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Review

# Cardiac to cancer: Connecting connexins to clinical opportunity

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ABSTRACT

**Gap junctions and their connexin components are indispensable in mediating the cellular coordination required for tissue and organ homeostasis. The critical nature of their existence mandates a connection to disease while at the same time offering therapeutic potential. Therapeutic intervention may be offered through the pharmacological and molecular disruption of the pathways involved in connexin biosynthesis, gap junction assembly, stabilization, or degradation. Chemical inhibitors aimed at closing connexin channels, peptide mimetics corresponding to short connexin sequences, and gene therapy approaches have been incredibly useful molecular tools in deciphering the complexities associated with connexin biology. Recently, therapeutic potential in targeting connexins has evolved from basic research in cell-based models to clinical opportunity in the form of human trials. Clinical promise is particularly evident with regards to targeting connexin43 in the context of wound healing. The following review is aimed at highlighting novel advances where the pharmacological manipulation of connexin biology has proven beneficial in animals or humans.**


## 1. Introduction

Evolution of the multi-celled organism demanded the coordinated integration of cell types and the intricate orchestration of the processes involved in cell coordination, synchronization, growth, differentiation, and programmed cell death. The formation of channels that directly link the cytoplasm of adjacent cells (gap junctions) or permit cell-extracellular communication (hemichannels), encoded by a set of highly evolutionarily conserved genes (connexins), appears ingeniously simple when considering the complex biology of the higher functioning organism. Scientific literature reveals the essential roles of these processes in cell and tissue homeostasis and angiogenesis, and by default, in a slew of pathological processes [1–3]. Research aimed at disclosing the dynamic, cell and disease specific roles involved in connexin transcription, translation, turnover, trafficking, and dysfunction barely scrape the surface in terms of deciphering the complexities associated with translating connexin biology to therapeutic opportunity. In reviewing the clinical promise associated with connexins, it seems best to keep in mind a quote by HL Mencken: "For every complex human problem, there's a solution that is simple, neat, and wrong. . .".

The canonical connexin is diagrammed as a tetraspan transmembrane protein with two extracellular loops, a cytoplasmic loop, and a cytoplasmic amino and carboxyl-terminus (N- and C-terminus). Six connexins oligomerize to form cored connexon transmembrane channels that couple with connexons on neighboring cells to form intercellular channels, which in turn aggregate to form gap junctions (GJs) [4,5]. Hemichannel recruitment to the preexisting GJ plaque is a critical determinant in the operational area, size, and stability of GJs [6]. The formation and dissolution of hemichannels and GJs is dynamic and regulates both junctional and non-junctional intercellular communication. It has been over half a century that the physiological relevance of these processes was first appreciated in studies that examined the electrical transmission of signals at the giant motor synapses in the crayfish [7]. We now know that intercellular communication is key in numerous processes critical for biological homeostasis including, but certainly not limited to, the rapid transmission of action potentials in heart and neuronal tissues [8–10], and the diffusion of metabolites, nucleotides, nutrients, and second messengers with roles in apoptosis, gene expression, inflammatory responses and cellular growth [11–28]. An additional level of complexity is bestowed in situations where heteromeric assembly of different connexin

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proteins in a single channel may result in unique and specific biophysical properties rendering preference with regard to passaging molecules [29,30].

Therapeutic intervention may be offered through the pharmacological and molecular disruption of the pathways involved in connexin biosynthesis, GJ assembly, stabilization, or degradation [31]. Chemical inhibitors aimed at closing connexin channels, peptide mimetics corresponding to short connexin sequences, and gene therapy approaches have been incredibly useful molecular tools in deciphering the complexities associated with connexin biology (Fig. 1). The therapeutic potential of these tools has only recently been evaluated in clinical trials. The following review will focus on discussing the translational relevance and clinical potential underlying the pharmacological manipulation of connexin biology.

## 2. Lessons learned from wound healing

The role of connexins in maintaining tissue homeostasis offers important lessons in both the scientific and commercial exploration of connexin–based therapeutics. A wide range of inherited human disorders stem from mutations in connexin genes (Table 1; reviewed in detail in [32,33]), highlighting the indispensable role of GJs in normal tissue function. Of the twenty-one identified connexin isoforms in the sequenced human genome, the epidermis expresses at least 10, including Cx26, Cx30, Cx30.3, Cx31.1, and Cx43, each correlated with a uniquely dynamic spatial and temporal expression pattern and functional role [34–37]. Given that the coordination and progression of wound healing and re-epithelialization is tied to a complex series of events that occur between various cell types, extracellular matrix components and signaling molecules, the critical role of connexins in regulating the metabolic coupling between cells and transfer of cell signaling molecules during the cutaneous injury response is palpable. These include roles in leukocyte diapedesis, re-epithelialization, wound contraction, fibroblast function, and collagen deposition and synthesis [38,39].

### 2.1. Connexin43 and therapeutic opportunity

The translational bridge connecting connexins to wound healing therapeutics was first made readily apparent with Cx43, the most highly expressed and widely studied connexin in human skin [39–42]. Experiments in murine wound models describe the transient expression pattern of Cx43 and GJ intercellular communication at the wound periphery following dermal injury. Following injury, Cx43 in the wound edge slowly downregulates over approximately 48 h, during which time keratinocytes adopt a migratory phenotype [37,39–42]. These same studies show that Cx43 downregulation is correlated with increased levels of TGF-β mRNA and collagen α-1, and decreased levels of chemokine ligand-2, tumor necrosis factor alpha (TNFα), infiltrating neutrophils and macrophages at the wound site, as well as the promotion of angiogenesis, fibroblast migration, and keratinocyte proliferation. Conversely, Cx43 expression is elevated in the blood vessels proximal to the wound site, with another rise several days post-wounding during granulation formation. The post-translational phosphorylation of serines (S364, S365, S325, S328, S330, S368, S279, S282, S262) in the C-terminal domain of Cx43 during these processes contributes to conformational changes and the formation or disruption of GJs and GJ intercellular communication depending on the site phosphorylated [43–46]. Additionally, Cx43 hemichannels release molecules such as glutamate, ATP, NAD+, prostaglandin E2, and glutathione, providing a paracrine route for intercellular

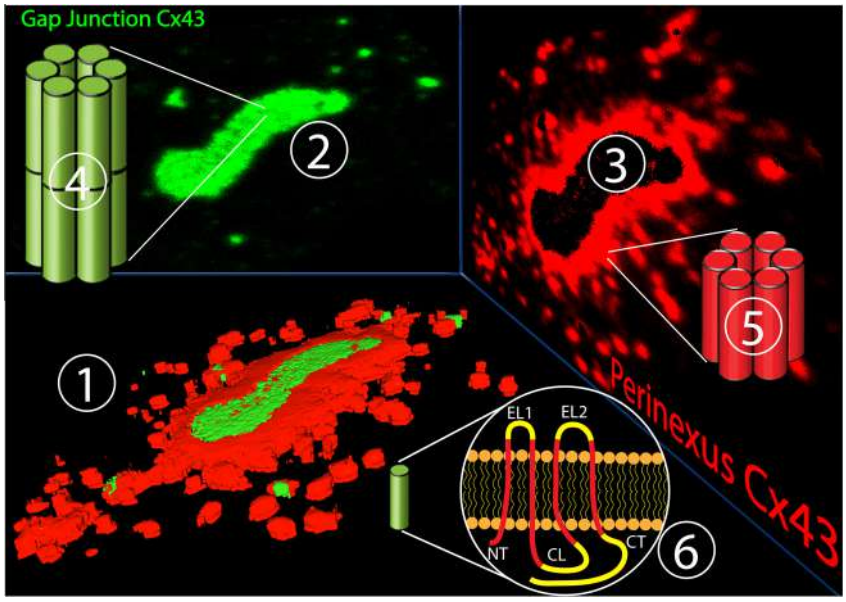


**Fig. 1.** Connexin-based therapeutics target both gap junctions and hemichannels through multiple domains on the Cx43 subunit. ① A three dimensional reconstruction of the Cx43 junctional complex generated from confocal optical sections of standard Cx43 immunofluorescence ②, and Cx43-Cx43 Duolink (Olink Bioscience, Uppsala, Sweden) ③. ② Standard Cx43 immunofluorescence highlights gap junctions, which are composed of aggregated intercellular channels ④. Drug targets may influence intercellular coupling by improving conduction in the heart or spatial buffering in the brain, for example. ③ Duolink imaging of Cx43 enables imaging of the perinexus, which is composed of diffuse hemichannels ⑤. Hemichannels are generally reduced in number or closed by connexin-based therapeutics as their opening in pathological systems participates in cell death signaling, and purinergic signaling of inflammatory cells. ⑥ Both intercellular channels ④ and hemichannels ⑤ are oligomers of connexin subunits. Each subunit has a cytoplasmic N-terminus (NT), 4 transmembrane domains, 2 extracellular loops (EL1 and EL2), a cytoplasmic loop (CL), and a cytoplasmic C-terminus (CT). Yellow areas indicate regions of the primary sequence that have been used to (or could be used to) generate connexin-based therapeutics. Specifically, Gap26 and Gap27 are mimetic peptides of the first and second extracellular loops respectively, and Gap19 is derived from the cytoplasmic loop [158]. ACT-1 mimetic peptide [25,64–66,96,99], the endogenous peptide discovered by Smyth and Shaw [177], and PEP-1 and PEP-2 mimetic peptides [173] are all found on the C-terminus. Furthermore, the C-terminus contains numerous phosphorylation sites that act as molecular switches for Cx43 dysregulation in injury.

**Table 1**
Connexin associated genetic diseases.

| Human disease | Clinical manifestation | Connexin protein |
|---|---|---|
| *Myelin-related diseases* | | |
| X-linked charcot-Marie-Tooth disease (CMTX) | Limb weakness; progressive demyelination of peripheral axons | Cx32 |
| Pelizaeus-Merzbacher-like disease (PMLD) | Mild peripheral neuropathy | Cx46<br>Cx47 |
| *Cardiovascular disease* | | |
| Idiopathic atrial fibrillation | Atrial arrhythmia and congestive heart failure | Cx40 |
| Visceroatrial heterotaxia | Heart malformations and visceral organ defects | Cx43 |
| *Skin diseases* | | |
| Vohwinkel syndrome | Palmoplantar keratoderma with ichthyosis and hearing loss | Cx26 |
| Keratitis-ichthyosis-deafness syndrome (KID) | Vascularizing keratitis with hyperkeratotic skin lesions and hearing loss | Cx26<br>Cx30 |
| Erythrokeratoderma variabilis (EKV) | Slow growing erythematous patches and static keratodermas; deafness w/Cx31 mutation | Cx30<br>Cx30.3<br>Cx31 |
| Clouston's hidrotic ectodermal dysplasia | Hypotrichosis, palmoplantar keratoderma, nail dystrophy, and/or hyperpigmentation | Cx30 |
| Psoriasis | Hyperproliferation of epidermal keratinocytes resulting in scaly, reddened skin patches, papules and plaques | Cx26 |
| Hystrix-like icthyosis with deafness (HID) | Severe ichthyosis and hearing loss | Cx26 |
| Bart-pumphrey syndrome | Hyperkeratosis over the metacarpophalangeal and proximal and distal interphalangeal joints and hearing loss | Cx26 |
| Non-syndromic and syndromic deafness | Sensorineural hearing loss; and in syndromic (associated with hyperproliferative skin disorders) | Cx26<br>Cx30<br>Cx31<br>Cx43 |
| Zonular pulverulent cataracts | Congenital cataracts | Cx46<br>Cx50 |
| Oculodentodigital dysplasia (ODDD) | Pleiotropic developmental disorder: includes ophthalmologic, dental, craniofacial, bone and limb disorders | Cx43 |

communication and the regulation of the key inflammatory responses that serve to coordinate wound repair and regulate neutrophil infiltration and fibroblast proliferation [18–28].

Studies aimed at genetically and molecularly targeting Cx43 have set the stage for the clinical development of connexin-based therapeutics. Kretz et al., demonstrated that Cx43-gene knockout mice had a faster rate of wound closure as compared to wild-type animals, suggesting that reductions in keratinocyte GJ intercellular communication is required for efficient mobilization and migration [47]. Similarly, topical application of a Cx43 antisense oligonucleotide (Cx43asODN) to incisional and excisional wounds accelerated Cx43 downregulation, resulting in enhanced wound healing, reduced neutrophil, macrophage, and leukocyte infiltration, and the reduction in overall area of granulation tissue formation [39,40]. These results were extended to reductions in scarring in murine neonate burn wounds and in enhanced reepithelialization of wounds in diabetic rat models [48,49]. Accelerated healing at skin wound sites was associated with increased TGF-β1 mRNA and collagen content, and a reduction in the mRNA levels of CC chemokine ligand 2 and TNF-α suggesting that an anti-inflammatory milieu is essential for improved healing [40].

Clinical efficacy has been indicated in randomized trials evaluating the potential of Cx43asODN in a thermo-reversible, slow release pluronic gel formulation (Nexagon™) in accelerating the closure of chronic venous stasis ulcers and diabetic foot ulcers (CoDa Therapeutics Inc., http://www.codatherapeutics.com). Current efforts of this group include the generation and optimization of bioactivated alginate microspheres coated with a pluronic gel containing their Cx43 antisense. Potential applications may include use as a dermal substitute [50]. Company reports indicate clinical safety and efficacy of Cx43asODN, however, as with any therapeutic approach predicated on gene silencing, targeting connexins with a wide-spectrum antisense designed to universally knock down activity may be associated with therapeutic risks including delivery and efficacy issues, off-target effects, and a higher probability of generating adverse physiological effects [51].

Connexin mimetic peptides corresponding to short specific sequences that directly interact with connexin extracellular loop domains offer specific and reversible modulation of gap-junctional communication (Fig. 1). As compared to pharmacological GJ blockers, peptide mimetics likely depend on GJ turnover and act more slowly. Early electrophysiological studies using aortic smooth muscle cell models and peptide mimetics of Cx40 and Cx43 suggested that connexin mimetic peptides do not disrupt GJ biogenesis, but directly impact channel gating [52,53]. Recent reports confirm that connexin peptide mimetics effectively block hemichannel opening and prevent hemichannel docking to form gap junctions without affecting their structure [54–56]. The utility of such peptides in unraveling the molecular mechanisms underlying epidermal morphogenesis is evident in a series of elegantly designed 3D living skin equivalent models where attenuation of Cx43-mediated communication via the use of mimetic peptides directly enhanced wound-closure [57].

Given the high degree of conservation in the extracellular loop of connexin isoforms the most commonly used connexin peptide mimetics, Gap26 and Gap27, are promiscuous, thus potentially hindering clinical development. These peptides were originally created to bind to the extracellular loop of cells displaying hemichannels, and mimic the docking of that hemichannel with another [58]. The intended effect was to open the hemichannel by simulating intercellular channel formation, but instead, the peptides are well documented to close hemichannels and block intercellular channel formation [59]. Based on studies that examined the timeframe for inhibition of hemichannels vs GJ channels, which demonstrated that hemichannels are blocked by Gap26 within 2–3 min while GJs require 30 min of exposure [59], it has been suggested that Gap26 and Gap27 do interact with the extracellular loops of hemichannels resulting in blockage of the channel pore followed by prevention of GJ channel formation, effectively inhibiting GJ communication [60]. It is important to consider that these peptides may have secondary effects through changes in Cx43 phosphorylation [61]. In wound healing models using normal human fibroblast

and keratinocytes, Gap27 increased S368 phosphorylation and enhanced cell migration and proliferation, without altering Cx43 protein levels [62]. However, these results could not be extended to *in vitro* diabetic cell models [61]. Gap junction blockers represent helpful molecular tools in defining gap junction function, but it is quite difficult to consider them as therapeutic drugs due to their non-specificity and potential side effects.

Therapeutic opportunity was discovered with a distinct Cx43 mimetic when a unique molecular tool designed to disrupt the interaction between Cx43 with its binding partner zonula occludens (ZO-1) was found to affect NIH-3T3 fibroblast migration in 2D scratch assays. ACT1 (alpha-connexin carboxyl-terminal peptide 1) is a synthetic peptide incorporating the C-terminal ZO-1 binding domain of Cx43 (RPRPDDLEI) linked at it N-terminus to an antennapedia penetration sequence (RQPKIWFPNRRKPWKK) to enable cell entry [63,64]. ZO-1 binding of the Cx43 C-terminus is directly linked to changes in cellular communication and gap junction remodeling during the process of wound healing [65]. Application of ACT1 in *in vivo* wound healing and ischemic cardiac injury studies indicated anti-inflammatory, anti-fibrotic, and tissue regenerative properties via GJ intercellular communication stabilization [42,64,66–68]. In murine skin and pig wound models, reductions in neutrophil infiltration and rate of wound closure were similar as to those reported with Cx43asODN [64].

ACT1's method of action with regards to tissue regeneration and the dampening of inflammatory responses is independent of Cx43 expression. ACT1 has a high binding specificity with the PDZ-2 domain of ZO-1 and competitively inhibits the interaction of Cx43 and ZO-1, which mediates increases in GJ size and stability with concomitant reductions in hemichannels [25,63,69]. As such, ZO-1 regulation of the cellular distribution of Cx43, and modulation of ZO-1's action provides a control point for dynamic switching between gap junctional communication and hemichannel communication [25]. By blocking Cx43/ZO-1 interaction, ACT1 favors enhanced GJ intercellular communication while reducing Cx43 hemichannel activity [25]. Molecular deciphering of ACT1's mechanism of action additionally reveals potential interaction with CCN3 – a matricellular protein implicated in glioma and tissue-injury response [70], 14-3-3 proteins [71], SH3-mediated interactions [72], various protein kinases [46], and with the cytoplasmic loop domain of Cx43 [73]. The functional consequences included an increase in the coordination of cellular communication, tempered inflammatory responses, enhanced wound re-epithelialization and reductions in the formation of excess fibrous connective tissue [25,42,64], suggesting therapeutic potential.

Similar results have been obtained in studies evaluating the impact of ACT1 incorporation into silicone implants which reported the attenuation of neutrophil infiltration, increased vascularity of the capsule tissue, reduced type I collagen deposition around the implant, and reduced persistence of contractile myofibroblasts [65]. The similarities to the effects of ACT1 in the skin are not surprising given that the foreign body response is a modified version of cutaneous wound healing [65]. These results suggest therapeutic opportunity for ACT1 as an enabling technology for modulating the wound-healing response to implants via promoting integration of implanted materials and tissue-engineered devices in the human body [74].

A topical formulation of ACT1 (Granexin™ Gel; FirstString Research Inc., http://firststringresearch.com) has been carried through four efficacious human clinical trials, including a Phase I clinical study to evaluate dose range, safety, and efficacy in promoting faster healing and scar reduction in full-thickness punch biopsy wounds; and three Phase 2 human clinical trials for the treatment of chronic diabetic foot ulcers, venous leg ulcers, and the reduction of scar formation in surgical incision wounds following laparoscopic surgery (http://online.wsj.com/article/PR-CO-20130715-904787.html?mod=googlenews_wsj). ACT1 application significantly accelerated the closure of chronic wounds, reduced scarring, and was not associated with immunogenicity or local or systemic drug-related adverse events. Pre-clinical toxicity studies reported clinical signs following intravenous administration of ACT1 at $\geqslant$10 mg/kg (Maximum Tolerated Dose), suggesting that systemic administration of ACT1 is available in other injury types where topical administration is not feasible (e.g. ischemic brain injury). Therapeutic promise is further supported by animal toxicology studies reporting that the clinical symptoms associated with high doses of ACT1, including piloerection, decreased activity, wobbly gait, prostration, and breathing abnormalities, were typically short in duration and resolved within 24 h. Clinically speaking, the relatively mild and reversible nature of these symptoms is encouraging but is likely linked to short half-life of the peptide. Whilst sufficient for dermal application protocols as pertaining to wound healing, applications requiring extended delivery of connexin-based peptide mimetics will require formulation optimization and may be associated with additional toxicology profiles.

Acknowledging how mechanism of action relates to therapeutic window of opportunity is a critical component in the evaluation of clinical potential. For example, application of connexin-based therapeutics (e.g. ACT1) whose mechanism of action in wound healing relates to the modulation of inflammatory pathways would be most efficacious if applied immediately following injury during initial inflammatory responses, where failure to resolve these responses leads to abrogation of the repair process and chronic non-healing states [75]. In radiation exposure, where atrophy and uncontrolled inflammatory responses may occur months or years later [76,77], therapeutic application during definitive care protocols may have the potential to restore tissue homeostasis.

It is important to recognize that the clinical exploration of other dermally expressed connexins has been suggested. Cx26 and Cx30 are both upregulated during epidermal regeneration, are much more strongly expressed in hyperproliferative than in normal epidermis, and likely play a significant role in keratinocyte differentiation [36,78]. These two connexins may form heteromeric gap junctions with distinct permeability properties [79]. Therapeutic opportunity for targeting Cx26 stems from evidence using murine psoriasis models where it was found that mice expressing Cx26 under an Involucrin (keratinocyte-specific) promoter stalled the epidermis in a hyperproliferative state and delayed reepithelialization [80]. Importantly, ectopic Cx26 expression increased ATP release; the authors suggest that decreasing Cx26 levels of epithelialized lesions may offer therapeutic benefit by reestablishing the epidermal barrier and modulating the skin inflammatory response.

### 2.2. Diabetic wound healing

The established efficacy of the aforementioned Cx43 antisense and C-terminal mimetic based therapeutics in the context of wound healing directly translates to subsequent evaluation in disease states characterized by chronic wounds and connexin dysregulation. Diabetic wounds are associated with a debilitating chronic phenotype and often remain refractory to conventional therapeutic intervention [81]. The onset and progression of diabetes is associated with significant alteration in connexin expression, phosphorylation, functionality, and degradation. These effects may be linked to extrinsic factors, such as abnormal glucose levels [82], as well as intrinsic factors such as disruption in proinflammatory cytokine or apoptosis-regulating gene expression [83]. Elevated Cx43 expression and GJ communication has been reported in diabetic keratinocytes and fibroblasts, respectively [36,84]. In streptozotocin-induced diabetic rat models, epidermal expression of Cx43 and Cx26 is significantly

downregulated while dermal expression of Cx43 is elevated [49]. Following injury, wound edge keratinocytes upregulate Cx43 and form a thickened bulb of non-migrating cells. Normal wound healing processes, characterized by the downregulation of Cx43, are significantly delayed and initiate at approximately 48–72 h post wounding [49]. The mechanistic link between the dysregulation of Cx43 expression/gap junctional intercellular communication and delayed diabetic wound healing remains to be deciphered. However, it is likely that Cx43 mediated cell apoptosis [85,86], regulation of inflammatory signals, growth factors, pro- or anti-oxidant molecules via the gating or opening of GJ channels, and Cx43 meditation of immunocompetent cells all may have roles. Recent evidence using cultured rat glomerular mesangial cells under high-glucose conditions link the AMPK/mTOR signaling pathway in regulating Cx43 expression and the pathogenesis of diabetic nephropathy [87].

It was preclinical studies in the diabetic C57BL/KsJ-$m^{+/+}$Lept$^{db}$ ($db^{+}/db^{+}$) mouse model that provided initial evidence for the wound-healing potential of the Cx43 mimetic peptide ACT1 in the treatment of diabetic skin wounds. As in previous murine models [64], ACT1 application significantly accelerated wound healing and tempered inflammatory responses in diabetic rodents. Similarly, topical application of Cx43asODN has been shown to effectively prevent Cx43 upregulation and keratinocyte clumping in streptozotocin diabetic rat models following wounding and doubled the rate of reepithelialization [49].

Human trials with ACT1 have solidified the clinical promise associated with targeting Cx43 to overcome the resistant healing phenotype associated with diabetic wounds. In Phase 2 clinical trials, topical application of ACT1 (Granexin™ Gel) to chronic diabetic foot ulcers resulted in a significantly greater reduction in mean percent wound area as compared to standard of care protocols. In these trials, therapeutically targeting Cx43 was not associated with immunological responses, local or systemic adverse events. Similar trials are currently underway for Cx43asODN (ClinicalTrials.gov Identifier NCT01490879). It is important to acknowledge that knocking out Cx43 in murine models has led to alterations in testicular gene expression [88] as well hematopoiesis [89], again reinforcing the need for caution when therapeutically applying antisense technologies. Additional investigation into the relationship between connexin expression and hypoglycemic drugs, cytokine expression, oxidative environment, and inflammatory responses in the context of diabetic wounds will aid in steps towards FDA approval.

The marked upregulation of Cx43 in the dermis of human chronic wounds appears to be a feature underlying impaired reepithelialization and wound healing. Detrimental Cx43 upregulation in dermal fibroblasts has also been described in venous leg ulcers. *In vivo* and *in vitro* targeting of Cx43 and N-cadherin resulted in increased Golgi polarization whilst reducing fibroblast proliferation and cell adhesion, and inducing cytoskeletal changes, increased lamellipodia protrusion, and activation of Rho GTPases [90]. These results suggest a GJ independent role for Cx43 in the stabilization of multiprotein complexes comprising N-cadherin that are required for cell–cell adhesion and adhesion-dependent actin dynamics. The authors suggest that these complexes must be broken down to facilitate efficient fibroblast migration. Similar to application to diabetic ulcers, preliminary clinical evaluation of the safety and efficacy of both ACT1 and Cx43asODN in accelerating the healing of chronic venous ulcers has been encouraging. It is likely that targeting Cx43 in other chronic wounds that are similarly characterized by chronic inflammation and retarded reepithelialization, including pressure ulcers, and post-surgical and traumatic ulcers will elucidate additional therapeutic promise.

### 2.3. Corneal wound healing

Connexin expression, mainly Cx26, Cx30, Cx31.3, Cx37, Cx43, and Cx50, has been well documented in the human cornea [91]. Notwithstanding the obvious physiological differences with regards to the processes that define dermal versus corneal wound healing, the connexin response in these tissues is remarkably similar. Despite the cornea being an avascular structure, both the skin and cornea have a collagenous stroma, and a stratified squamous cell epithelium. Furthermore, the cytokines and growth factors involved in wound repair are similar, including IL-1, TNFα, EGF, PDGF, and TGF-β [92]. Additionally, during wound healing corneal stromal keratinocytes and skin dermal fibroblasts play equivalent roles. Following corneal wounding, connexins, including Cx43, are rapidly downregulated at the migrating wound edge and in regenerating migrating corneal epithelial cells [93]. Given the similarities in Cx43 expression patterns during wound healing in the epidermis and corneal epithelium, therapeutic extension of connexin targeting agents can be easily justified. In rat corneal scrape injury models the over-expression of Cx43 using adenovirus inhibited corneal endothelial wound healing, while knockdown with Cx43 siRNA or Cx43 antisense sped it up and inhibited endothelial-mesenchymal transition [94], which has important roles in corneal fibrosis following injury [95]. Clinically, corneal fibrosis translates to the loss of corneal clarity and visual acuity. Corneal therapeutics offering accelerated healing in addition to the prevention of fibrosis would be of great value.

In the corneal epithelium, in association with enhancing the rate of Cx43 decrease following wounding and increasing the rate of recovery, application of Cx43asODN or the Cx43-based mimetic peptide ACT1 in rat corneal injury models reduced hyperplasia and fibrosis [93,96]. The evaluation of endothelial-mesenchymal transition pathway genes, including keratin 8, keratin 19, and TGFβ2, in *in vivo* corneal wound healing studies applying ACT1 suggest that immediately after wounding a transmigration stage of re-epithelialization occurred, rather than the proliferation of cells from the limbal region. ACT1 treatment of corneal wounds therefore directly mediates the upregulation of epithelial cell migration [96].

Clinical potential of a Cx43asODN Poloxamer407 gel formulation has been evaluated in a small study incorporating 5 patients with severe non-responsive ocular surface burns. Treated eyes were associated with reduced inflammation, and recovery of the vascular bed and limbal reperfusion prior to epithelial recovery [97]. Phase 2 clinical trials evaluating the efficacy and safety of a Cx43asODN formulation in the treatment of persistent corneal epithelial defects are currently recruiting (ClinicalTrials.gov Identifier NCT01165450).

Effective topical ocular drug delivery of peptide and protein drugs presents a particular challenge in the formulation of effective corneal therapeutics. Susceptibility to physical and chemical alteration (i.e. denaturation, aggregation, oxidation, hydrolysis, etc.) following physiological fluctuations may limit bioabsorption across the corneal epithelium. In response to this challenge, following initial efficacy studies outlining the therapeutic potential of ACT1 in accelerating the healing of corneal wounds, a biocompatible, sustained, controlled release formulation involving ACT1 loaded alginate poly-l-ornithine microcapsules has been formulated [96,98]. This formulation renders the Cx43-based mimetic peptide resistant to physical and chemical degradation and extends the peptides ocular half-life in order to maintain the peptides integrity while achieving the appropriate ocular drug concentration. Furthermore, this formulation is associated with prolonged precorneal retention and controlled ACT1 release. Application of microcapsule-based connexin therapeutics will likely prove fruitful in a greater therapeutic context where microcapsules may be specifically engineered to provide targeted delivery.

Clinical opportunity for targeting connexins in the context of corneal wound healing may be particularly relevant in individuals with pathologies that are directly associated with corneal epithelial defects. Diabetes is associated with a number of corneal complications including reduced barrier function, thickness, corneal sensitivity (neuropathy), and tear secretion, as well as changes in endothelial cell morphology and density, and diabetic keratopathy. In normoglycemic persons, minor epithelial defects rapidly heal; however, individuals with diabetes present with corneal epithelial defects that are associated with abnormal corneal re-epithelialization that remain resistant to conventional therapeutic regimens. Preclinical studies using the Goto-Kakizaki Type 2 diabetic rat model and a streptozotocin-induced Type 1 diabetic rat model have demonstrated the safety and efficacy of a sustained release ACT1 microcapsule eye drop formulation in significantly accelerating corneal re-epithelialization and regulating anti-inflammatory proteins, including TNFα and the CXCR3 chemokine ITAC following injury in diabetic rats [99].

Clinical opportunity has been suggested for other corneal expressed connexins. Cx31.1 forms non-functional channels in the upper suprabasal and superficial layers of the corneal epithelium and appears to have key roles in regulating apoptotic pathways and maintaining corneal homeostasis [100]. Application of Cx31.1 antisense oligonucleotides in *ex vivo* rodent and human corneal models results in decreased epithelial cell apoptosis and increases in corneal thickness. The mechanism of action is likely linked to roles for Cx31.1 in the reduction in superficial cell death and shedding [101]. Additional research investigating the mechanism is required to fully elucidate the clinical potential of targeting Cx31.1 in the cornea. The evaluation of alternative molecular tools that are aimed at regulating Cx31.1 activity as opposed to abolishing protein expression may prove beneficial.

### 2.4. Acute lung injury

Given the relative heterogeneity that is associated with cellular composition of the lung, it is not surprising that connexins are expressed differentially depending on cell phenotype/function and stage of differentiation (reviewed in [102]). Defining connexin expression in the lung is further complicated by the potential of coexpressed connexins to (a) form heteromeric gap junction channels and to (b) 'personalize' permeability and gating profiles as required [103]. Connexin dysregulation has been linked to the etiology and progression of a number of lung diseases including acute lung injury, cystic fibrosis, asthma, and pulmonary arterial hypertension.

Acute lung injury is associated with the loss of alveolar-capillary membrane integrity, excessive transepithelial neutrophil migration, release of pro-inflammatory/cytotoxic mediators, impaired arterial oxygenation and ultimate risk of hypoxemic respiratory failure. During the acute phase of lung injury, increased pulmonary vascular permeability is associated with an upregulation of Cx43 and Cx46 expression and the downregulation of Cx40 [104,105]. The critical role for Cx43 in promoting and regulating inflammatory signals has been demonstrated in murine models lacking endothelial Cx43 and in Cx43+/− mice where Cx43 expression was linked to the propagation of calcium waves along pulmonary vessels, the promotion of leukocyte adhesion to the vascular surface, and the recruitment of neutrophils to the alveolar space following lipopolysaccharide (LPS) challenge [106]. Alternatively, Cx40 prevents neutrophil adhesion and contributes to anti-inflammatory signaling pathways. Blockers of GJ intercellular communication, such as the long-chain alcohol, heptanol, inhibited the diffusion of endothelial calcium oscillations along the capillaries induced by pressure elevation in perfused lung; translating to an increased risk of pulmonary edema in acute lung injury [107]. These data imply therapeutic opportunity in regulating the propagation of pro-inflammatory signaling via connexin manipulation from a variety of angles during acute lung injury.

Anti-Cx43 strategies have been assessed in LPS models of acute lung injury where intratrachael administration of the Cx43 mimetic peptide Gap26 resulted in attenuation of the inflammatory response and reduced neutrophil recruitment from blood circulation to the lungs [105]. Additional therapeutic innovation has recently been inspired in the suggestion that the protective effects of Cx-based mimetic peptides in acute lung injury may be mediated in part by decreased ATP release from pannexins [108]. Pannexins are structurally similar but phylogenetically unrelated to the connexin family and may offer a novel therapeutic strategy in managing pulmonary inflammation.

Alternatively, recent *in vivo* studies suggest that stabilizing endothelial and epithelial barriers may be a strategy in the treatment of acute lung injury [109–111]. Small molecule inhibitors of the calmodulin activated protein kinase, myosin light chain kinase (MLCK), improved outcomes in animal models of acute lung injury. MLC activation caused cell contraction leading to the formation of gaps between cells, loosening adherence, and tight junction dysfunctions, enabling inflammatory mediators and infectious agents to enter the surrounding tissue [112–115]. Furthermore, modulation of ZO-1 function may have beneficial effects towards restoring barrier function. ZO-1 phosphorylation has roles in tight junctional opening in alveolar epithelial cells and the permeability of the blood-air barrier [112,116].

Therapeutic promise for Cx43 peptide-mimetics in the treatment of acute lung injury has recently been investigated. ACT1 treatment of bronchial epithelial cells and lung microvascular endothelial cells exposed to LPS and hydrogen peroxide, key stressors associated with acute lung injury induction and ventilator complications, stabilized tight junction integrity and improved transepithelial electrical resistance (Atkinson et al., 2014, in prep). In an *in vivo* lethal murine LPS-induced acute lung injury model, pre-treatment of mice with ACT1 significantly improved survival rate as compared to untreated controls. In a sub-lethal murine model, intranasal pre-treatment of mice with ACT1 30 min prior to LPS exposure resulted in a significant reduction in neutrophil, macrophage, and albumin in lavage fluids, improved lung pathology scores, and reduced KC/MIP-1 chemokine levels 18 h post LPS instillation as compared to control treated animals. Translational extension of these observations suggests that ACT1 therapy in acute lung injury may contribute to a reduction in inflammatory cell infiltration, fluid and pathogen transportation. ACT1 mediated regulation of ZO-1 binding, Cx43 phosphorylation and hemichannel activity likely all have roles that will provide further insight into therapeutic opportunity.

## 3. Potential for connexin-based therapeutics in the heart

Like the transient and defined expression pattern of connexins during wound healing, studies of humans with ischemic heart disease and matching animal models reveal changes in connexin expression and subcellular localization in the injured heart [117–120]. These similarities underlie the translatability of connexin-based therapeutics from the skin to the heart [121]. The heart depends on the spatial order of GJ organization, where connexins transmit and synchronize the electrical impulse that initiates cardiomyocyte contraction from beat to beat. The three main isoforms expressed in the heart are Cx40, Cx43, and Cx45 [122]. Cx43 is the most widely distributed in the heart, being the primary if not only isoform expressed in ventricular myocytes [117,118], where it largely localizes to the intercalated disk (ID; the end-to-end abutment of two connected cardiomyocytes). As such, this section will focus

on Cx43-based therapeutics pertaining to the treatment of cardiopathologies.

A hallmark of cardiac pathology is a poorly understood process termed GJ remodeling [117–120]. In general, remodeling describes a process by which GJs are redistributed from the ID to the lateral borders of cardiomyocytes, and this phenomenon is common to diseases ranging from ischemic infarction to congestive heart failure [123]. The form that lateralized Cx43 takes – intercellular channels or hemichannels – is currently a matter of speculation, but either situation would likely be arrhythmogenic [6]. Specifically, in one case, lateralized GJs would alter anisotropic conduction, providing a substrate for arrhythmia [124,125]. In the other case, under pathological conditions Cx43 hemichannels open, promoting cardiomyocyte cell death [126].

A separate but related issue is the fibrosis and scarring that come with end-stage heart failure and myocardial infarction, respectively. These phenomena may affect conduction in the heart in a number of ways. First, fibrosis that occurs due to aging creates collagenous septa between atrial myocytes that reduce coupling and are proarrhythmogenic [127]. A similar situation plays a role in arrhythmia due to heart disease – "replacement" fibrosis serves to maintain the structural integrity of the heart as cells die, but interstitial collagen deposition impairs conduction [128]. In the case of infarction, the myocardium becomes necrotic, eliciting an innate inflammatory response followed by scarring and fibrosis of the infarct border zone myocardium [121]. The fibrotic border zone becomes a substrate for reentrant arrhythmia due to a combination of scarring, and structural remodeling of both myocytes bundles and GJs [129].

Connexins are involved in these processes not only through altered conduction, but also, as in the realm of wound healing, via the role they play in inflammation and scarring. ATP released from connexin hemichannels in the vasculature and damaged myocytes targets neutrophils to the infarct site [74,130]. Subsequently, connexins play a key role in monocyte-endothelial cell adhesion [131], fibroblast migration and differentiation to myofibroblasts [40,132], and collagen deposition [38,133]. Additionally, recent developments suggest that GJs formed between myocytes and fibroblasts play both physiological and pathological roles in conduction and arrhythmogenesis. For an excellent current review of that subject we refer the reader to the work of Kohl and Gourdie [134].

### 3.1. To the heart of it: connexin targeting cardio-therapeutics

It is into this complex mix that connexin-based therapeutics enter the fray. Treatments that enhance GJ communication might improve slowed conduction, but could also potentiate alternative conduction pathways (e.g. myocyte-fibroblast connections), promote the bystander effect, or enhance hemichannel ATP release. Conversely, treatments that gate hemichannels closed are often the same drugs that inhibit GJ communication. Currently, no connexin-based therapeutics are in use in the clinic for the treatment of either arrhythmia or heart disease in general, but there are several in the pipeline, many with published results in animals, and a multitude of potential targets.

Most of the connexin-based therapeutics are peptides, a subset of which are Cx43-mimetic peptides. Of the non-mimetic peptides, there appear to be 2 classes [135]: those based on antiarrhythmic peptide (AAP) – an endogenous peptide isolated from the bovine atrium [136]; and those based on RXP-E – a peptide identified by phage display to bind the Cx43 C-terminus containing the amino acid sequence R-X-P where X is any amino acid [137]. The AAP derivatives include HP-5, AAP10, ZP123 (the more stable D-amino acid analogue of AAP10, aka rotigaptide and GAP-486), and GAP-134 (a dipeptide derivative of ZP123, aka ZP1609 and danegaptide) [135]. Studies in animals show that these peptides have antiarrhythmic properties in a variety of models [138–142], and most recently GAP-134 has been shown to reduce infarct size in a porcine left anterior descending (LAD) artery occlusion model of myocardial infarction [143]. Phase II clinical trials were initiated with ZP123 (ClinicalTrials.gov Identifiers NCT00137293 and NCT00137332), but were terminated to prioritize clinical trials for GAP-134 (NCT00510029, NCT00543946, and NCT00783341) [135]. ZP123 showed promising results, and GAP-134 will likely prove to be an equally good antiarrhythmic with the added benefit of oral bioavailability [135].

Mechanistic studies have indicated a mode of action involving increased GJ communication. Clarke et al. demonstrated that ZP123 increases GJ communication [144], and although there is no direct evidence of improved GJ intercellular communication with GAP-134 treatment, GAP-134 did prevent conduction velocity slowing in a canine pericarditis model [145], indicating that the peptide does have a positive effect on communication. Perplexingly, while AAP10 enhanced Cx43 hemichannel-mediated ATP release in cultured cardiomyocytes [130], both GAP-134 and ZP123 reduced dye uptake in C6 glioma cells [142]. Whether ZP123 and GAP-143 inhibit or enhance hemichannel function in myocytes remains to be seen, but cell type-specific responses may be at the "heart" of the matter. The reason for this comes down to the general mode of action for AAPs. As reviewed by De Vuyst et al. [135], these peptides activate an unknown GPCR that in turn activates PKCα. In myocytes, other PKC isoforms, such as PKCε, may be responsible for differential effects [135].

The RXP peptides also show promise, although no published animal work has been done at this point. RXP-E, CyRP-71 (a cyclic variant of RXP-E), and ZP2519 (a peptide based on the minimum core active structure of the RXP peptides) all have been demonstrated to prevent acidification-induced closure of gap junctions (a situation encountered during ischemia [146]) [137,147,148]. Furthermore, RXP-E prevented action potential propagation block in monolayers of neonatal rat ventricular myocytes [149]. Unlike the AAPs, the RXP peptides function through direct interaction with the Cx43 carboxyl-terminus [137,149], in the region responsible for pH-dependent dimerization [150]. Further work will be needed to determine if the RXP peptides will be beneficial as cardio-therapeutics.

Similarly, all of the Cx43-mimetic peptides function by direct interaction with Cx43. The best characterized of these peptides are the extracellular loop peptides, Gap26 and Gap27. Studies on cardiac function with Gap26 and Gap27 have been conducted in cultured cells and animals. In culture, Gap26 has been demonstrated to block hemichannel-mediated dye uptake and significantly improve cell viability in cardiomyocytes exposed to simulated ischemia [126]. In Langendorff-perfused rat hearts subjected to LAD occlusion, addition of Gap26 reduced infarct size by ~50% [151], and application of either Gap26 or Gap27 reduced infarct size up to 61% in an *in vivo* rat model of myocardial infarction by LAD occlusion [152]. These results may seem paradoxical given that Gap26/27 also affect intercellular channel formation, but in these studies the peptides were delivered as a single bolus injection, suggesting they were only present in the heart long enough to block hemichannels. That being said, it is important to note that caution needs to be used, especially in the heart, when applying blockers of GJ channels or knocking down connexin expression. Arrhythmia is a likely outcome of potent GJ inhibition, as suggested by work showing that cardiac-specific deletion of Cx43 causes sudden arrhythmic death in mice by 2 months of age [153].

As the above discussion suggests, the dichotomy between blocking hemichannels vs GJ channels is a controversy that is especially relevant in the heart. As discussed elsewhere in this article, blocking GJ channels can have beneficial effects in injury by

preventing the spread of damage through the bystander effect. This may also be true in the heart, as it has been found that mice with heterozygous deletion of Cx43 display reduced infarct volumes [154]. However, the trade-off of reduced GJ coupling for decreased bystander effect may not pay – mice heterozygous for Cx43 also display conduction slowing [155]. Pharmacological inhibition of connexins also results in conduction slowing [156,157], suggesting that connexin blockers should only be applied cautiously, and systemic administration should be avoided.

Gap19 is another Cx43-mimetic peptide that shows promise as a cardio-therapeutic. It is a non-apeptide derived from the larger L2 domain of the Cx43 cytoplasmic loop [158]. Previous studies showed that a full L2 peptide fused to the membrane-permeable TAT sequence maintained hemichannels in a closed state when subjected to mechanical stimulation by binding to the Cx43 carboxyl-terminus [159]. Unlike the TAT-L2 peptide, Gap19 itself is membrane permeable due to a combination of its small size and the inclusion of the KKFK membrane translocation motif [158]. In a comprehensive study, Wang et al. have showed that Gap19 also binds to the Cx43 C-terminus, specifically inhibits Cx43 hemichannels and ATP release without affecting GJ communication, increased cell viability of cardiomyocytes exposed to simulated ischemia, and reduced infarct size in mice subjected to LAD ligation [158]. These results suggest that Gap19 may be an ideal candidate to treat myocardial infarction.

The last of the mimetic peptides to be discussed is ACT1. As discussed above, it is a mimetic of the Cx43 carboxyl-terminus that blocks Cx43/ZO-1 interaction [63]. Importantly, it has been shown that Cx43/ZO-1 interaction is enhanced in ischemic and dilated cardiomyopathy in association with an increase in the relative amount of non-junctional (hemichannel) Cx43 as compared to Cx43 in GJs [160]. It is also important to this discussion to reiterate that the effect of ACT1 on GJs is to increase the number of intercellular channels at the expense of hemichannels, consequently enhancing GJ communication while simultaneously reducing hemichannel function [25]. These effects were associated with a structure adjacent to the GJ called the perinexus where hemichannels reside ([6,25]; also, see Fig. 1). Recent work demonstrates that Cx43 interacts with the voltage-gated sodium channel $Na_v1.5$ (the channel responsible for the upstroke in the cardiomyocyte action potential) in the perinexus [161], where it may play a role in conduction [162]. Preliminary work indicates that this interaction may be enhanced by ACT1 [163], suggesting that ACT1 has effects on cardiomyocytes through multiple mechanisms.

*In vivo* work with ACT1 has demonstrated a positive effect on the injured myocardium. O'Quinn et al. demonstrated that application of ACT1 in a mouse model of cryoinfarction reduced Cx43/ZO-1 colocalization and GJ remodeling in the infarct border zone, and significantly reduced the severity of pacing-induced arrhythmias in isolated perfused hearts [66]. In addition, ACT1 enhanced phosphorylation of the S368 residue (pS368) on the Cx43 carboxyl-terminus. Cx43 phosphorylated on S368 may help to preserve Cx43 at the ID [66,67,119]. pS368 may have also had a beneficial effect on preservation of border zone myocytes as pS368 has been shown to enhance selective permeability [119], which has been suggested to create a distinct communication compartment [45] that might insulate border zone myocytes from the bystander effect and prevent them from becoming a substrate for arrhythmia.

No clinical studies assessing the cardioprotective effects of ACT1 have yet been conducted. However, as discussed above, preclinical and clinical trials demonstrate that ACT1 was well tolerated in animals and was not associated with any adverse events or immunogenecity. This suggests that ACT1 may be an ideal candidate for repurposing as an antiarrhythmic.

In addition to the peptide-based treatments, genetic engineering approaches have recently been explored in animal models. Bikou et al. induced atrial fibrillation in a porcine model by atrial burst pacing, and found that Cx43 protein content was reduced, leading to a deterioration in cardiac function [164]. Adenoviral Cx43 gene transfer by injection into the atria followed by epicardial electroporation resulted in increased Cx43 protein levels and prevention of reduced cardiac function during burst pacing. Similarly, Igarashi et al. introduced Cx43 and Cx40 to the atria of Yorkshire swine by gene painting of adenoviral vectors, and atrial fibrillation was induced by burst pacing [165]. Unpaced animals displayed no changes in atrial conduction, but animals subjected to atrial fibrillation displaced reduced and lateralized Cx43 expression that could be restored by gene therapy. Importantly, expression of both Cx43 and Cx40 improved conduction and prevented atrial fibrillation [165]. Whether connexin gene therapy will make it to the clinic remains to be seen, but these studies provide an encouraging start.

A number of other therapeutic targets are implicated by basic science research. The Cx43 carboxyl-terminus has a number of protein modification and protein interaction sites that regulate its life-cycle and channel gating [67]. Inhibition or enhancement of a number of the protein interaction or modification events may prove to be beneficial in the treatment of cardiac pathologies. However, this is a subject far too broad for the limited scope of this article, and we refer the reader to the following reviews for a full discussion: [45,67,166–168]. Nonetheless, a few recent discovers are noteworthy. Dunn and Lampe showed that Akt phosphorylates Cx43 on S373, which in turn leads to dissociation of Cx43/ZO-1 interaction and the formation of larger GJs [169], suggesting that this phosphorylation site regulates the same molecular switch as ACT1 [25]. Conversely, Src phosphorylation of S279 and S282 have been shown to mediate Cx43 endocytosis [170,171], indicating that targeting of Src-mediated Cx43 phosphorlation may prevent injury-mediated endocytosis of GJs. Supporting this concept, Mestroni and Nguyen review a number of studies in which Src inhibitors show antiarrhythmic properties [172], and Gangoso et al. describe a Cx43/Src interaction inhibiting peptide (PEP-1 and PEP-2) that reverses the glioma stem cell phenotype [173]. Taken together, these studies suggest point to S373, S279, and S282 as potential targets of Cx43-based molecular therapeutics.

On the other hand, targeting Cx43 trafficking to the membrane may also be an effective way to combat Cx43 dysregulation in cardiac pathology. The Shaw lab demonstrated that Cx43 trafficking to the membrane is dependent on both microtubules [174], and actin [175], and that these delivery systems are both disrupted during ischemia [175,176], indicating therapeutic potential. Excitingly, recent work by Smyth and Shaw determined that multiple carboxyl-terminal isoforms of Cx43 occur in the heart, and that these truncated Cx43 isoforms are the result of internal translation of Cx43 mRNA [177]. Importantly, it was found that expression of a 20 kDa isoform in particular is necessary as a chaperone to traffic Cx43 to the cell surface [177]. Treatment with synthetic versions of these endogenous peptides, or gene therapy approaches that enhance their expression, may turn out to be effective therapies to combat the reduced Cx43 expression that is common to cardiac disease.

## 4. Potential for connexin-based therapeutics in the central nervous system

Twelve years following the 1952 discovery of GJs in electrical coupling between heart cells, electrical coupling between glial cells was noted in the leech central nervous system [178]. Given that the human central nervous system (CNS) is one of the most

complicated assemblies in all of biology in terms of its size, structure, arrangement, and interconnectedness the clinical potential of connexin-based CNS therapeutics demands extensive discussion that extends beyond this review. As a starting point, and in terms of the brain's interconnections, it is the neuronal synapses that are usually called to mind – the human brain contains about 85 billion neurons [179], and 150 trillion synapses [180]. GJs are formed between neurons where they function in synaptogenesis, neuronal differentiation, migration, and neural circuit formation and maturation during development, and in mature brains it is thought that they modulate synchronized oscillatory activity [181].

What also needs to be appreciated, is that the brain contains a number of supporting glial cells approximately equal to the number of neurons [179], and these cells are also heavily interconnected by GJs [182]. In particular, astrocytic GJs form between astrocytes at blood vessels and synapses where they are thought to regulate the blood–brain barrier and provide a homeostatic environment for neurons, respectively [183]. Furthermore, a number of physiological roles for hemichannels in the CNS have emerged, from calcium wave propagation [184] to ephaptic coupling in the retina [185]. Recently, astrocytic processes terminating at synapses have been speculated to form a "tripartite synapse" with astrocytes modulating and even participating in synaptic transmission through the release of gliotransmitters [186]. Whether connexins are a component of the tripartite synapse remains to be seen.

Within this context, a general paradigm appears to have arisen for the response of connexins to injury in the CNS. Specifically, astrocytic GJs and hemichannels generally appear to be oppositely regulated by injury signals - proinflammatory cytokines, ROS/RNS, and ATP [182]. In the setting of ischemia, opening of astrocyte hemichannels and further release of ATP [182] and glutamate results in neuronal cell death directly through opening of neuronal pannexin channels [187], and recruitment of inflammatory leukocytes that exacerbate cerebral damage [188]. Importantly, these processes, and changes in connexin channel function and expression in general, may also be integral to a number of diseases including Alzheimer's, Huntington's, and Parkinson's disease [189,190]. With this in mind, connexins – Cx43 in particular, as it is the primary connexin expressed in astrocytes [189] – are a vital target for a new class of connexin-based therapeutics in the CNS.

### 4.1. Connexin targeting CNS therapeutics: proceed with caution

Before entering into a discussion of connexin-based CNS therapeutics, it must be acknowledged that pharmacological blockers of GJ channels have been associated with adverse neurological effects. For example, mefloquine is an antimalarial formerly marketed in the US as Lariam®. It is routinely used in connexin basic science studies as a channel blocker (e.g. [25]), but has also been shown to have the following clinical psychiatric side effects: anxiety, panic attacks, paranoia, persecutory delusions, dissociative psychosis, and anterograde amnesia [191]. A case study of the extreme hallucinogenic and amnesic effects of mefloquine has recently been popularized by David S. MacLean in an episode of This American Life (http://www.thisamericanlife.org/contributors/david-maclean) and his book, *The Answer to the Riddle Is Me* [192]. These effects appear to result from blocking of GJs between inhibitory neurons in the limbic system, thus preventing synchronization. Along these same lines, an extracellular loop mimetic peptide was shown to have beneficial effects following global cerebral ischemia in near-term fetal sheep at low doses, but at high doses resulted in cell swelling, acidosis, and increased mortality [193]. These results suggest that, like the heart (see previous section), in the brain blocking hemichannels may be beneficial in pathological scenarios, but GJ blockade will likely have deleterious effects. Therefore, we recommend caution in applying connexin-based therapeutics in the nervous system.

To our knowledge, only one clinical study has been performed involving connexin-based therapeutics in the CNS. Tonabersat is a novel benzoylamino benzopyran compound, that has been shown to block GJs [194]. Pre-clinical animal trials have shown that it does not display adverse effects in the heart or CNS, even at high doses, but clinical trials reported dizziness, nausea, vertigo, somnolence, paraesthesia, abdominal pain and palpitation in patients, and in one study cardiovascular effects were noted in several patients [194]. Despite these negative effects, Tonabersat was effective in relieving migraine headaches in two of the three clinical trials [194].

A number of animal studies have been performed that also highlight connexins as a viable drug target. Initial studies with Cx43 antisense in pluronic gel showed that application to the spinal cord of rats achieved a rapid, long-lasting knockdown of Cx43 [195]. In later work by the same group it was found that application of Cx43 antisense in two different models of spinal cord injury (compression and partial transection) reduced astrogliosis, vascular permeability and neutrophil infiltration, and improved performance in locomotion tests [196]. Preliminary data using a biodegradable patch formulation of the Cx43-based ACT1 peptide has shown promise in neuronal preservation, and reduced astrogliosis and microgliosis in a rat spinal crush injury model. More recently, Yoon et al. showed that application of Cx43 antisense to organotypic brain slice cultures improved neuronal survival and maintained normal cell and organ morphology at 14 days in culture compared to controls [197]. Taken together, these data indicate that Cx43 participates in neuronal cell death and recruitment of leukocytes in CNS injury, and that Cx43 knockdown by antisense is an effective remedy.

Work by two separate groups using a weight-drop model of spinal cord injury in transgenic mice with Cx43 deletion support these findings. Specifically, Chen et al. found that Cx43 transgenic mice subjected to spinal cord injury displayed less neuropathic pain in association with reduced astrogliosis [198]. In an independent study, Huang et al. generated spinal cord injury by weight-drop in Cx43 deletion mice and found that ATP release, astrogliosis and microglial activation, and lesion size were all reduced while motor recovery was significantly improved compared to controls [199]. These data suggest that Cx43 hemichannel mediated ATP release is a critical factor in injury and recovery in CNS damage.

Along these lines, a homologue of the connexin mimetic peptide Gap27 has been shown to have beneficial effects in CNS injury. In an organotypic hippocampal slice model of epileptiform lesion, low concentrations (5–100 μM) of the peptide blocked cell death during exposure to bicuculline methochloride (BMC; a competitive $GABA_A$ receptor antagonist used to induce the lesion), while high concentrations (500 μM) exacerbated cell death [200]. Conversely, during recovery from BMC the high concentration of the peptide was the most effective at reducing cell death indicating that hemichannel mediated injury in the CNS is most prominent during the insult itself, but that GJ communication has a bystander effect during recovery periods (e.g. reperfusion). Similarly, systemic application of the Gap27 homologue after ischemic retinal injury induced by high intraocular pressure resulted in increased retinal ganglion cell survival, and reduced vascular leakage and astrogliosis compared to controls [201]. Importantly, *in vitro* assays of endothelial survival and hemichannel function following hypoxia showed that connexin channel blockers, including the mimetic peptide, reduced hemichannel activity and increased cell viability supporting the concept of hemichannel mediated injury.

Connexin hemichannels and GJ channels also appear to play a large role in perinatal ischemic brain injury. Early work by de

Pina-Benabou et al. showed that application of carbenoxolone to hippocampal slice cultures subjected to oxygen-glucose deprivation, or to rat pups subjected to intrauterine hypoxia–ischemia, reduced neuronal death and long-term neuronal damage respectively [202]. As discussed above, it has been shown that low doses of extracellular loop peptide were found to be beneficial in a fetal sheep model of ischemic brain injury [193]. Specifically, EEG power was significantly higher following ischemia in the low dose peptide group both immediately following ischemia, and 5 days onward post ischemia. These results were supported by later work by the same group, but interestingly, it was found that the peptide was only effective when applied 90 min after ischemia, but not during ischemia, suggesting that timing of treatment is as much an issue as dosage with connexin-based therapeutics [203].

Non-peptide connexin targeting pharmacological treatments may also have therapeutic application in the CNS. In one instance, quinine (a Cx36-specific channel blocker [204]) was applied to the retina after mechanical trauma inflicted with a 28 gauge needle [205]. In treated retinas, reductions in retinal cell death were associated with caspase downregulation. Interestingly, the broad-spectrum connexin channel inhibitor carbenoxelone was far less effective in blocking cell death and caspase downregulation. Given that Cx36 is a primarily neuronal connexin isoform [181], these results suggest that a neuron-to-neuron bystander effect is an important factor in retinal neurodegeneration.

Future directions for connexin-based therapeutics are suggested by the basic science literature. ZO-1 has been found to colocalize and coimmunoprecipitate with Cx47 (an isoform expressed in oligodendrocytes) [206] and Cx36 [207] in the mouse brain, colocalize with Cx36 in the monkey retina and the perinexus of Cx50 and Cx57 in the rabbit retina [208], and colocalize and coimmunoprecipitate with astrocytic Cx30 and Cx43 [209]. Importantly, pulldown assays showed that Cx47 [206] and Cx30 [209] interact with the second PDZ domain of ZO-1 (as does Cx43 [210]), while Cx36 interacts with the first PDZ domain [207]. These data suggest that ZO-1 may regulate glial cell GJs in a similar manner to that described for Cx43 in which ZO-1 limits the rate of hemichannel accrual into GJ plaques [25]. If this is the case, then peptides that inhibit Cx43/ZO-1 interaction, such as ACT1, may prove to also have beneficial effects in CNS injury by limiting hemichannel-mediated neurodegeneration.

## 5. The connexin cancer conundrum

At about the same time that GJs were being described in the leech CNS, a critical role for connexins, GJs, and GJ intercellular communication in tumorigenesis and/or cancer intravasion and extravasation was recognized [211]. Initial expression and functional analyses defined connexins as putative tumor suppressors and restoration of depressed connexin expression and GJ intercellular communication inhibits tumor cell growth [211–215]. In a seemingly paradoxical twist, it is now evident that connexins are 'conditional tumor suppressors' and have additional roles in cancers that are type and stage specific and may be regulated through upstream phosphorylation events or may be entirely independent of GJ intercellular communication [214,216–221]. The differential and dynamic role that connexins have in cancers is extensive and has been the subject of recent reviews [212,222].

Therapeutic opportunity has been suggested in harnessing the bystander effect, whereby cells transmit signaling moieties to their neighbors through GJ intercellular communication. Such an approach suggests the ability to amplify therapeutic efficacy by selectively targeting a subset of tumor cells that serve to pass-on cytotoxic signals. Given that changes in connexin levels may directly translate to changes in the efficacy of the bystander effect, modulating connexin expression has become a successful strategy in "suicide gene therapy". In suicide gene therapy, also termed gene-directed enzyme prodrug therapy, intratumoral delivery of suicide genes that facilitate the activation of specific prodrugs into their cytotoxic effector derivatives, is enhanced by simultaneously using inducers and stabilizers of GJ intercellular communication. Transfection of Cx32 genes simultaneously with the herpes-simplex virus-derived thymidine kinase (HSVtk), which converts ganciclovir (GCV) into GCV-monophosphate whose subsequent phosphorylation yields the toxic pro-apoptotic metabolite, GCV-triphosphate, revealed cytotoxicty in HeLa cells [223]. Therapeutic efficacy of these types of approaches has been challenged by the rate-limiting steps associated with phosphorylation-mediated activation, limited cytotoxicity in slow-growing tumors, poor biochemical profiles that limit application (poor lipophilicity prevents crossing of the blood–brain barrier), and necessity to use at systemically dangerous dosing levels. Recent attempts at novel enzyme prodrug systems have involved catalytically enhancing variants of human thymidylate kinase, where pannexin1 was suggested to form the functional GJ that mediated bystander killing events [224]. Potential to harness the bystander effect extends to radiation protocols where the transmission of apoptotic signals and redox active molecules may diffuse from irradiated to non-irradiated cells [225], as well as in chemotherapeutic, immunotherapeutic, or hormonal therapies, where connexin expression and GJ communication potentiates tumor sensitivity and therapeutic efficacy [226–228]. Alternatively, the bystander effect can be incorporated in strategies that are aimed at targeting the tumor microenvironement, where for example, Cx43 and Cx26 GJs formed between endothelial and tumor cells can be used to facilitate the elimination of endothelial cells by cytotoxic T lymphocytes, thus preventing tumor progression [229,230]. An elegant and promising approach in the treatment of gliomas has been suggested recently in research using cisplatin loaded nanogels conjugated with monocloncal antibodies against the extracellular loops of Cx43 and BSAT1, where nanogel treatment significantly increased the median survival of rats in an experimental model of glioma [231].

The clinical potential of restoring connexin expression is evident in the number of research publications proposing that targeting the transcriptional, translational or posttranslational regulation of connexins in cancer may have therapeutic potential [212,222,232]. Forced expression of Cx26 and Cx43 in prostate cancer cells using recombinant adenoviral constructs showed efficacy in enhancing cell sensitivity to doxorubicin treatment and TNFα-induced apoptosis, respectively [233]. The efficacy of HDAC inhibitors has been linked to global affects on GJ expression and increases in GJ communication [234]. Therapeutics that target the kinases that mediate connexin phosphorylation, especially with regards to Cx43 [45], present an alternative strategy. Treatment of colon cancer cells with the plant flavenoid, kaempferol, enhanced endogenous Cx43 expression and phosphorylation, while post-transcriptional regulation of GJs through internal ribosomal entry site-dependent synthesis presents an alternative approach to restoring connexin expression [235,236]. Taken together, these studies suggest that a general strategy for combating cancer tumorigenesis and metastasis may be the enhancement of Cx43 GJ function.

Clinical trials focusing on the validation of enzyme/prodrug gene therapy prove therapeutic potential via the bystander effect. These include clinical Phase 1 and 2 studies involving adenovirus mediated delivery of HSVtk combined with GCV in high grade gliomas as well in prostrate cancer patients [237,238]. These studies addressed safety, toxicity, and gene delivery issues but missed the mark in terms of deciphering the anti-tumor effects of connexins. What is largely lacking is correlation of these studies to the formation of GJ and connexin expression. Furthermore, there are no

clinical studies that evaluate the potential of specific, targeted connexin-based cancer therapeutics. This is likely the result of studies that reveal the context-dependent effects of connexins in cancers. Talhouk et al., recently completed a series of studies in breast cancer cell lines that reveal the context-dependent tumor suppressor effects of Cx43 expression with regards to culture conditions and the assembly of GJ complexes with α-catenin, β-catenin, and ZO-2 [239]. Furthermore, the cautionary tale associated with connexin modulation involves acknowledging the effects of the intervention in terms each of the stages involved in cancer progression. Studies aimed at defining the functional involvement of connexin hemichannels in cancer cell migration and metastasis are needed. Given the complexities associated with defining connexin mechanism as it relates to tumor type, microenvronment, and stage, there are significant questions that need to be asked and answered prior to the approval of the first targeted connexin-based cancer therapeutics.

## 6. Conclusion and thoughts on the future

The pathways that drive the onset and progression of disease reflect the combined effects of genetic susceptibility, environmental stimuli, and hormonal and physiological cues from other organ systems. Given that all these pathways can be linked in some form or another to connexin regulation or dysregulation it is not surprising that the therapeutic opportunity in targeting connexins is expansive. The topics that were not addressed in this review include immunological disorders, hearing loss, and gastrointestinal diseases, and are by no means of less clinical importance. Furthermore, therapeutic opportunity in the roles and functions of Cx43 in germ cell development and spermatogenesis, in coordinating activities occurs in the smooth-muscle cells of the uterine wall, and in developmental situations such as breast tissue in pregnancy and lactation when there are surges in the levels of specific GJ proteins, are exciting, relatively unexplored therapeutic avenues. Of additional clinical interest is the potential utility of connexins as biomarkers. Cx43 expression has been linked to lower early recurrence rates and better prognosis in patients with hepatitis B-related hepatocellular carcinoma after radical hepatectomy [240].

While the success of small peptide connexin therapeutics, such as ACT1, is undeniable, these peptides are often associated with short half-lives due to rapid degradation and require chemical modification or packaging technologies to enhance bioavailability, specificity and pharmacological efficacy. To enhance peptide stability in the body the addition of synthetic side chains may prove fruitful. Phosphodiester modifications involving replacing a non-bridging oxygen by a sulfur atom in the backbone of connexin antisense or missense oligodeoxynucleotides may protect compounds from nuclease degradation, enhance cellular uptake and increase their efficacy as target-specific agents [241]. Adenoviral approaches geared at the development of viral-based vectors aimed at providing a continuous secretion of small peptide connexin mimetics offer opportunity for the long-term expression of new genes in human cells at high efficiency without causing genetic mutation or strong immune reactions. The search for effective non-peptide analogs of the connexin mimetic peptides may prove to be a fruitful new avenue for rational drug design. Alternatively, gene editing, where connexin genes may be targeted at specific sites in the genome of cells, may offer a future therapeutic strategy. Another strategy yet to be fully investigated is the therapeutic application of non-coding RNAs (microRNAs). Cx43 is targeted by specific microRNAs and modulation of microRNA function may have potential in variety of disorders. Given the ubiquitous, transient, and complex nature of connexin expression, additional drug optimization strategies need to address concerns with regards to selectivity, stability, and off-target effects.

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