Translating connexin biology into therapeutics

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Abstract

It is 45 years since gap junctions were first described. Universities face increasing commercial pressures and declining federal funding, with governments and funding foundations showing greater interest in gaining return on their investments. This review outlines approaches taken to translate gap junction research to clinical application and the challenges faced. The need for commercialisation is discussed and key concepts behind research patenting briefly described. Connexin channel roles in disease and injury are also discussed, as is identification of the connexin hemichannel as a therapeutic target which appears to play a role in both the start and perpetuation of the inflammasome pathway. Furthermore connexin hemichannel opening results in vascular dieback in acute injury and chronic disease. Translation to human indications is illustrated from the perspective of one connexin biotechnology company, CoDa Therapeutics, Inc.

Keywords

Connexin, Research Translation, Connexin Patent, Inflammation, Vascular leak

1. Introduction

Gerald Kidder was a pioneer in connexin gene knockout mouse studies and was largely responsible for driving through the currently applied connexin gene nomenclature. As Dr Kidder reaches retirement after a notable career in gap junction research that included many other highlights, it is an appropriate time to look at the potential for connexin channel research to improve human health and wellbeing. It is 45 years since gap junctions were first described [1, 2], 30 years since gap junction blockade with antibodies was first demonstrated to disrupt development [3], nearly 30 years since the inaugural gap junction meeting in Asilomar in 1987, now held biennially, and 20 years since cardiac malformations were described in Connexin43 (Cx43) knockout mice by Kidder and others [4]. Connexin channels are key structures for cell-to-cell communication. It is now well recognised, however, that connexin hemichannels can form pathological pores and their manipulation promises significant therapeutic benefit. In this review we discuss some approaches being taken to translate biomedical research and the challenges faced, with our own work used as an example.

The commercialisation pathway for research translation when integrated into the university system has been viewed at times with suspicion owing to potential conflicts of interest. It has matured in recent years, however, and should be seen as a welcome emerging funding opportunity. For example, the United States Defence Department offers funding for basic research projects and the recent announcement of a five-year collaboration between The Johns Hopkins University and Bayer HealthCare for the development of ophthalmic therapies against retinal diseases is a key example of a university/corporate research partnership. The balancing of corporate versus university researcher expectations has not been without its issues. The interaction most commonly means that the corporation will own any outcomes, have exclusive rights to development, or have first right of refusal, but university technology transfer offices have done well to ensure that these agreements are written to provide an equitable return for the institution as well as for the inventors and researchers. These interactions require confidentiality agreements but it is possible to work within the scope of the arrangements and still ensure that laboratory discussions can be free and creative without fear of losing other researcher's rights. If done correctly, the funding interactions should be a win-win situation – technology progresses and these advances have a chance to benefit the public through development by the company of candidate diagnostics and/or therapeutics.

While commercially supported research comes with definite financial and resource benefits, there is a trade-off as the commercialisation route can potentially narrow the innovation funnel whereby the breadth of research becomes focused on the immediate commercial questions and needs. However, having accepted research funding, there is also an obligation to transfer innovation that shows potential for improved health outcomes. This can also result in ensuring that useful new data and information are available for use at the FDA and in Patent Offices. Importantly, it can also lead to new discoveries or improvements that themselves are suitable for commercial development and patenting.

2. Patenting Research Outcomes

Filing a patent application for a noteworthy invention or other advance is the primary route to attract commercial or corporate engagement as it is generally the only basis that underpins the opportunity for a financial return following years of development risk and expense. A patent grants the right to exclude others from using the invention claimed in the patent. It is granted by a country to an inventor, or assignee, for a defined period of time in exchange for detailed public disclosure of an invention. The owner or licensee of the patent are granted a period of time, most commonly 20 years, in which to commercialise the invention within the granting state and so recoup costs and gain a return on their investment before others can copy them. Extensions may be possible and in the United States the Hatch-Waxman act encourages the manufacture of generic drugs but also provides a period of exclusivity such that with approval of a generic version of a new molecular entity cannot be approved for five years. Thus, a drug approved for market near the end of its 20 year patent life will nonetheless have a period of exclusive coverage.

In order to be patentable an invention must be useful, novel and non-obvious. In order to be novel, an invention cannot have been previously disclosed, as in a publication or at a meeting or conference. Confidential disclosures do not defeat novelty. A patent may cover various types of inventions, including products, machines, compositions of matter, manufacturing processes, improvements of any of the foregoing, and more.

Patenting may not prevent others from using an invention for future research – it typically means that products cannot be commercialised based upon that intellectual property without obtaining the right to do so. This may include purchase of the intellectual property by assignment whereby another party ends up owning the rights to the intellectual property for a fee. However it is common

to license a patent whereby the original owner retains the property but licenses another party to use it for a fee. These fees may include upfront cash payments, milestone payments where a sum of money is transferred as the patented invention progresses through the various development stages to market such as clinical trials, new drug registration or first sales in market, royalty payments (usually a percentage of net revenues) or a combination of these.

Academic researchers are usually part of a team but there will be only one or several persons involved in making the invention. The “light bulb” moment typically referred to as “conception.” Legally they are the inventor(s) but it is possible and often desirable to have an income sharing agreement with the rest of the research team. The inventor may be named on the patent but recognition is given to the fact that the invention is reduced to practice and/or further developed based on the combined efforts of a team.

A key limitation for the academic researcher is the need to disclose results, for example to publish or present at conferences, in order to meet annual performance review requirements, grant funder expectations, or graduate student needs for career advancement. This needs to be balanced against the desire that patent applications not be filed too early, before supporting evidence of sufficient strength is in hand, or before adequate commercialisation provisions or plans are in place. Companies on the other hand can more easily maintain confidentiality and triage the preparation and filing of patent applications, and can run additional experiments to generate further data specifically for inclusion in a patent application. Companies have employees sign confidentiality agreements and typically will own all intellectual property made or developed by employees during the course of their employment, including all data they generate.

Patents go through three phases, (1) a provisional patent application, most commonly filed in the researcher’s own country, followed by (2) a PCT (Patent Cooperation Treaty) application a year later. The Patent Cooperation Treaty is an international patent law treaty that provides a unified procedure for filing patent applications in each of its contracting states. In general, depending on the country, eighteen or more months following a PCT patent application, a decision must be made where to file (3) National Phase patent applications (e.g., in the US, European Patent Office, etc.), with costs rising accordingly. The original patent attorney will contract a second agent in each of the countries in which the invention is to be protected with resulting legal fee escalation, translation costs in many cases, and annual patent fees for each country covered. If the intellectual property is not moving towards commercial application, these costs can become challenging and continuation

of certain patent applications may need to be considered. It is of note that that the application will by that time be published and thus remains a prior disclosure (prior art) and cannot be attempted to be patented again. This is a concern for university technology transfer offices that do not have the resources to maintain patents and therefore may have to drop patents before the technology is transferred. This means that good research may effectively be wasted unless someone is prepared to commercialise without patent protection. Unfortunately, for pharmaceutics especially, that is extremely unlikely as the cost of developing prescription pharmaceutical drugs is remarkably high (averaging over $2.6 billion, according to the Tufts Center for the Study of Drug Development). An investor will require a period of market exclusivity in order to recoup costs and make a profit before generic drugs (which take no risk, and do not face any significant development costs and can thus be brought to market at a fraction of the expense) become available.

Companies will aim to obtain broad patent claims as far as possible commensurate to the nature and scope of the invention. For first-in-class or platform technologies, patent protection may cover a wide range of potential indications, such as uses for therapeutic and disease applications, but patents may also be quite specific. The “value” of the patent will depend upon many things, including strength, claim scope, potential market size, how far the product has been developed (how close to market) and the remaining term, and factors such as whether orphan drug status is likely where the market may be smaller but the regulatory pathway less onerous.

The gap junction community is not behind in this regard – a search for “connexin” and related terms at the U.S. Patent & Trademark Office website reveals nearly 500 issued and pending US patent applications as shown in the Table below. And a search in Google Patents reveals over 1200 utility patents and applications that refer to “connexins,” and 27 that refer to “hemichannels.”

Table 1. The number of US patents issued related to Connexins or connexin channels

	Issued US Patents	Published US Patent Applications
Claim language includes:		
CONNEXIN	78	259
GAP JUNCTION	21	89
HEMICHANNEL	2	19
	101	**367**

Disruptive technologies, which may completely alter the way a disease is perceived or treated, can be more difficult to progress as investors and pharmaceutical companies need to be convinced of the innovative approach, but their novelty may open far wider market opportunities. Once patents have been obtained, the translation driven researcher and his/her company or licensee faces "the valley of death" – the gap between commercial funding and the clinical development of a new compound to the level where it can be approved by a regulatory authority for sale. A potential new drug faces target validation, lead product identification and optimization, including formulation and packaging. Furthermore, preclinical development including toxicity testing by independent contractors and product stability assessment, Phase I (safety), Phase II (efficacy in a selected population) and Phase III (efficacy in the wider population) testing are required prior to regulatory approval. For a more detailed outline the reader is referred to *Science Business: The promise, the reality and the future of biotech* [5].

In the example of CoDa Therapeutics Inc., there are fifteen years of research and discovery behind its work with positive results in over 25 different animal models, 55 publications on gap junction channel modulation from its scientific co-founders, and nine years of preclinical and clinical development at the time of writing. Thus, considerable investment has also been put into patents related to the company's products and to protect its intellectual property. The aim is to patent both initial and new molecules and indications, extend patent opportunities by patenting improvements, and to prepare applications in a manner that reduces the risk of copying or the presence of minor loopholes that enable others to lodge undermining or competitive patents. In the case of CoDa Therapeutics Inc. the company has over thirty patent families covering its three lead connexin channel targeting compounds, indications, methods of use, compositions of matter, formulations,

dosing regimens, articles of manufacture, and more. These include eighteen patent families related to Nexagon® (an antisense product) and related compounds, eight related to Peptagon™ (a peptidomimetic product) and related compounds, two related to HCB1019 (a small molecule) and related compounds. The company has a number of patent families directed to dermal applications of its products, and six patent families, for example, relating to ocular disorders and conditions. Patent protection extends out to 2035 for newer patents.

3. CoDa Connexin Compounds in Development

CoDa Therapeutics Inc. has three connexin-related compounds in development. The first is an antisense oligodeoxynucleotide that binds to the messenger RNA (Watson-Crick binding) to block connexin protein translation. **Nexagon®** is a 30 nucleotide single strand DNA sequence specific to Cx43,although the company's intellectual property covers other connexin isoforms and all antisense approaches, for example RNAi or morpholinos. When first founded in 2004 in New Zealand and later incorporated in the USA in 2006, CoDa Therapeutics Inc. faced resistance to antisense approaches. This arose from the antisense boom and bust in the late 1980's and early 1990's that saw several companies fail and many investors lose their investments. Those failures resulted from the need to modify antisense for systemic delivery, which reduced specificity and increased toxicity, and an inability to target the oligonucleotides to their site of action such as a tumour. The first antisense drug in the market was Fomivirsen (Vitravene) licensed by the FDA in 1998, an antiviral drug used to treat cytomegalovirus in immunocompromised patients. It was injected into the eye and so the delivery issues were overcome by having a compartmentalised delivery site. CoDa Therapeutics Inc. uses an unmodified DNA backbone oligomer. The half-life of unmodified oligodeoxynucleotides in serum is about 1-2 minutes and in the cell cytoplasm about 20 minutes (for review see [6]). Nexagon® is formulated in a thermoreversible Poloxamer 407 gel which is a component of other FDA-approved products and can be delivered cold. The gel sets as it warms to physiological temperatures. The target indications, such as skin and ocular surface, enable topical delivery to the site of action with the gel providing continual delivery over several hours with negligible systemic uptake. Even high dose intravenous injection of the 30-mer API has revealed no adverse effects, and the clinically packaged product has proven stable over 48 months (unpublished data). Nexagon® has been applied to several hundred patients with positive outcomes in clinical trials for venous and diabetic leg ulcers, and in the treatment of non-healing ocular burns (chemical and thermal).

Peptagon™ is a connexin peptidomimetic that mimics a portion of extracellular loop two of the Cx43 protein (termed Peptide5 in research publications – see [7]). Peptagon has shown efficacy in blocking Cx43 hemichannels when delivered topically (see for example [8]) or systemically [9]. When delivered directly into the ventricles of the brain of a pre-term sheep following hypoxia induced by closure of the carotid arteries so mimicking the hypoxia that can occur during child birth and giving rise to cerebral palsy, the peptide reduces seizures and results in faster return to normal sleep cycling whilst giving significantly improved EEG outcomes, all strong clinically relevant signs [10]. Mode of action / site of action studies are close to publication and Peptagon™ clinical development will be initiated in 2016.

CoDa Therapeutics Inc. third compound, **HCB1019**, is a promising new hemichannel blocker acting by a new mechanism and is completing preclinical and nonclinical evaluation.

4. Finding the Therapeutic Target

Translation requires a level of integration and pragmatism. In the case of connexin biology changes in cytoplasmic calcium concentration or connexin phosphorylation modulation may be challenging as therapeutic approaches unless they can be achieved whilst avoiding effects on other cellular processes and pathways. Some of the most interesting approaches have targeted connexin expression or the channels themselves. These can in general be split into three main classes: Approaches targeting transcription / translation modulation (such as antisense approaches), peptidomimetic approaches (such as Gap26, Gap27, Peptide5; Gap19 and ACT-1) and non-specific compound or small molecule approaches including fatty acids, volatile anaesthetics (such as halothane and ethane), alcohols (octanol, heptanol), steroids such as 18β-glycyrrhetinic acid and its water soluble derivative carbenoxolone, or quinine and derivatives [11]. These approaches are listed above in their order of specificity, with antisense being very specific to connexin targets, peptidomimetics being connexin-specific though not always isoform-specific, and the non-specific compounds often affecting multiple channel types, with some being considered to be “dirty” drugs.

The peptidomimetics can be grouped into those that act cytoplasmically (targeting connexin amino or carboxyl – terminal tails, or the intracellular loop) [12, 13] or externally (those targeting extracellular loops one or two) on the connexin protein [7, 14, 15]. The advantage of cytoplasmically acting peptides is that they may again display a level of connexin isoform specificity and in the case of Gap19, has been reported to close hemichannels but maintain cell-cell communication [16]. There

is mounting evidence that connexin hemichannels and gap junction channels for Cx43 at least are inversely modulated (see for example [17-22]), which has some elegance to it.

Conversely, peptides targeting the cytoplasmic C-terminal region may have far wider effects owing to the complexity of protein-protein interactions in the Cx43 tail. The peptide ACT-1 has been reported to play a number of roles and, for example, may [23] or may not [24] impair malignancy. For both antisense and cytoplasmic acting peptides, transmembrane permeability is required which makes delivery and targeting more challenging, especially for systemic delivery indications. Both the Nexagon® and ACT-1 molecules, for example, have been targeted in clinical trials by using topical application and gel delivery systems. Of the peptides acting on the extracellular loops to regulate connexin channel function, Gap26, Gap27 and Peptide5 (the active pharmaceutical ingredient in Peptagon™) are the most characterised with around 100 reports describing the effects of administration of these peptides in a range of cells and tissues [25]. Peptide5 has been delivered topically [26], intra-ocularly [27], and into the cerebro-spinal fluid [28] and systemically with significant success [9]. The use of multiple approaches has also been used to confirm target applicability and specificity. In spinal cord injury, for example, antisense [29], topically delivered peptide [26] and systemic peptide approaches (in preparation) have yielded remarkably similar and significant benefits across different injury models. In the case of antisense, the upregulation of new connexin hemichannels following injury is prevented, although this approach also leads to a transient reduction in cell-cell coupling. In a retinal ischemia-reperfusion model both systemic delivery and intraocular injection of Peptide5 reduced vascular leak and inflammation, promoting almost total neuronal survival [9, 27, 30].

Since 75 – 95% of new drugs fail during development and before reaching market, it is essential to manage risk which is where simpler, targeted, delivery strategies, and supporting data from multiple models and connexin channel modulators, can provide a level of investor comfort.

5. Connexin Translation Targets

In our own work, using all three classes of connexin channel modulator and multiple animal models, *ex vivo* organotypic models, and *in vitro* models, we have discovered four key areas for therapeutic application of connexin channel modulation after injury or in chronic disease. These are cell migration (epithelial recovery and granulation tissue formation), oedema (cell swelling), inflammation (and the inflammasome pathway), and vascular haemorrhage or dieback. The latter

three in particular, and because they are linked, provide a common therapeutic target across multiple injury types and chronic conditions. In the retina, for example, these three elements appear to instigate injury, injury spread and chronic disease progression in conditions such as age related macular degeneration, diabetic retinopathy, glaucoma and macular oedema (for review see [31]). In all cases, the connexin hemichannel is the therapeutic target identified for reduction of disease onset and progression.

5.1 The connexin hemichannel as a therapeutic target

In the course of translating connexin modulation to the clinic our understanding of the biology has continued to deepen over the years. When considering therapeutic approaches and research translation of inventions to products, the connexin hemichannel provides an example where it is possible to integrate knowledge and propose a working hypothesis that supports the therapeutic opportunity. The invertebrate cell-cell communication channel forming proteins are the innexins, but at the interface between invertebrates and vertebrates the pannexin membrane channel forming protein family evolved retaining about 25 – 33% homology, but not having a role in cell-cell communication (for review see [32]). Instead a new connexin family of proteins arose to take over as the structural proteins of cell-cell communication channels. Pannexins having external complex glycoproteins [32] and are not considered able to dock to form a cell-cell communication channel. Instead pannexin channels directly link the cell cytoplasm with the extracellular milieu, and must therefore be tightly regulated in order to play their physiological roles in normal tissue development and homeostasis without disrupting normal cell activities. This does not exclude the possibility that in injury and disease things can go wrong, and pannexin channels, for example, have been reported to play a role in apoptosis where their opening may be a regulated response leading to an ATP and UTP “find me” signal for phagocytes [33].

There is less direct evidence though for connexin hemichannels having normal physiological roles. ATP release through hemichannels plays a role in purinergic signalling but nonetheless requires a triggering event such as mechanical stress, quinine-induced hemichannel opening, lowered extracellular Ca^{2+} or the application of transmembrane voltages [34-37]. Under pathological conditions such as ischaemia, there is a large drop in extracellular Ca^{2+} concentration that is associated with hemichannel opening, and oxidative stress causes membrane depolarization to also trigger hemichannel opening [38, 39]. Hemichannel opening also occurs in response to metabolic inhibition [17, 40], treatment with pro-inflammatory cytokines such as IL-1β or TNF-α, or by

lipopolysaccharide stimulation [20, 41]. In the cochlea of the ear, Connexin26 hemichannels were thought to contribute to signaling [42], but new evidence suggests that it may be pannexin channels playing this role. Deletion of the main connexin isoforms in the cochlea, Connexin26 and Connexin30, doesn't have any effect on ATP release and endocochlear potential generation whereas Pannexin1 deletion virtually abolishes ATP release under physiological conditions [43]. A "normal" physiological role for hemichannels may be in the retina where ephaptic signalling plays a role in contrast enhancement in fish and reptile species [44-47]. Even here, the relevant connexin isoforms are not present in the mouse and either a different isoform is involved or a different channel type entirely as in the cochlea [48]. Where there is hemichannel mediated release of ATP in the rodent retina (from Muller glial cells for example), it is in response to osmotic stress and glutamate [49].

The open connexin hemichannel, compared to most other membrane channels such as those formed by aquaporins, or sodium and potassium channels, forms a large, relatively non-selective pathological pore [50] and both increased connexin protein expression and increased hemichannel opening probability contribute to lesion spread and the maintenance of chronic disease conditions. Inhibiting gap junction channels in the CNS following injury compromises astrocytic function and increases neuronal injury [51], and the sustained loss of Cx43 after brain injury in inducible knockout animals exacerbates the size of the injury [52, 53]. Conversely, when Cx43 hemichannels are triggered to open after injury, they contribute to cell death, for example astrocytes [17] and vascular endothelial cells [9]. Tissue oedema in the first instance is caused by cytoplasmic swelling since prevention of Cx43 expression, or more specifically hemichannel block, almost completely stops swelling in tissues as diverse as spinal cord [29], optic nerve [54] and skin wounds [55]. Cx43 gap junction channels and hemichannels are oppositely regulated under inflammatory conditions [20], with the adverse outcome reflecting both uncoupled gap junctions and open hemichannels. Enhanced Cx43 hemichannel opening leads to elevated purinergic signalling mediators including ATP in the extracellular space [37]. ATP release may be in an entirely uncontrolled manner, as in necrosis, although some degree of control has been suggested [37, 56]. Nonetheless, extracellular ATP has been shown to rise in both ischaemia [57] and epilepsy initiated seizures [58], and acts as a potent activator of the ATP-gated purinoceptors expressed on the surface of many cell types including astrocytes and microglia [59], leukocytes [60] and endothelial cells [61]. Furthermore, ATP induces its own release through connexin hemichannels with expanding activation of purinergic receptors [62] and so is seen as a key player in the 'inflammasome' pathway [63-65]. Figure 1 summarises the steps in this pathway with connexin hemichannel opening playing a role in both inflammasome pathway initiation and propagation (for further review see [86], in press). Indeed, one "physiological

role" for connexin hemichannels may be in inflammatory cells (leukocytes, neutrophils, macrophages) which express high levels of Cx43 in wounds sites and release ATP but, unlike other cell types, are not themselves adversely affected. The role of connexin hemichannels in injury and disease, and the benefits of hemichannel regulation, will be illustrated in more detail in specific examples below.

5.2 Vascular leak and vasculopathies

Another example of knowledge integration - vascular leak or die back - warrants some discussion, especially as it pertains to inflammation. It is widely assumed that vessel leak results from tight junction disruption or changes in cell adhesion. In many cases this is no doubt correct, but after injury, whether trauma, ischemia, inflammatory cytokine induced or infection and at the edge of chronic wounds, connexin hemichannel mediated vascular leak is prevalent with hemichannel opening leading directly to endothelial cell loss [9, 66]. This has been investigated in detail in embolic stroke where there is '*disintegration of the endothelial layer itself, thereby allowing unhindered extravasation of blood-borne molecules at different time points*' [67] and in focal cerebral ischemia where four distinct stages of blood-brain barrier breakdown with ultimate loss of endothelial cells occurs [68]. In areas where there is blood-brain barrier breakdown the endothelium becomes ruffled or discontinuous. The Danesh-Meyer et al (2012) study showed blood vessel truncation, clumping of endothelial cells and loss of vessel wall within four hours of retinal ischemia and reperfusion, with vessel leak reduced by 86% with a single intraperitoneal injection (systemic delivery) of the hemichannel blocking Peptide5 at the point of reperfusion [9]. Extravasation of large molecules (FITC tagged bovine serum albumin) is also seen 4 mm either side of a spinal cord crush injury [29] with Cx43 antisense preventing this from occurring. In experimental cerebral malaria models vascular leak and petechial haemorrhaging is the major lethal event [69], with systemically delivered Peptide5 via intraperitoneal injection showing significantly delayed clinical signs and opening a window for disease intervention [66]. In both Alzheimer's and Parkinson's diseases, chronic neuroinflammatory disorders, microvasculature is lost indicating capillary dysfunction [70, 71]. In a skin ischemia model, a flap of skin on the back of a mouse is pinched for 1.5 hours creating two zones of ischaemia-reperfusion injury. Vascular haemorrhage starts within four hours and continues over at least 24 hours (Figure 2). Even in tumours, there is a long embedded dogma that neovascularisation feeds a tumour. This, however, can be questioned if the vascular pathology observed is interpreted in light that tumours are hypoxic, drug delivery is restricted by poor blood flow, and brain tumours are inherently difficult to treat reportedly owing to the blood brain barrier

despite there being leaky blood vessels. These all imply that neovascularisation does not actually give rise to functional vessels. Patients receiving reduced anti-angiogenic treatments that lead to increased blood flow have longer survival than those who do not gain increased blood flow [72]. The cancer vessel argument is expanded in Zhang et al (2014) [73], but in the discussions here related to injury and disease, a truncated vessel and endothelial cell proliferation should not be assumed to mean sprouting and vessel growth; it is more likely to indicate die back with disorganised endothelial cell clumping. Cell rupture as a result of Cx43 expression after injury is not restricted to endothelial cells though, but occurs in many cell types, for example neuronal and glial cells after spinal cord injury (Figure 3).

Associated with vascular leak is inflammation, either by resident cells (astrocytosis and microglial activation following CNS trauma), or by blood born cells invading the injury site. Neutrophil and macrophage infiltration is for the most part tightly controlled, with Cx43 expression associated with macrophage infiltration and endothelial cell activation [74] and, conversely, trans-endothelial inflammatory cell migration reduced by gap junction inhibitors *in vitro* [75]. However, in a rat model of bacteraemia, latex particles perfused into inflamed lungs end up clustered within tissue in a pattern similar to that of neutrophils [76]. This means that the inanimate latex beads, which do not have the specific endothelial adhesion properties of neutrophils, are still able to penetrate the endothelium. Vascular die back in response to injury may therefore bypass the conventional chemoattraction, rolling adhesion, endothelial transmigration pathway, instead allowing for the rapid accumulation of large numbers of leukocytes at a site of injury that simply “fall” through gaps in the vessel wall. Connexin channel regulation, whilst preventing vascular disruption, significantly reduces neutrophil counts after spinal cord injury [29] in skin burns [77] and by up to 80% adjacent to skin wounds [55, 78]. These cells themselves, once in the injury site express significant amounts of Cx43 (Figure 4 shows Cx43 labelling on macrophages after a laser burn to the optic nerve), contributing to cytokine and ATP release and the inflammasome pathway (Figure 1). The connexin hemichannel mediated mechanism of vessel dieback (and associated lesion spread) may have evolved to enable the dumping of large numbers of leukocytes into a wound site to reduce the risk of infection, and to also isolate the damaged tissue, reducing the risk of virus spread and septicaemia. Over the course of many hundreds of animal experiments and numerous human clinical trials, however, we have never observed infection after Cx43 knockdown or hemichannel block. Of note too, most chronic disease conditions, whether age related macular degeneration, diabetic retinopathy, Alzheimer’s disease, Parkinson’s disease, diabetic or venous leg ulcers, stroke, cardiac ischemia, inflammatory arthritis or others, are primarily diseases of aging and occur post-

reproduction age; there is no evolutionary selective pressure to ameliorate their incidence and with aging, inflammation in response to injury is more sustained.

6. Some Examples of Connexin Translation to Human Utility

6.1 Reducing Cx43 levels improves healing of epithelial tissues

In a rat cornea photorefractive keratectomy model transient down-regulation of Cx43 using antisense delivered in the thermoreversible Poloxamer 407 gel significantly reduced oedema with control treated corneas swelling to triple their usual thickness at day one post injury, and remaining double thickness at days two and three [79]. Cx43 specific antisense treated corneas, on the other hand, maintained normal corneal thickness, had less epithelial hyperplasia, reduced inflammation (myofibroblast differentiation and proliferation) and re-epithelialised faster. In rat incision and excision skin wounds transient down regulation of Cx43 using antisense delivered in Poloxamer 407 gel significantly improved outcomes [55]. Ranking of wounds by masked observers indicated a significant reduction in swelling, redness and gape. Neutrophil counts assessed one day post wounding showed a reduction in numbers at the wound site by about 20% but up to 80% adjacent to the wounds site. As a result, by day seven, granulation tissue was reduced by 40% with a smaller, less distorted scar in final analysis. In skin burns Cx43 antisense reduces the spread of tissue damage and again reduces neutrophil infiltration around the wound following injury [77]. As with incisions and excisions wound closure is accelerated, and scarring reduced, with a greater number of dermal appendages than in controls. Down-regulation of Cx43 at wound edges correlates with both keratinocyte and fibroblast migration, whereas abnormal overexpression of Cx43 perturbs healing in the streptozotocin diabetic rodent impaired healing model [80]. It is of note then that at the edges of human chronic wounds Cx43, 26 and 30 are strikingly upregulated in the epidermis, as well as Cx43 in the dermis [81]. It is also possible that in some cases, aberrant heteromerization of Cx43 and Cx26 may underlie pathology, for example in keratitis-ichthyosis-deafness syndrome [82] and palmoplantar keratoderma and deafness [83], thereby extending the potential influence of Cx43 antisense and peptide technologies to disorders ascribed to other Cxs. Dysregulated connexin expression appears to be common feature in chronic skin wounds.

Translating to Humans:

Venous leg ulcers account for two million working days lost per annum and $3 billion in treatment costs per year in the United States alone. In one clinical trial, CoDa Therapeutics Inc. treated venous leg ulcers with Nexagon® in a four week study with 98 patients at multiple sites. The result was a 69% reduction in venous leg ulcer size within four weeks, and 31% of wounds completely healed, five times greater closure than vehicle alone [85].

6.2 Reducing inflammation reduces scar tissue deposition

As discussed above reducing Cx43 expression within a wound significantly reduces granulation tissue volume and scar tissue deposition [55, 77]. In the eye glaucoma is a progressive optic neuropathy and the second leading cause of irreversible blindness in the world. In cases where topical medical therapy and/or laser treatment are unable to control intraocular pressure, surgical intervention is used to improve aqueous fluid egress from the anterior chamber of the eye. A trabeculectomy is the most widely used incisional surgery to form a drainage canal, with or without the insertion of a drainage tube. In some patients excessive scarring closes off the drainage canal and treatments with the cytotoxins mitomycin-C or 5-fluorouracil are used to reduce inflammatory cell viability and scarring. Where that fails repeat treatments are limited owing to the risk of corneal melt.

In a rabbit trabeculectomy model Cx43 antisense was used to reduce inflammation and fibrosis following insertion of a drainage tube [84]. A single application of the antisense injected into the drainage bleb significantly reduced Cx43 upregulation at 0.5, 1 and 1.5 mm either side of the tube. Anterior chamber reaction resolved within five days instead of eight in the controls and smooth muscle actin labelled was reduced owing to reduced myofibroblast activation. Scarring, assessed by two independent masked observers, was significantly reduced with clear drainage tubes in controls but scar tissue growing a significant distance into and blocking the drainage tube in control animals.

Translating to Humans:

In two patients presenting with high intraocular pressure which was not controlled with repeat needling and 5-fluorouraci or mitomycin-C applications, a re-needling was carried out and Nexagon® (anti-Cx43 antisense) injected into the conjunctival drainage bleb on a compassionate use basis (these are the only patients so far treated for this indication).
The *first patient* was a 48-year old female with previous ocular trauma and failed corneal graft secondary to persistently raised intraocular pressure. There were multiple interventions but aggressive scarring continued. This patient received two doses of Nexagon® 10 days apart and has sustained normal intraocular pressure. The *second patient* was a 32-year old male with inflammatory glaucoma secondary to trauma, planned for corneal transplant if pressures could be stabilized. He had a revised Baerveldt tube inserted but had aggressive scarring blocking the outflow and persistent raised intraocular pressure. Treatment with three doses of Nexagon® over 6 weeks led to clinically reduced scarring in the eye with retention of a fully patent drainage tube and sustained normal intraocular pressure.

6.3 Preventing vascular die-back enables healing of chronic wounds

Vascular haemorrhage is a feature of acute and chronic wounds. In the spinal cord crush wounds discussed above extravasation of a large molecule (FITC - bovine serum albumin) was seen 4 mm either side of the lesion, but vessel leak was virtually eliminated by the application of Cx43 antisense to transiently down-regulate Cx43 expression. In the eye, retinal ischemia can be induced by cannulating the anterior chamber and raising the intraocular pressure to 120mm mercury. The retina becomes pallid and one hour later the cannula is removed and reperfusion of the retinal vessels confirmed by ophthalmoscopy [9]. The inflammatory response (astrocytosis and increased Cx43 expression) is not even across the retina, but in patches correlating with vascular leak (ascertained using systemic perfusion of Evans Blue dye). A single intraperitoneal injection of hemichannel blocking Peptide5 reduced vascular leak at the peak 4 hour post-reperfusion period by 86% and astrocytic glial fibrillary acidic protein was kept at uninjured retinal levels, whereas it's expression was doubled in scrambled peptide controls. About one third of the control treated animal's retinal ganglion cells are subsequently lost at seven and twenty-one days post-ischemia whereas Peptide5-treated animals did not have a significant loss of neurons. The mechanism of endothelial cell loss is connexin hemichannel opening, as shown with *in vitro* endothelial cell hypoxia-reperfusion studies,

where Peptide5 at hemichannel blocking concentrations is able to provide complete protection [9, 66].

Translating to Humans:

In the eye, prolonged inflammation and lack of epithelial recovery in non-healing corneal epithelial wounds may lead to corneal opacity, blindness or enucleation. Ormonde et al (2012) described the application of Nexagon® (anti-Cx43 antisense) to five patients with severe ocular surface burns (persistent epithelial defects) that had failed to respond to established therapy for between seven days and eight weeks prior to treatment [87]. One or two doses of Nexagon® were delivered in Poloxamer407 gel under an amniotic membrane graft or a bandage contact lens. The drug reduced inflammation within one to two days, and all five achieved stable corneal healing. It is of note that recovery of the vascular bed (and limbal reperfusion) preceded epithelial recovery. The first patient has maintained 20-30 vision at ten years post treatment.

7. Conclusion

Transient down regulation of Cx43 or block of connexin hemichannels after injury significantly reduces oedema, inflammation, lesion spread and scar tissue deposition and can speed epithelial recovery. This has been demonstrated in numerous models by our group, but also by many others working with connexin channel modulators. In this review we have focused on our own work in order to demonstrate some current efforts toward translation of connexin biology to human therapeutic application. There are also other companies with a focus on connexins, such as FirstString Research (USA; anti-Cx43 ZO-1 binding site proteins), Theranexus (France) and Zealand Pharma (Denmark; connexin phosphorylation compounds).

The future for the gap junction community is exciting, with connexin hemichannel modulation as one example showing promise for various acute injury and chronic disease indications. This is just the tip of the iceberg as seen from our perspective, but we hope that despite Gerald Kidder's retirement, the field will continue to thrive and that some of the approaches outlined in this article and others will prove useful to treat human diseases, disorders and conditions.

8. Acknowledgements and Conflict of Interest

We thank Ann Wu for images used in Figure 3 and Taras Papchenko for the images used in Figure 4. We acknowledge Professor Helen Danesh-Meyer for the trabeculectomy compassionate use data.

David Becker and Colin Green are co-founders of CoDa Therapeutics, Inc. and hold stock in that company. They are consultants for CoDa Therapeutics, Inc. Bradford Duft and Anthony Phillips are employees of CoDa Therapeutics inc. All of the authors are named as inventors on one or more patents and patent applications owned or controlled by CoDa Therapeutics, Inc. that relate to the therapeutic regulation of connexin channels.

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10. Figures and Captions:

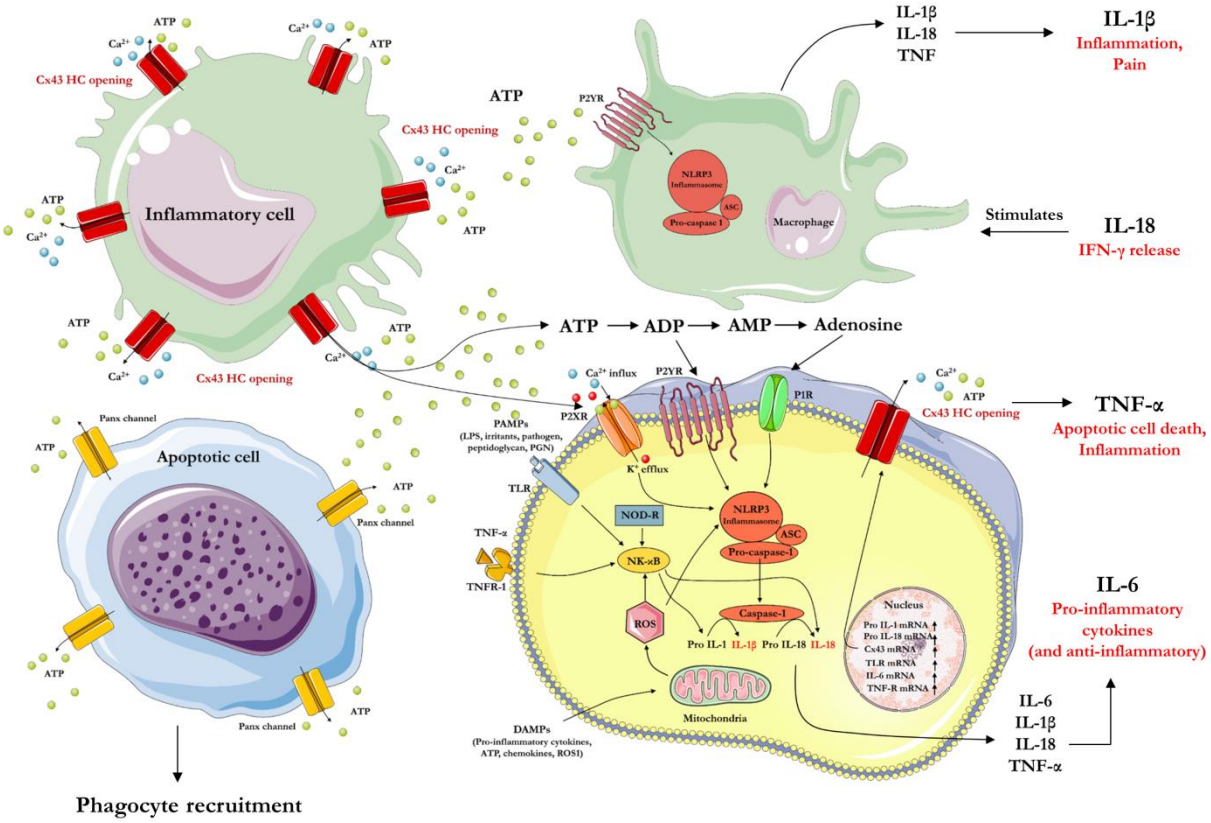

Figure 1: A diagram illustrating Cx43 hemichannel roles in the inflammasome pathway. ATP is released into the extracellular space following injury and cell death. Multiple cell types release ATP, including reactive inflammatory cells, astrocytes, microglia and neurons in the CNS. The extracellular ATP (and ADP and derivatives) activate cell surface P2 purinergic receptors (P2YRs; GPCRs with seven transmembrane-spanning motifs) or nucleotide-gated ionotropic P2X receptors (P2XRs), which are ion channels. The nucleotides are further catalysed to adenosine, which activates cell surface P1 purinergic receptors. Binding of ATP to the P2X channels trigger a flux of Na^+, Ca^{2+} and K^+ ions across the pore. Activated P1 and P2 receptors trigger the intracellular signalling molecule, NLRP3, which binds to apoptosis-associated ASC. ASC in turn interacts with protease caspase1 (pro-caspase1) to form the inflammasome complex. Activated caspase-1 cleaves pro-inflammatory cytokines to their active forms, IL-1β and IL-18. In addition to the purinergic receptor mediated pathway, pathogen-associated molecular patterns (PAMPs - such as irritants, peptidoglycans or bacterial LPS) and cell-

derived damage-associated molecular patterns (DAMPs – such as inflammatory cytokines or chemokines) act as signalling molecules, binding to specific receptors such as the Toll-like receptors (TLRs), NOD-like receptors (NOD-R) and Tumour necrosis factor receptor 1 (TNFR-1). Activation of these receptors initiates the pro-inflammatory nuclear factor (NF-κβ) pathway to initiate cytokine release. Mitochondria are also at the centre of the cell death pathway that can be triggered by stimuli such as DAMPs, leading to elevation of reactive oxygen species (ROS) and the activation of the NF-κβ pathway. Inflammation alters gene expression, including upregulation of Cx43 mRNA, resulting in greater numbers of hemichannels at the cell surface where increased hemichannel opening leads to further ATP release and regulates the release of inflammatory cytokines. These cytokines may not pass through hemichannels directly, but hemichannel block significantly reduces their levels relative to untreated tissues. Pannexin channel opening on the other hand may be a regulated response leading to an ATP and UTP "find me" signal for phagocytes during apoptosis [33].

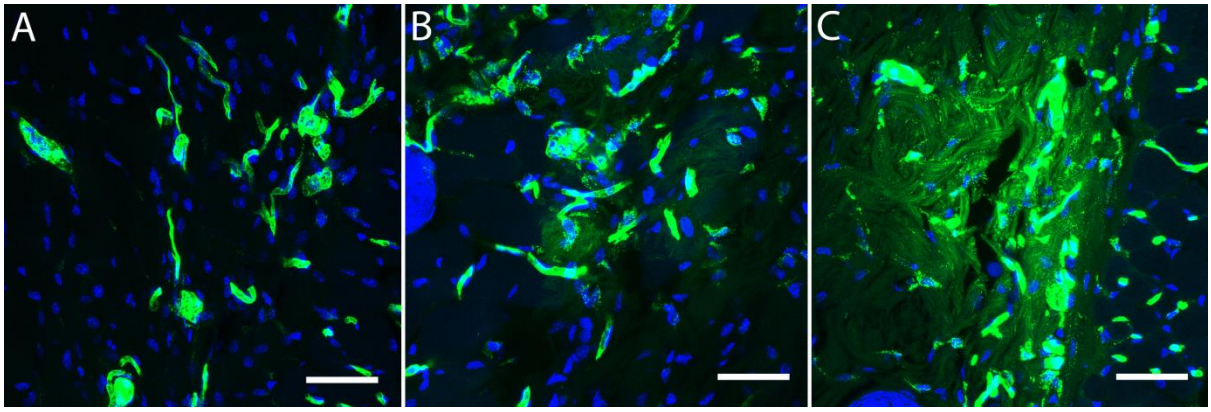

Figure 2: Confocal microscope images of skin 24 hours post-ischaemia. A flap of skin on the back of the mouse was pinched for 1.5 hours creating two zones of ischaemia-reperfusion injury. FITC-BSA was injected into the tail vein 20 minutes before the mouse was killed. In uninjured control skin (A), the FITC-BSA (green) is restricted to the blood vessels (cell nuclei are blue). Images (B) and (C) show examples of small and extensive areas of vessel leak respectively at the end of the 24 hour reperfusion period. Vessel leak starts within 4 hours of ischaemia-reperfusion injury. Scale bar = 50 μm.

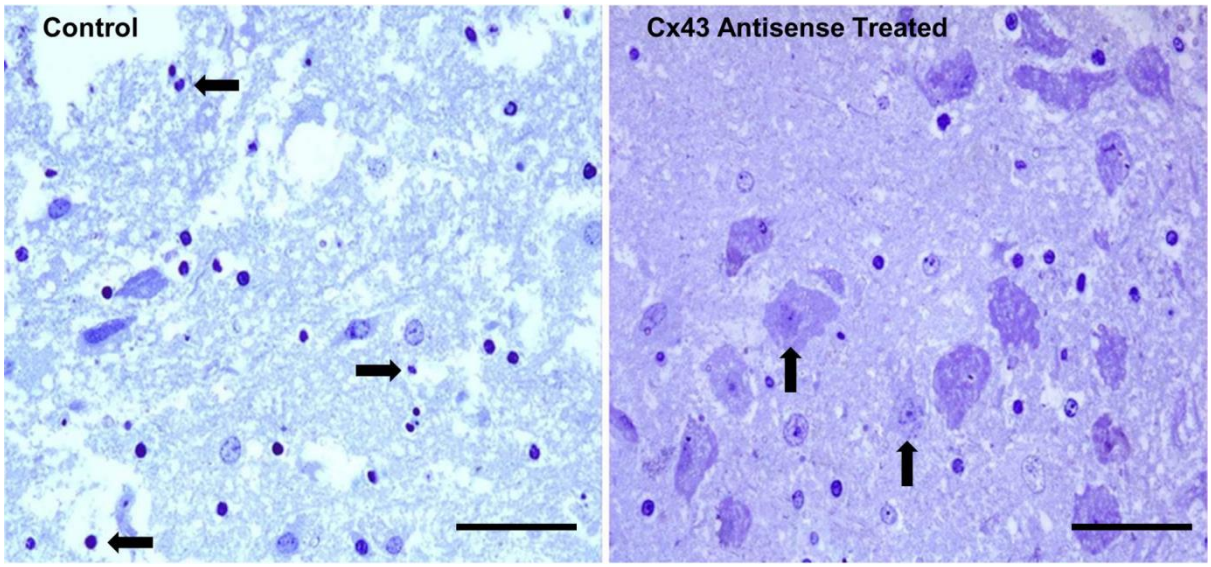

Figure 3: Toluidine blue stained resin sections of rat ex vivo spinal cord segments after 24 hours of air-liquid interface organotypic culture. In control segments (left image) cell oedema has occurred as a result of the excision injury, leading to rupture. Dense, pyknotic nuclei are seen (horizontal arrows) with no surrounding cytoplasm. Spaces are left in the tissue where cells have swollen and burst. In contrast, in segments treated with Cx43 antisense oligodeoxynucleotides (right image), blocking Cx43 protein translation and hemichannel formation, cells remain intact with nucleoli evident in the nucleus and cell cytoplasm intact (vertical arrows). Scale bar = 50 µm.

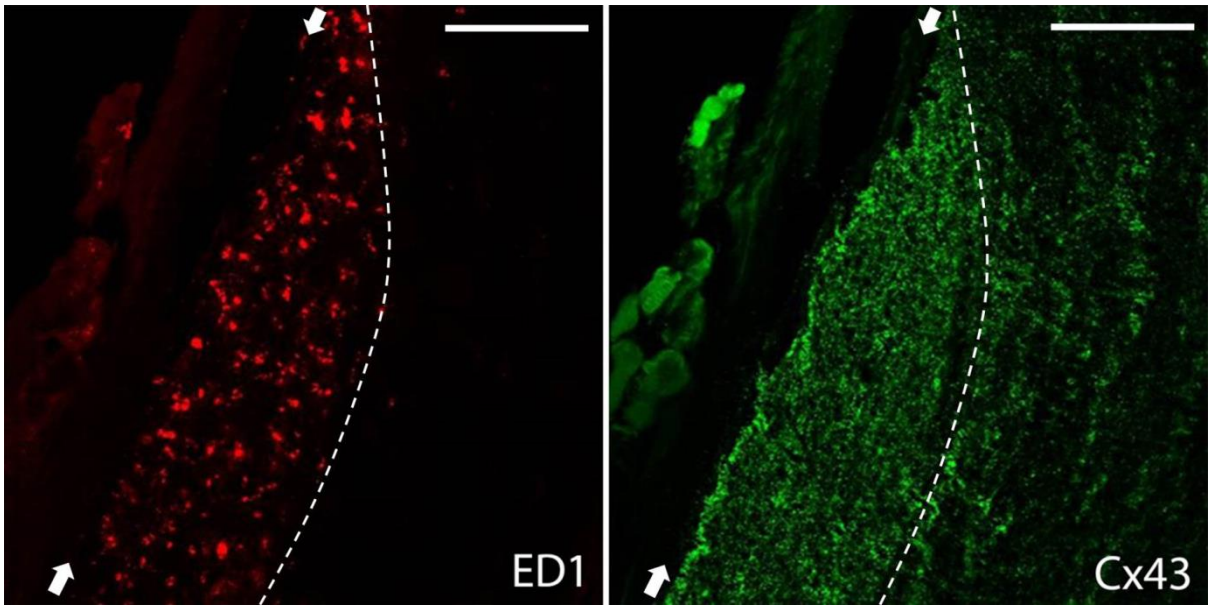

Figure 4: A rat optic nerve *in vivo* laser burn injury. In this type of CNS injury, blood born macrophages (ED1 positive labelled cells – left image) migrate into the spreading lesion from the surface. These cells express large amounts of Cx43 (right image) consistent with their role in the inflammasome pathway, releasing ATP and inflammatory cytokines. The arrows indicate the outer edge of the optic nerve and the dashed line the lesion that has formed 5 days post-injury. On the inner edge of the lesion, within the white matter tissue of the optic nerve, GFAP expression is also upregulated (not shown). Scale bar = 10 µm.