also highlighted pannexins as promising new therapeutic targets (Box 2). In this article, we describe the evolution of connexin biology, highlighting the unique cell biological features of connexins and their overall role in healthy physiology. We summarize their known and putative roles in disease processes that lay the foundation for their thoughtful consideration in therapeutics.

The successes and failures of first generation connexin therapeutics designed to treat acute and chronic diseases and injuries, including ischemia reperfusion injury, diabetic ulcers and epidermal and corneal wounds are discussed. Given that no less than four companies have initiated clinical trials, this article is both timely and aims to provide an overview of the approaches used in some of the most promising next generation therapeutics. We also assess the challenges ahead in the selective targeting of specific connexin family members and connexin mutations as customized therapeutics become more commonplace.

Discovery of gap junctions and connexins

Gap junctions were first discovered in the early sixties when Robertson reported cell synapses in the brains of goldfish [8]. Pioneering work by Revel, Karnovsky, Benedetti and Emmelot [1,9,10] describe the existence of a 20 angstrom “gap” between closely opposed membranes visible via thin-section electron microscopy, but it was Revel and Karnovsky that most frequently get credit for coining the term “gap junction” [1]. Parallel studies reported the existence of low resistance intercellular channels between contacting cells and intercellular exchange of molecules [11–13]. Over the following two decades there was extensive refinement of structural insights into gap junctions and further characterization of the low resistance channels found within these lattice structures. In the early eighties, studies focussed on identifying the molecular constituents of gap junctions. This led many laboratories to work on the biochemical characterization of gap junctions, effort that was accelerated by the identification of the first gap junction protein in 1986 [14,15]. In 1987, Beyer and colleagues cloned and sequenced a 43 kD gap junction protein from the heart and resurrected a previously used nomenclature to describe this gap junction protein as connexin43 (Cx43), which subsequently became widely adopted by the gap junction community [16]. A parallel nomenclature that utilized the “GJ” abbreviation followed by a Greek letter designating the sub-family and a number taken from the order of its discovery emerged for both protein and gene names, only the latter of which is still widely used today [17–19]. Thus, connexin proteins are designated with the abbreviation “CX or Cx” followed by the predicted molecular mass of the human connexin gene in kilodaltons. For example, the human Cx43 protein is encoded by the *GJA1* gene [2].

The next two decades saw the discovery of the entire connexin gene family totaling 21 members in humans (Figure 1) [2]. This golden-age of connexin biology was filled with many surprises that began with the realization that healthy physiology requires connexin expression in every tissue type in the human anatomy. While this might have been reasonably predicted for cells arranged in solid tissues, it was not obvious they would play important roles in circulating cells of the hematopoietic system that even include megakaryocyte-derived platelets [20,21]. Most cells within tissues were also found to express more than one member of the connexin family with some often simultaneously expressing 2 or more connexins [7,22,23]. Since 6 connexins oligomerize to form a “connexon” or “hemichannel” that meets head-to-head with a connexon in an adjacent cell, this result immediately raised the issue of the potential complexity of connexon channel formation as many connexins were found to selectively oligomerize with other connexin members within a connexon [22,24,25]. For instance, when two compatible connexins are co-expressed in two adjacent cells, 196 different arrangements of channels could in principle be assembled from the two connexin hexamers docked between the cells [26]. The connexin family can be sub-divided into sub-families (α) based on sequence similarity (Figure 1) and most connexins interact primarily with members of their own sub-family group.

Subsequent studies set out to fully identify the members of the metabolome that actually passes through a gap junction channel and how one channel would differ from another [27–29]. This led to some success by identifying subsets of molecular sizes, shape and charges of transjunctional molecules, but new approaches to fully uncover the intricacies of how channels differ and select for biologically relevant transjunctional molecules are awaited. The arrival of the first high resolution crystal structures have begun to provide new insights into the molecular structure of the gap junction channel [30,31] but much remains to be done. Further, the unique roles and physiological importance of connexin hemichannels as conduits for small molecule exchange between the cytosol and extracellular milieu has become more complex to interrogate with the discovery of pannexin channels that potentially have some overlapping roles (Box 1). However, connexin hemichannels and tightly regulated pannexin channels have unique regulatory properties especially when considering mechanisms linking these channel types to disease processes.

Life cycle and turnover dynamics of connexins

Prior to the identification of connexins, fully assembled gap junctions were estimated to have a half-life of 5 hours [32], which was remarkably faster than predicted given their complex organization as revealed by electron microscopic imaging and biochemical purification. After the discovery of connexins as the constituent gap junction channel protein, and production of appropriate antibodies, more accurate pulse-chase labeling experiments revealed that most connexins had a half-life of 1–2.5 hours, which reflected their entire life-cycle from synthesis to degradation [33,34]. This rate of turnover was 10–25 times faster than other well-studied integral membrane proteins that reside at the cell surface. This finding raised the question of why connexins would need such an energy-demanding, rapid rate of renewal, and it was postulated that this may allow for a level of channel regulation that could rapidly respond to cellular cues and signals requesting increased or decreased gap junctional intercellular communication (GJIC). While a short

connexin half-life remains the rule-of-thumb for the majority of the connexin members examined thus far, exceptions do exist such as Cx46 present in enucleated lens fibers [35] and Cx30 in keratinocytes [36]. Connexins present in these specialized tissues have been reported to have longer half-lives and may even survive throughout the life time of the cell (e.g., Cx46) or until the cell further differentiates (e.g., Cx30).

Given that the formation and removal of gap junctions is a dynamic process, there has been a concerted effort to map out the mechanistic details of how connexins fold, oligomerize, traffic, dock, cluster, function, internalize and degrade (Figure 2). Several recent reviews document these events in considerable detail and calculations indicate that most cells in the human body will completely renew their gap junctions in a remarkably short 24 hour period [7,23,37–39]. Connexins tend to follow a classical secretory pathway by co-translationally inserting into endoplasmic reticulum (ER) membranes (Figure 2) where they proceed to undergo folding and intramolecular disulfide bond formation [25,40]. While many connexins oligomerize in the ER, others, like Cx43, appear to delay this process until reaching the *trans* Golgi network [41]. Reasons for this delay remain elusive but one argument suggests that this delay would prevent any aberrant and premature opening of connexin hemichannels or connexons in the ER membrane, which could destroy the integrity of the ER luminal compartment. Connexins frequently use the assistance of microtubules to traffic to the cell surface and may randomly appear at the cell surface or be directed via microtubule anchoring sites to the edges of pre-existing gap junctions [42–44]. Docking of hemichannels is highly regulated and appears to occur quickly as most imaging technologies fail to detect a cohort of undocked hemichannels at the cell surface. The concept of whether Cx43 hemichannels routinely open to connect the cytoplasm with the extracellular milieu is frequently debated as documented conditions for hemichannel opening are not typically present during homeostasis (i.e., very low extracellular calcium) [37,45,46]. However, there is good evidence for a hemichannel-rich “perinexus” region surrounding fully assembled gap junctions that may have functional importance [47,48], and there is a growing body of literature implicating the hemichannel in specific pathologies [37,49]. Gap junction channels proceed to aggregate from the outer edges into semi-crystalline arrays where the majority of extraneous lipids are excluded from the gap junction plaques resulting in a detergent-resistant structure with the older channels being found in the central region of the gap junction (Figure 2)[50,51]. In short order, these structures or fragments of these gap junctions are removed from one of the two contacting cells as double membrane structures called “annular junctions” [52], rebranded some years ago as “connexosomes” [23,53–55]. This apparently unique process allows for hundreds of channels to be eliminated from the cell surface in a single event allowing rapid turnover of gap junctions particularly in response to growth factors or autophagy. Once internalized, there is little evidence that connexins recycle to the cell surface for reuse, but rather connexosomes have been shown to fuse with lysosomes presumably for their ultimate destruction [23,56,57].

Physiological functions of connexins

The physiological importance of connexins in human anatomy can best be illustrated by assessing their role in early development through to healthy tissue homeostasis in adults. As early as the 8 cell stage [58], developing embryos express connexins and initial GJIC begins to

play a role in shaping and regulating normal development [59]. As embryogenesis continues and the three primary germ layers form, exquisite genetic programming allows for the spatial and temporal expression of select members of the connexin gene family [59]. Connexin gene knockout studies in mice have revealed that ablation of some connexin genes can be tolerated during development (i.e., Cx30 [60]) while loss of others is lethal (i.e., Cx45 [61]) early in the developing embryo. The reasons for this dichotomy are not fully understood, but likely rooted in compensation mechanisms attributed to other co-expressed connexins.

As organogenesis begins, some connexin members are expressed during early stages, only to be eliminated in the adult or to segregate to specific cell types as a second cohort of connexins are expressed that sustain organ function. For example, in the fetal heart, Cx40 is broadly expressed only to be restricted to the atria and conduction system in the adult, whereas Cx43 expression serves to orchestrate synchronized ventricular muscle contraction [62,63]. In contrast, Cx43 is abundantly expressed in the embryonic myoblasts only to be eliminated in adult skeletal muscle as myoblast fusion and extensive nerve innovation removes the need for GJIC during muscle contraction [64]. On first pass, there seems to be a correlation between more connexin family members being expressed in anatomically complex organs like the brain where 9 connexins are expressed [65], compared to the human myocardium where we find 3 [37]. This could be linked to the number of different cell types found in complex organs. However, this relationship is not consistent as the epidermis of the skin expresses upwards of 9 connexins principally in a single cell type, the differentiation-competent, keratinocyte [66]. Here connexin gene expression is turned on and off as a consequence of cell programming changes that occur as keratinocytes enter a terminal series of differentiation changes that ultimately lead to cell death.

At birth and through to the adult, connexin family members like Cx43 are abundantly found in numerous cell types and over half of the human organs [23]. Still other organs and tissues become highly restrictive in which members of the connexin family are expressed. For instance, liver hepatocytes only express Cx26 and Cx32, which serve an important role in liver homeostasis and potentially during regeneration [67,68]. Still other organs like bone express Cx43, but also a rarer connexin, Cx45 [69]. Collectively, connexins can be mapped to every tissue and cell type but their expression levels are highly regulated owing to the physiological activity of the tissue. An example of a physiologically responsive tissue is the uterus where the gap junction content in smooth muscle cells increases by a factor of 10 just prior to labor onset [70–72] in order to coordinate contractions.

Connexins in disease

Connexins are linked to disease or wound healing through several different mechanistic paradigms. First, connexin-mediated intercellular communication is often up- or down-regulated in response to cellular cues that in turn drive connexin gene expression, connexin assembly, or connexin turnover. This scenario has been most widely studied in cancer prevention, onset and progression but likely impacts the severity of other, less well examined disease processes [3,73]. A second connexin-linked disease/injury paradigm is based on the concept that connexin expression acts a “brake” in wound healing and that a transient down regulation would act to facilitate the healing process [37,74]. An intriguing corollary to this

notion is that a rapid spike in connexin expression or activity could stimulate healing of chronic wounds [75,76]. At the channel level, the community continues to interrogate the differential positive and negative roles of GJIC and the connexin hemichannel that may act as a pathological pore during wound healing, inflammation [49], and other diseases as discussed in a recent review [37]. In the case of inflammation, there is growing evidence that connexins play important roles in chronic and acute inflammation in neurological, cardiovascular and lung tissues as reviewed elsewhere [37,49,77]. Thus, reducing connexin-mediated inflammation might be a key step in moderating wound response and disease. Third, connexins as a causal driver of disease was confirmed with the findings that connexin gene mutations lead to over two dozen developmental abnormalities and chronic disease conditions [6,7]. Pannexins have also been linked to disease, but, thus far, this linkage is much more limited and indirect compared to that of connexins (Box 2).

Connexin regulation in cancer

The notion that connexins and gap junctions might be linked to cancer first came about shortly after their discovery in the mid twentieth century. The first studies showed that tumor cells lacked the electrical coupling and metabolic cooperation found in their healthy counterparts [78,79]. Later, tumour promoters were found to block GJIC [80]. These findings sparked a fifty year investigation into the role connexins play in cancer prevention, onset, progression and metastasis [3]. Studies in mice and humans indicated that in many early stage tumors, connexins are lost or mislocalized and their restoration leads to improved growth control and even partial reversion of epithelial to mesenchymal transition events (Figure 3) [81–84]. The *in vivo* ablation of connexins (e.g., Cx26, Cx32, Cx43) in mice supported the role of connexins as tumor suppressors, but in many of these models, increased susceptibility to tumorigenesis required mice to be challenged with a tumor initiator [3,85–88].

The potential complexity of the role connexins play during tumorigenesis became more apparent when several reports supported multiple GJIC-independent roles for connexins [89–91]. For example, Cx43 was found to interact with many other proteins that regulated diverse cellular processes (the interactome) [92] (Figure 3) and was reported to be expressed in mitochondria [93–95] and the nucleus [96] or to function as hemichannels that link the cytoplasm with the extracellular space [37,97]. Still other studies revealed that connexins (e.g., Cx26, Cx43) may become highly expressed in metastatic tumor sites leading to enhanced tumour cell adaptation and survival through exchange of metabolites [98–100]. In a mouse line where Cx40 was ablated, tumor angiogenesis and growth were reduced and mice exhibited better overall survival suggesting a critical role for endothelial Cx40 in tumor progression [101]. These latter studies resulted in a reassessment of the therapeutic potential of upregulating connexins in cancer, as connexin inhibition might be more effective in some situations. These opposing effects of connexins in cancer is thought to be linked to the specific tumor type and stage of disease.

Clinically, the central question rests on whether connexins are a meaningful and appropriate druggable target to consider in cancer treatment. Some fifty years after linking gap junction loss to cancer, the answer remains unclear, with over 1500 papers reporting strong reasons to target connexins in customized cancer treatments, but some reasons for avoiding them [3]. The

next approach to assess the role of connexins in cancer may involve epidemiological studies that longitudinally track tumorigenesis in human subjects harboring loss-of-function connexin mutants. This can be best illustrated in the hearing loss community as the most common *GJB2* (Cx26) gene mutation is 35delG, which results in effectively a Cx26 knockout in tens of thousands of patients worldwide [102,103]. Given that Cx26 is highly expressed in the human female breast [104], it is now possible to consider assessing this cohort of patients for breast cancer susceptibility. Going forward, this human strategy will likely override the use of animal models, as it will also account for ethnic variations and the genetic diversity found in society.

Connexins in injury and wound repair

Although keratinocytes at different stages of differentiation express multiple different connexins [66], Cx43 appears to be the most prevalent connexin in human skin and it plays a regulatory role during early stages of wound healing. Notably, the expression and phosphorylation status of Cx43 in the dermis and epidermis of the skin change during epidermal wound repair [105–107]. A steady state level of cell proliferation in the basal layer replaces exfoliated skin supplying cells to cover the wound bed. It has been well established that skin wounding stimulates cellular signals that activate Cx43-based GJIC to synchronize the early steps of cell migration to fill the wound bed [108,109]. One day after skin wounding in both rodents and humans, Cx43 levels decline, returning to homeostatic levels after wound closure [108–110]. Not surprisingly, altering Cx43 expression levels has been shown to affect wound repair [75,107,108,111–115]. Wound closure is delayed in diabetic rat skin as Cx43 expression remains abnormally high [115]. Conversely, mice with reduced levels of epidermal Cx43 show more rapid healing [113]. Specifically targeting Cx43 in wounded tissue via topically applied agents offers the advantage that one can concentrate drug treatment to the site of injury and potentially avoid off-target effects that Cx43-directed drugs might have on organs such as the brain and heart where Cx43 is also highly expressed [116].

Recent discoveries have shown that Cx43 also plays a critical role in corneal wound repair. The connexin-rich cornea is often subject to chemical or structural injury as well as damage from keratoplasty or other surgical procedures. In the absence of proper wound repair, vision can be negatively impacted. While mechanistically distinct in several ways, like epidermal responses during wound repair, corneal wound repair first involves cell migration into the wound bed followed by cell proliferation. In the case of severe wounds, cell migration and proliferation may be insufficient to fully heal the cornea leading to a surgical need for corneal grafts or transplantation. Using rat corneal scrape models, two different groups showed that Cx43 antisense application promoted wound closure and reduced stromal edema and inflammation [117,118]. In addition, a peptide mimicking the last 9 amino acids of the Cx43 C-terminus (aCT1) reduced inflammation and increased corneal wound closure rates in wild type rats [119] and in a streptozotocin-induced rat model of type I diabetes [120]. These results clearly indicate that the cornea is a viable target for Cx43 modulating drugs.

Connexins in cardiac disease

Through their ability to allow ion flow, gap junctions play a critical role in impulse propagation in the heart [121,122], and connexin ablation in mice can lead to arrhythmias and death [123–125]. Intercalated discs joining cardiomyocytes are rich in gap junctions, mechanical junctions and ion channels that coordinate and regulate the synchronous beating of the heart. Cx43 is the predominate connexin in the ventricle [126], and reductions in Cx43 reduce conduction velocity [123,124,127,128]. Loss or dysregulation of Cx43 occurs during many cardiac pathologies including hypertrophy, failure, arrhythmia and ischemia reperfusion injury (IRI) [37,129]. Recent evidence has suggested that Cx43 hemichannels and Nav1.5 found in the perinexus surrounding gap junctions play a key role in conduction [130,131] and are more dispersed in humans with atrial fibrillation [132]. Ischemia can cause loss of Cx43 from the intercalated discs with its relocalization to the lateral edges of the myocyte, a process referred to as lateralization (e.g. [133]). IRI occurs during reoxygenation of ischemic tissues. Cx43 plays a critical role in the phenomenon of preconditioning wherein short bouts of ischemia preceding a longer ischemic interval can actually protect the heart from IRI and preserve intercalated disc localization of Cx43 [134–136]. Preservation of Cx43 at the intercalated disc may not only maintain conduction velocity but also the normoxic Cx43 interactome as ischemia has been shown to change the proteins that interact with Cx43 potentially affecting cardiac function [137]. Given the huge economic consequences of cardiac diseases, Cx43 is an attractive drug target to consider especially acutely during reperfusion of the heart.

Connexins in inherited diseases

Over the past quarter century the number of diseases or developmental abnormalities causally linked to connexin gene mutations has risen to 28 [6,7]. On one hand, this should be expected given the ubiquitous distribution of connexins. On the other hand, one might predict that the number of diseases might be much higher given the generic role connexins play in cellular function. Connexin redundancy and compensation likely plays some role in protecting tissues and organs from disease in patients harboring connexin gene mutations, as we know that most cells express multiple connexins [23]. Another important consideration is that many connexin-linked diseases are inherited in an autosomal dominant fashion, where the unaffected gene allele may serve to provide sufficient connexin function to protect organs from developmental defects and disease [6,7]. This might be best illustrated in the heart where patients with Cx43 activity predicted to be less than 50% of healthy subjects do not typically exhibit heart abnormalities or susceptibility to heart disease [39,138].

The most common of the connexin-linked diseases is sensorineural hearing loss linked to *GJB2* (Cx26), *GJB6* (Cx30) and *GJB3* (Cx31) gene mutations [102,103]. Several theories exist as to how aberrant connexin communication in the cochlea leads to deafness, but defects in metabolite and/or ion exchange ultimately result in loss of hair cells during development or early in life [139–141]. Another sensory organ affected by connexin gene mutations is the lens, where *GJA3* (Cx46) and *GJA8* (Cx50) mutations lead to congenital cataracts [142–144]. Both *GJB1* (Cx32) [145,146] and *GJC2* (Cx47) [147,148] gene mutations lead to the rare demyelinating diseases, X-linked Charcot Marie Tooth disease and Pelizaeus-Merzbacher-like disease,

respectively. In the case of Cx32, loss of function leads to axon demyelination due to perturbation in the Schmidt-Lanterman clefts, where metabolites are unable to pass across the myelin sheath from the cytoplasm lining the peripheral of the Schwann cell to cytoplasm adjacent to the axon [149]. Thus, in this unique setting, Cx32 is mediating intracellular communication rather than classical intercellular communication. The most diverse set of connexin-linked diseases affect the skin as over 10 clinically distinct skin diseases that vary in severity from being lethal in young children, to those that result in chronic morbidities that can be managed throughout a normal life expectancy [150–152]. The variability of these skin diseases is due to the fact that upwards of 9 connexins are differentially expressed in the strata of the human epidermis and mutations in any one of 5 connexins expressed in the epidermis have been reported to lead to skin disease [7,66]. As an example, leaky Cx26 hemichannels in Cx26-G45E mutant mice that model keratitis-ichthyosis-deafness syndrome, act as a pathological pore in this devastating disease [153]. Surprisingly, *GJA1* (Cx43) gene mutations almost exclusively cause oculodentodigital dysplasia, a rare disease with related developmental abnormalities [154,155]. This finding is somewhat remarkable given the broad expression of Cx43 in humans and the fact that mice lacking Cx43 die at birth [156]. It is likely that other connexin gene mutations will be discovered that are linked to rare diseases as mutations in the *GJA5* (Cx40) and *GJA1* genes have been shown to be associated with arrhythmias; however the data sets are small and causal relationships are highly debated [37,63,157,158].

Any attempts to treat patients that have connexin-linked diseases would require a customized and targeted approach where the nature of the connexin defect is well understood. With over 500 specific connexin gene mutations reported in the literature, this would require an understanding of how each mutation (or class of mutations) is altering connexin function and whether they cause gain-of or loss-of function. Studies have shown that there are at least 10 different mechanisms underpinning how specific connexin mutations cause disease [7,37,159]. These range from loss-of-function phenotypes where connexin mutants fail to traffic or misassemble into non-functional channels, or gain-of-function effects where connexin mutants aberrantly expand their capacity to interact with co-expressed connexin family members or form leaky hemichannels [6,159]. Any treatment regimen might also consider the unaffected connexins that are expressed in an affected tissue/organ as drug design could involve upregulating an endogenous connexin to compensate for the mutant connexin defect. For autosomal dominant connexin-linked diseases, strategic approaches might consider using CRISPR/Cas9 to target and ablate a mutant connexin gene allele. This approach has merit as we have seen that mutant mice harboring 50% of normal connexin gene dosage are often disease-free [156,160] and patients heterozygous for *GJB2* (Cx26) mutations are commonly disease-free [161–163]. However, in order for the repair of a gene to occur, the CRISPR/Cas9 components would have to reach the targeted cells, which presumably would not be 100% successful and would lead to mosaicism with unknown consequences. Further caution may also be necessary as a recent study suggests that genome-editing may be most efficient in cells where p53 is inhibited raising concerns that CRISPR/Cas9 intervention may favor cells more prone to tumorigenesis [164].

Current approaches to connexin therapeutics

To date, most connexin drug development programs focused on modulating gap junctions for therapeutic purposes have targeted Cx43. This might be expected as Cx43 is ubiquitous and represents the most well-studied gap junction protein in healthy tissues and in disease. In addition, Cx43 is an early responder to many types of injury [37,111]. Several different strategic approaches, with regard to how and where in the life-cycle to target Cx43, are being pursued (Figure 4 and Table 1). Depending on the disease indication, approaches include: targeting Cx43 present in hemichannels versus gap junctions or in possibly different Cx43-expressing cell types, developing peptide mimetics, as well as repurposing specific drugs in combination with connexin modulators. While anti-connexin antibody targeting strategies are early in development as possible therapeutics, evidence is beginning to emerge where Cx43 hemichannel and GJIC attenuating antibodies targeting the extracellular loops may have therapeutic value in cancer and other diseases [165,166]. However, antibodies normally would have to be tested in animal systems and then presumably humanized before clinical trials in patients could proceed. Below we review some of the most advanced therapeutic strategies and diseases being targeted, highlighting recent successes and challenges faced.

Treatment of epidermal wounds

Two companies have targeted Cx43 in epidermal wounds: CoDa Therapeutics and FirstString Research (Table 1). CoDa first developed a 30-mer antisense oligonucleotide (AsODN), which knocked down Cx43 expression, based on research from the laboratories of Colin Green (University of Auckland, New Zealand) and David Becker (Nanyang Technological University, Singapore). In animal models of epidermal skin wounds and spinal cord injury, neutrophil counts and subsequent macrophage invasion into wound sites were reduced in the presence of the AsODN [114,167,168]. The AsODN underwent Phase 2 testing (NCT00820196; NCT01199588; NCT01490879) for venous leg ulcers and diabetic foot ulcers. Results from NCT00820196 were summarized as a “69% reduction in venous leg ulcer size within four weeks, and 31% of wounds completely healed, five times greater closure than vehicle alone” in a review highlighting CoDa’s achievements [74]. CoDa has been restructured as OcuNexus Therapeutics Inc., and they have taken the development of AsODN (renamed as Nexagon®) forward for multiple applications in conditions affecting the eye that are described later.

aCT1 (also known as αCT1, trade name Granexin® gel, FirstString) is a peptide that mimics the last 9 amino acids of Cx43 (AA374–382) preceded by a cell internalization sequence. Granexin gel is currently being tested in chronic wound indications including diabetic foot (Phase 3, NCT02667327, NCT02666131) and venous leg ulcers (Phase 1, NCT02652572) (https://firststringresearch.com/). This compound was originally discovered and developed as a research tool to study cardiac conduction system development and ischemic cardiac injury in Robert Gourdie’s laboratory (Virginia Tech Carilion Research Institute, Virginia, USA) [75,76,169,170], based on previous reports showing that Cx43 bound ZO-1 through this C-terminal region [171,172]. In early preclinical work, aCT1 was shown to reduce scar area and inflammatory neutrophil infiltration and promote restoration of dermal histoarchitecture and

mechanical strength after skin wounding [75]. Later in prospective, randomized, multicenter clinical trials in diabetic patients with foot ulcers (CTRI/2011/09/001984) [173] and in patients with chronic venous leg ulcers (CTRI/2011/09/001985) [174], aCT1 treatment was shown to significantly reduce the ulcer area and median time-to-complete ulcer closure [175]. A within patient-controlled multicenter randomized trial with aCT1 showed significantly less scarring in bilateral incisional laparoscopic surgery wounds (CTRI/2011/09/002004) [176].

The mechanism of action underlying aCT1's role in wound repair, particularly as it pertains to injury type, remains to be fully elucidated. The role that GJIC plays in this process, which cell type(s) is actually affected by aCT1 treatment and the molecular processes that subsequently translate to improve healing in multiple models of injury, remain unknown. Many possibilities exist including cells of the dermis, epidermis (keratinocytes), macrophages, neutrophils, and others. Early studies showed that an interaction between Cx43 and ZO-1 causes a reduction in gap junction size and that aCT1 prevents this interaction allowing larger gap junctions to increase communication between cells in culture (Figure 4) [177,178]. Such changes in gap junction size may be mediated by Akt phosphorylation of Cx43 at S373, as this phosphorylation event was increased in human skin upon wounding and was found to eliminate the interaction between Cx43 and ZO-1 in cultured cells leading to the expected increase in gap junction size and communication [106]. aCT1 treatment also increased Cx43 phosphorylation at a key regulatory serine 368 that is phosphorylated by protein kinase C [169], potentially affecting channel or hemichannel conductance or other regulatory processes. Since GJIC is important for both initiation and coordination of migration of keratinocytes in early phases of wound repair [107], it is possible that aCT1 reinitiates this activation phase making it more effective. This might be particularly true in diabetic or chronic wounds as they often show abnormal Cx43 expression [179–181]. Alternatively, aCT1's effect might mostly be related to its anti-inflammatory properties and instead target macrophages or neutrophils. aCT1 also has been reported to reduce the number of Cx43 hemichannels in the plasma membrane by promoting their incorporation into gap junctions [178] potentially reducing pathology associated with hemichannel activity. Nevertheless, consistent with the premise that sustained GJIC and inhibition of hemichannels promotes cell and tissue health, it will be intriguing to determine how FirstString Research's drug promotes healing in clinically demanding wounds.

Treatment of eye wounds and inflammation

OcuNexus has an extensive focus in eye wounds and inflammation and has three drug candidates at various stages of development to treat different conditions of the eye: Nexagon® (AsODN), Xiflam (tonabersat) and Peptagon™ (Peptide5). AsODN treatment reduced scarring in a rabbit eye glaucoma trabeculectomy model [182] and a Phase III trial for treatment of non-healing corneal wounds from chemical or thermal injury, based on previous compassionate use in a number of patients, is being planned (https://ocunexus.com) [183]. Tonabersat is a cis benzopyran compound that had been reported to reduce the expression of Cx26 in the brain [184,185]. Tonabersat has been used in randomized trials for the treatment of acute migraines but did not show statistical improvements over placebo in one study [186]; however, it did prove effective in a second international two dose, double-blind, randomized study, suggesting that it may prove beneficial in treating migraine headaches as an oral

medication [187,188]. Xiflam is being repurposed as a connexin hemichannel blocker (Figure 4) [189] to be tested in retinal disease trials such as macular degeneration and diabetic retinopathy (https://ocunexus.com). In another approach, a synthetic peptide mimicking a segment of the second extracellular loop of Cx43, Peptide5, is also under development as a specific inhibitor of Cx43 hemichannels [190]. In the central nervous system, Peptide5 reduced vessel leak and associated inflammation and significantly enhanced neuronal (retinal ganglion cell) survival after retinal ischemia-reperfusion injury [191,192]. In preterm sheep ischemia and asphyxia models, Peptide5 prevented seizures, restored sleep cycling and provided neuronal protection with improved functional electroencephalography outcomes [193–195]. Mechanistically, both Peptide5 and Xiflam were shown to inhibit Cx43 hemichannels [189–192,196–199], and the inflammasome pathway [200,201], which has been implicated in many chronic inflammatory diseases characterized by inflammation and/or vessel hemorrhage and ischemia [199]. A central theme beginning to emerge here that may extend beyond the eye, is the possibility that hyperactive hemichannels leads to many cellular pathologies linked to inflammation and cell death [202–204]. Collectively, the drugs OcuNexus has in the therapeutic pipeline should help define Cx43's role in healing wounds of the eye and eye diseases.

FirstString is also investigating therapies for eye wounds and ocular disorders. Indeed, aCT1 has been reported to reduce inflammation and increase corneal wound closure rates in preclinical studies [119,120]. As a result, aCT1 is now being developed as an eye drop formulation for application after corneal injury/surgery, treatment of diabetic keratopathy and treatment of age-related macular degeneration [205]. Connexin Therapeutics is also apparently pursuing targeting connexins in glaucoma, but more detailed information on their approach is not publically available.

Nervous system disorders

Theranexus is developing drug candidates for the treatment of nervous system diseases, focusing on the role played by astrocyte connexins in response to psychotropic drugs. The lead drug candidate THN102, which contains modafinil (a first line treatment for narcolepsy) and low-dose flecainide (an antiarrhythmic agent), is being developed to treat narcolepsy. In wild-type and narcoleptic orexin knockout mice, modafinil administered with flecainide enhanced the wake-promoting and pro-cognitive effects of modafinil [206]. Modafinil was shown to increase Cx30 mRNA and protein expression in mouse cortex but had little effect on Cx43 [207]. THN102 underwent phase 1 testing in healthy sleep-deprived volunteers (NCT03182413) and now is being tested in people with narcolepsy in a phase 2 trial (NCT02821715). Theranexus is also testing a combination of donepezil and the anti-malarial drug mefloquine (THN201) for disorders linked to Alzheimer's disease in preclinical models (https://www.theranexus.com/). Mefloquine is a potent Cx36, Cx50 and Cx43 channel blocker [208–210]. The combination of the Alzheimer's drug amitriptyline and mefloquine is also being tested for neuropathic pain treatment in preclinical models [210]. With respect to connexins, Theranexus's focus is to repurpose and use combinatorial drug approaches to assess clinical benefits that may be at least partially rooted in changes in connexin expression or functional status.

Arrhythmia and other cardiac issues

In the early 2000's, Zealand Pharma developed ZP123 (Rotigaptide), a more stable hexapeptide rotation-inversion of AAP10, which had been shown to have anti-arrhythmic activity [211]. Several studies confirmed that ZP123 increased GJIC [212–214], increased conduction [215–217], reduced arrhythmia [218], reduced infarct size and reduced ischemia reperfusion injury (IRI) [219] in cell culture and/or a variety of animal models. ZP123 was licensed to Wyeth Pharmaceuticals in 2003 and was tested in two phase 2 clinical trials (NCT00137332, NCT00137293) on ventricular tachy-arrhythmia, although no outcomes appear to have been published. Based on ZP123, Zealand next developed the dipeptide ZP1609 (danegaptide), which has been shown to reduce infarct size following IRI in pigs [220], decrease cardiomyocyte hypercontracture [221] and exert antiarrhythmic effects on atrial fibrillation in dogs [217,222]. In 2011, Zealand recovered rights to ZP1609 and launched a phase 2 proof-of-concept clinical trial testing its ability to reduce injury following myocardial infarction. In 2018, the published results showed no effect of danegaptide on the primary endpoint of reducing IRI damage [223]. Although the company is still interested in connexins as therapeutic targets, neither of these peptides appear to still be in development (https://www.zealandpharma.com/). Clearly the peptides tested by Zealand Pharma had biological effects in some cellular and animal systems, but detailed mechanisms of how they functioned, whether they were functionally equivalent in all settings and how they affected their apparent targets were never fully elucidated.

Clinical challenges in connexin therapeutics

Given that healthy human physiology requires an appropriate level of functional gap junctions at all times and this level changes during healthy aging and during adaptive physiological changes, any therapeutic strategies to regulate connexin functional levels need to be carefully considered. Early arguments postulated that increased connexin expression and gap junction coupling would have no detrimental effects regardless of whether or not it led to increased health or a better response to disease treatment. However, mice over-expressing Cx43 in the heart were found to have even more developmental abnormalities than that found in Cx43 knockout mice, which survived to birth before quickly dying [156,224–227]. Deletion of another cardiac connexin, Cx45, is embryonically lethal [61] whereas mice lacking the third cardiac connexin, Cx40 are hypertensive and exhibit cardiac hypertrophy [123,125]. Thus, when considering the heart alone, the ablation of three distinct connexins yielded distinct deleterious outcomes, while overexpression of Cx43 is equally detrimental. These observations indicate that exact and intricate regulation of connexin levels is critically important in organ function.

Contemplation of the potential therapeutic value of connexin-targeted drugs must also consider whether findings in preclinical rodent models extend to humans. The available data is mixed: in some cases, genetically-modified mice recapitulate the connexin-linked disease found in humans such as congenital cataracts linked to Cx46 and Cx50 and neuropathies linked to Cx32 [228] while, in other situations, this is not the case. For example, Cx26 ablation in mice is embryonically lethal due to a malfunctioning placenta [229], but in humans [230,231], children have normal longevity with hearing loss [232,233]. In another case, mice lacking Cx30

appear relatively normal with the possibility of mild developmental defects [60,233,234], and patients lacking Cx30 may also be disease-free with the possibility of hearing loss, likely due to the correlated down regulation of Cx26 in the cochlear. In a third scenario, heterozygosity for an A88V mutation in the *GJB6* gene encoding Cx30 in humans [235] presents with a skin disease called Clouston syndrome, but the equivalent heterozygous mutant mouse is essentially skin-disease free [236], even though Cx30 is expressed in the same strata of the epidermis in both human and mouse skin.

Another major obstacle in connexin therapeutics is drug targeting and delivery. That said, 1st generation connexin therapeutics have frequently targeted accessible injuries or diseases that can be easily reached by topical applications such as skin or corneal wounds. These approaches not only ensure effective drug dosage to the wound but tend to limit systemic off-target effects where connexins in healthy tissues/organs may be affected. In another approach, hyaluronic acid coated albumin nanoparticles have shown some promise in the effective delivery of a Cx43 mimetic peptide for the treatment of a rat model of retinal ischaemia-reperfusion injury [237]. In still another intriguing study, human monoclonal antibodies designed to block hyperactive mutant Cx26 hemichannels in skin disease might serve as a strategic approach to alleviate drug targeting difficulties as we look toward next-generation therapeutics [238]. In the case of mutant connexins that cause disease, it is possible to envision a time where the mutant allele in affected cells is selectively ablated or corrected early in development or shortly after birth, thus avoiding life-long organ damage (Figure 3).

Outlook

There remains no doubt that connexins and gap junctions are vitally important for human health, and we envision at least three avenues for development of potential connexin therapeutics. First, unequivocal evidence has demonstrated that connexin gene mutations lead to numerous diseases from mild, well-tolerated conditions to severe, life-long morbidities and devastating, life-shortening illnesses. As with all genetic-linked diseases, it is one thing to know the genetic cause of a disease, but it is another to mechanistically fix the biological problem and cure the disease. To this end, genotyping children with hearing loss for *GJB2* and *GJB6* gene mutations is now standard practice, with the next step being the development of customized therapeutics. Perhaps the hope here will rest on the versatility and utility of CRISPR/Cas9 technology that is only beginning to be considered for the treatment of human disease. Given the developmental nature of these defects, one might need to apply gene editing technologies in combination with in vitro fertilization for cases where preimplantation genetic diagnoses revealed disease causing connexin gene mutations. Clearly, there is much to consider before it will be ethically acceptable to edit early embryos given the possibility of off-target effects and unexpected harmful outcomes.

The second area of optimism in connexin therapeutics going forward would be the development of oral medications that could selectively up- or down- regulate (depending on where clinical benefit is identified) the targeted connexin, inhibit pathological connexin hemichannel function or modulate GJIC in disease or during recovery from injury. Oral medication strategies have entered the pipeline for Zealand Pharma, OcuNexus and Theranexus, although achieving sufficient efficacy, tissue selectivity and limiting off-target

effects represent significant challenges. Here we await news of a major success that would drive future endeavors into oral connexin-targeting medications. In the age of personalized medicine, any connexin therapeutics may need to be used in combination with current FDA approved medications, a practice frequently used in cancer treatments.

Arguably the most promising connexin therapeutics currently in development might be those designed to initiate, stimulate or accelerate healing of acute or chronic surface wounds. Here, connexin modulators can be effectively applied to the wound with minimal risk to the patient and of off-target effects. Clinically, there is a huge demand for improved therapeutics for common wound morbidities such as diabetic skin ulcers and to reduce scarring. Given the ongoing clinical trials, the community remains optimistic that connexin modulators will prove beneficial in future therapies as we anticipate the first connexin-targeted drug entering the marketplace.

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Glossary

Perinexus	A zone at the periphery of gap junctions that is enriched in connexons/hemichannels where Cx43-ZO-1 interaction can occur
ZO-1	Zonula Occludens-1 is a MAGUK (membrane-associated guanylate kinase) scaffolding protein that interacts with Cx43 and tight junction associated proteins
IRI	Ischemia reperfusion injury occurs when blood supply (oxygen) returns to tissue after a period of ischemia (hypoxia). Damage results from oxygen restoration and resulting inflammation and oxidative damage
ID	Intercalated discs are specialized membrane structures at the ends of cardiomyocytes that contain desmosomes, adherens junctions, sodium channels and gap junctions that allow depolarization waves to transmit from one cell to its neighbor
Connexin	A tetraspanning membrane protein that is the molecular constituent of gap junctions

Connexons	A hexamer arrangement of connexins that contains the same or different connexin proteins arranged into an oligomer
Hemichannels	Connexons that have the ability to open for the passage of small molecules
Connexosomes	Double-membraned structures that have formed from the internalization of the gap junction components from contacting cells
GJIC	Gap junctional intercellular communication is the process where contacting cells harbouring functional gap junctions exchange small molecules
AsODN	Antisense oligodeoxynucleotides are short chain nucleotides that can be designed to target connexin encoding RNA to block protein expression
Peptide mimetics	Short peptide sequences of usually 8–24 amino acids that correspond identically to segments of connexin proteins that can be used to modulate connexin function

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Box 1: Pannexins and pannexin channels

At the dawn of the new millennium and with the growing understanding of the complement of genes encoded within the human genome, a new family of proteins called pannexins (*PANX*) were discovered that resembled the invertebrate gap junction protein family of innexin proteins [239,240]. Owing to early reports that pannexins may form gap junction intercellular channels [4,241], interest grew as to how they compared to the better-characterized connexin family. Currently, pannexins are thought to function mainly as tightly regulated channels connecting the cytoplasm and extracellular space. The pannexin family is small, containing only three members (Panx1, Panx2 and Panx3) with Panx1 being the most widely expressed and best studied [240,242–244]. Although it is tempting to extend insights into the life-cycle and function of Panx1 to include Panx2 and Panx3, caution must be exercised, as these pannexins likely have some unique properties. Panx1 is thought to be co-translationally imported into the endoplasmic reticulum where it quickly folds, oligomerizes and, unlike connexins, is N-linked glycosylated to a high-mannose glycoprotein before final editing in the Golgi apparatus to yield the complex N-glycans [245–247]. Once oligomerized, these large-pore channels are predicted to allow the passage of many small molecules [248–250]. Panx1 has been reported to serve a role as a calcium leak channel reducing the calcium load in the endoplasmic reticulum [251]. A COPII-dependent mechanism has been proposed to play a role as Panx1 employs the cytoskeletal network to pass through the classical secretory pathway [252,253] before being delivered to the cell surface. Once at the cell surface, it is thought that the primary role of Panx1 involves the release of small molecules, including ATP, from the cell [254–258]. Many of these Panx1 channel features are shared with connexin hemichannels, but these two channel types have many distinquishing characteristics, and investigators continue to interrogate the members of the metabolome that can pass through both channel types and those that are unique to each. Transient gating of Panx1 channels has been reported to occur by a number of processes resulting in channels acquiring a series of intermediate open states [259]. Alternatively, permanent opening of the Panx1 channel was observed after caspase cleavage during apoptosis [255]. Finally, unlike most connexins, the evidence to date suggests that Panx1 is long-lived at the cell surface before following a classical internalization pathway toward its destruction in lysosomes [260,261].

Box 2: Pannexins in disease

To date, only one germ-line mutation in the *PANX1* gene has been associated with human developmental abnormalities, and in this case, multi-organ defects were identified in keeping with what might be expected given the ubiquitous distribution of PANX1 in the human anatomy [262]. However, elevated or hyperactive pannexin levels have been associated with over a dozen diseases that include melanoma [263], ischemia [264–266], stroke [267,268], seizures [269], colitis [270], migraine headaches [271], HIV infection [272], osteoarthritis [273], epilepsy [274–276] among others [277]. It remains unclear if elevated pannexin levels are causal in any of these diseases but that does not preclude the fact that attenuating pannexin function may improve treatment. As an example, targeting Panx1 may have utility by blocking ATP release from microglia as it has been shown to alleviate the symptoms of opiate withdrawal in rodents [278]. In another study, Panx1 channel activation promoted epileptic seizure, while pharmacological inhibition or ablation of these channels led to reduced convulsions in a mouse model of temporal lobe epilepsy, indicating that blocking PANX1 channels in humans may have therapeutic value in the treatment of epilepsy [275]. In cases where pannexin levels drive the disease state, this raises the possibility that peptide mimetics, knockdown strategies or pharmacological inhibitors that specifically target pannexins may prove beneficial in treatment [274,279–281]. Supporting the notion that Panx1 ablation or down regulation can be tolerated in mammalian physiology, Panx1 null mice tend to be disease-free unless challenged with an insult or injury [258,282,283]. Central to even considering blocking pannexin channels in disease treatment, it is critical to acquire a more thorough understanding of what passes through pannexin channels. To that end, ATP/UTP readily pass through Panx1 channels [254,255], but it is likely that this is the tip of the iceberg when considering the extent of the metabolites that may exit, or even enter, cells through pannexin channels. Most drugs that affect pannexins also affect connexins and potentially other membrane proteins, with the exception of peptide mimetics like ^{10}Panx1 [274,280]. The anti-gout medication, probenecid [284] has been repurposed as a “specific” inhibitor of Panx1 channels to study the functional role of Panx1 in animal models. In one report, probenecid treated female mice had smaller infarct volumes in a permanent middle cerebral artery occlusion stroke model [267]. Another potential interesting and versatile drug, the quinolone antibiotic trovafloxacin formerly used to treat bacterial infections, has been demonstrated to be a potent inhibitor of Panx1 by blocking ATP release [285]. Further, an antihypertensive drug, spironolactone has been found to be an inhibitor of Panx1 channels raising the possibility that this unexpected effect might be important in regulating blood pressure [286]. A peptide analog encompassing a motif within the intracellular loop of Panx1 has also demonstrated therapeutic potential in blood pressure control in mice through its action in regulating vasoconstriction [287]. Finally, while carbenoxolone is FDA approved for the treatment of gastrointestinal ulcers and inflammation and is widely known to block pannexin channels, it also blocks connexin channels at high concentrations and has even entered clinical trials for Huntington’s disease (see Oxalys Pharmaceuticals, http://drugprofiles.informa.com/drug_profiles/24384-oxd-4) [288]. In this latter case, the effect of carbenoxolone *in vivo* could be widespread and include benefits or side effects from broad inhibition of pannexin and connexin channels [255,280,289]. Moving forward, more

specific inhibitors of pannexin channels are likely necessary if pannexin-targeted therapeutics are to proceed to clinical trials.

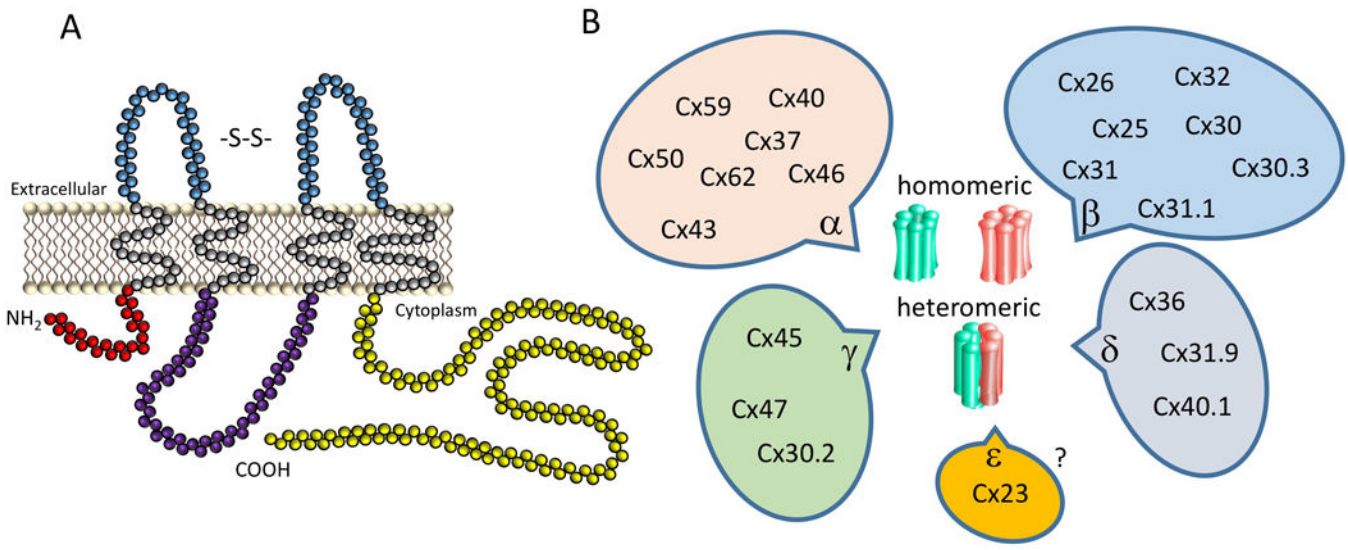

Figure 1: Connexin topology and the connexin family.

A: Connexin topology within the lipid bilayer illustrating the main functional domains and the connexin gene subclasses. The connexin polypeptide passes through the membrane 4 times (gray), resulting in both the amino and carboxyl terminal domains being exposed to the cytoplasm and connected by two extracellular and one cytoplasmic-exposed loops [290]. The highly conserved amino terminal domain (red) plays a role in regulating the channel pore [30,291] while the two conserved disulfide-linked extracellular loops (blue) govern hemichannel docking between adjacent cells [292]. The cytoplasmic loop (purple) size varies extensively amongst connexin subtypes and it has been reported to participate in pH gating of the channel for at least one connexin [293]. The cytoplasmic tail (yellow) length and amino acid sequence is the most varied region amongst the connexin family members. This domain is frequently phosphorylated in many connexins and responsible for binding the vast majority of the connexin interactome [294,295]. **B:** The connexin family is divided into 5 subclasses (α, β, γ, δ, ε) based on homology. Connexins may oligomerize into homomeric or heteromeric hexamers with other connexins within the same subclass and, on occasion, with connexins from other subclasses leading to highly diversified channel arrangements [296]. While most connexins are known to form functional channels, it remains unclear if this is the case for Cx23 [297].

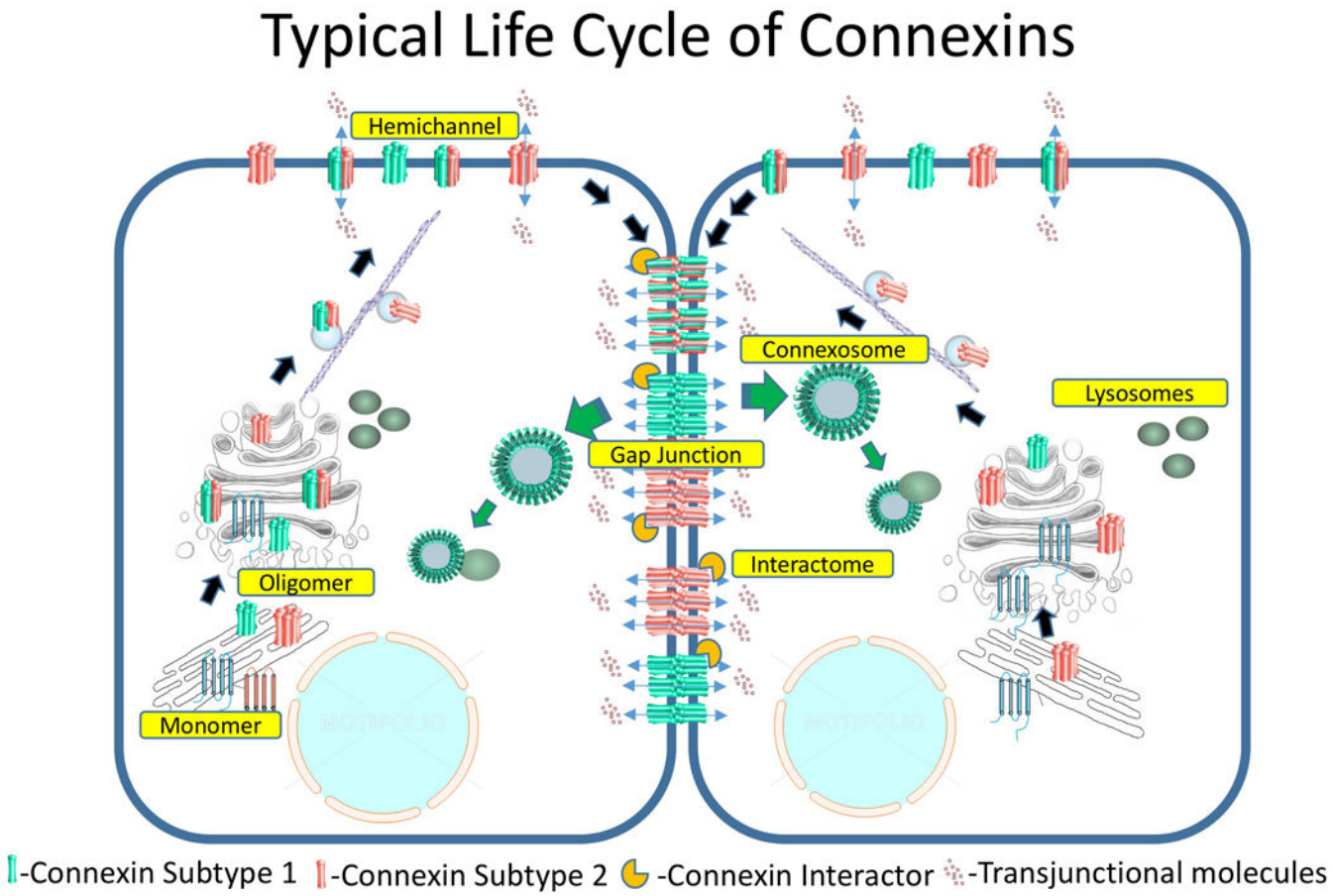

Figure 2: Canonical life cycle of connexins.

Two connexins (pink, blue) are shown to co-translationally insert into the endoplasmic reticulum where they appear as monomers or proceed to co-oligomerize into homomeric or heteromeric single membrane channels, while being resident in the endoplasmic reticulum or after transport to the Golgi apparatus. After constitutive or microtubule-facilitated delivery to the cell surface, connexins may function as hemichannels or proceed to form homotypic or heterotypic gap junction channels that cluster into fully assembled gap junctions. Typically, after a short residency at the cell surface, gap junctions, or fragments of gap junctions, are internalized as unique double-membraned connexosomes before their ultimate degradation in lysosomes. Throughout the typical short life expectancy of connexins, they interact with a large interactome that facilitates their assembly, turnover and function.

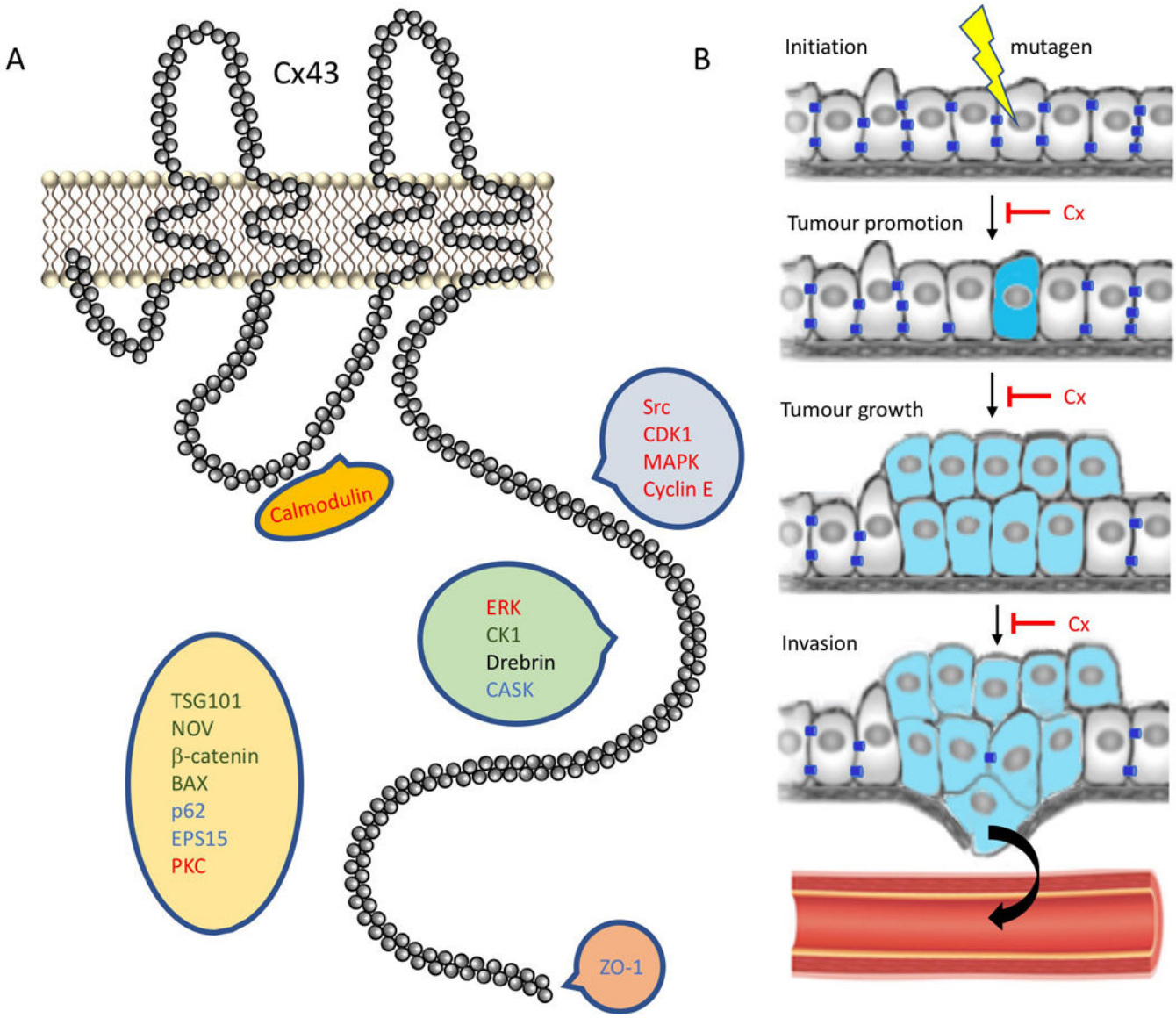

Figure 3: Links between connexins, the connexin interactome and early stage tumourigenesis.

A: Cx43 interacts with many proteins including some (noted here) that have been reported to be involved in tumourigenesis. Cx43 binding proteins are indicated as enhancing (red type), reducing (green type), or playing unclear roles (blue) in tumourigenesis but their mode of action may, in part, be due to their interactions with Cx43. Calmodulin interacts with the intracellular loop, while the others interact with motifs encoded within the C-terminal tail. The names of the Cx43 interactors are positioned near the site of the C-terminal tail where they bind, except TSG101, NOV, β-catenin, BAX, p62, EPS15 and PKC as their specific binding sites within the C-terminal domain are less well defined. A more complete list of Cx43 interactors can be found in other publications [137,295,298]. **B:** Connexins present in gap junctions (primarily Cx26, Cx32, Cx43) have been widely reported to inhibit the promotion, growth and invasion stages of tumourigenesis, but may have more complex roles in later stages of disease progression that is tumour type dependent [3].

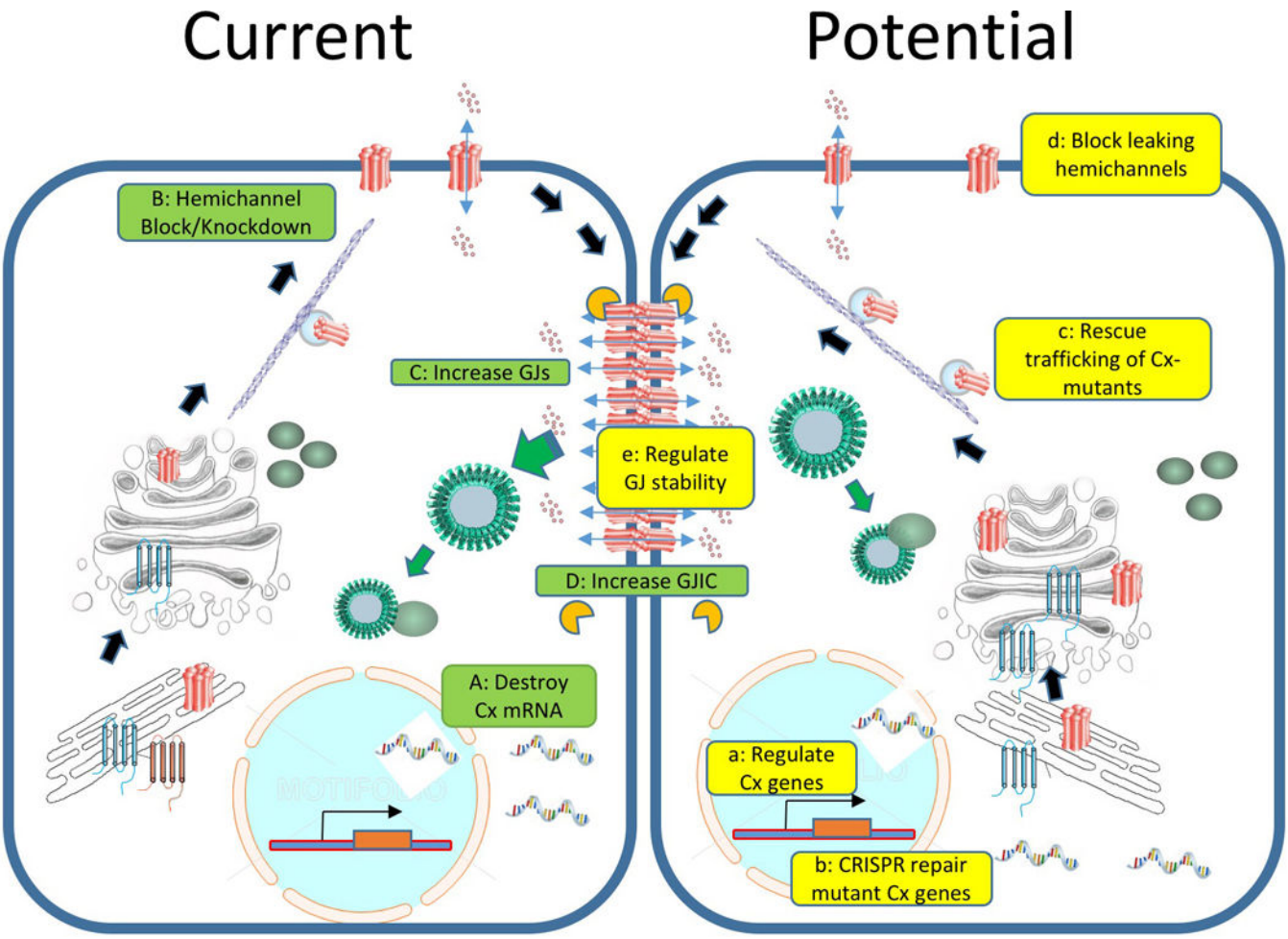

Figure 4: Current and potential connexin modulating therapeutic strategies.

Given the short half-life of connexins, therapeutic strategies can be designed to modulate their expression and/or function at multiple points throughout their life cycle. The purple boxes and numbers represent strategies that have entered therapeutic testing or under development. These include; targeting connexin mRNA levels by using antisense technology to reduce connexin protein levels (A), ultimately leading to a reduction in connexin hemichannels, which can also be blocked by select inhibitors (B), regulating GJ size by promoting hemichannel recruitment into GJ (C), and increasing GJIC (D). Blue boxes and numbers denote potential future therapeutic strategies. It is possible to imagine and design drugs that will drive the expression of connexin encoding genes (a) in cases where the expression of a second connexin family member may rescue the pathology invoked by a disease-linked connexin gene mutation. In the age of CRISPR/Cas9 technology, it is also possible to consider gene editing to repair disease-linked connexin gene mutations (b), or possibly rescue trafficking defective connexin mutants so they make it to the cell surface where they may still retain sufficient channel function to prevent disease (c). Novel antibody targeting or other strategies that block leaky mutant or dysregulated connexin hemichannels may have therapeutic value (d), as would regulating the overall stability of gap junctions that have assembled at sites of cell-cell contact (e).

Table 1:

Selected connexin-targeted agents currently in clinical trials

Condition/Wound/ Disease	Target	Drug Effect	Approach	Product	Company	Clinical Trial Number	Phase of Trial
Skin wounds diabetic, non-healing	Cx43	Decrease Cx43 levels	antisense	AsODN	CoDa Therapeutics	NCT00820196 NCT01199588 NCT01490879	Phase 2 Phase 2 Phase 2
	Cx43	Decrease Cx43-ZO-1 interaction	Peptide mimetics	aCT1	FirstString Research	NCT02667327 [173] NCT02666131 CTRI/2011/09/001985 [174]	Phase 3 Phase 3 Phase 2
Scar reduction	Cx43	Decrease Cx43-ZO-1 interaction	Peptide mimetics	aCT1	FirstString Research	CTRI/2011/09/002004 [176]	Phase 2
Corneal wounds	Cx43	Decrease Cx43 levels	antisense	AsODN	OcuNexus	Planned	
	Cx43	Decrease Cx43-ZO-1 interaction	Peptide mimetics	aCT1	FirstString Research	Pre-IND	
Retinal injury disease	Cx43	Decrease hemichannels	Antisense, peptide mimetics	AsODN, Peptide 5	OcuNexus	Planned	
Cardiac ischemic injury, arrhythmia	Cx43?	Increase GJIC	Modified Peptide	Rotagaptide, danagaptide ZP1609	Zealand Pharma	NCT00137332 NCT00137293 NCT01977755 [223]	Phase 2 Phase 2 Phase 2
Narcolepsy	Cx30? GJIC	Regulate GJIC	Repurposed drugs	THN102	Theranexus	NCT03182413 NCT02821715	Phase 1 Phase 2