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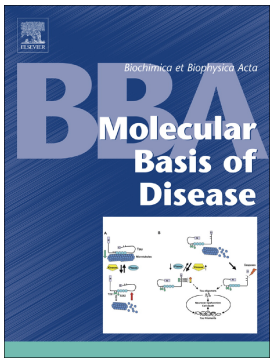

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Harnessing the Therapeutic Potential of Antibodies Targeting Connexin Hemichannels

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Abstract

Background: Connexin hemichannels have been implicated in pathology-promoting conditions, including inflammation, numerous widespread human diseases, including cancer and diabetes, and several rare diseases linked to pathological point mutations.

Methods: We analysed the literature focusing on antibodies capable of modulating hemichannel function, highlighting generation methods, applications to basic biomedical research and translational potential.

Results: Anti-hemichannel antibodies generated over the past 3 decades targeted mostly connexin 43, with a focus on cancer treatment. A slow transition from relatively unselective polyclonal antibodies to more selective monoclonal antibodies resulted in few products with interesting characteristics that are under evaluation for clinical trials. Selection of antibodies from combinatorial phage-display libraries, has permitted to engineer a monoclonal antibody that binds to and blocks pathological hemichannels formed by connexin 26, 30 and 32.

Conclusions: All known antibodies that modulate connexin hemichannels target the two small extracellular loops of the connexin proteins. The extracellular region of different connexins is highly conserved, and few residues of each connexins are exposed. The search for new antibodies may develop an unprecedented potential for therapeutic applications, as it may benefit tremendously from novel whole-cell screening platforms that permit *in situ* selection of antibodies against membrane proteins in native state. The demonstrated efficacy of mAbs in reaching and modulating hemichannels *in vivo*, together with their relative specificity for connexins overlapping epitopes, should hopefully stimulate an interest for widening the scope of anti-hemichannel antibodies. There is no shortage of currently incurable diseases for which therapeutic intervention may benefit from anti-hemichannel antibodies capable of modulating hemichannel function selectively and specifically.

Keywords: antibody drug discovery; cancer; inflammation, rare diseases.

1. Connexins, connexons and intercellular gap junction channels

Twenty connexin genes are expressed both in humans (identified by a "*GJ*" prefix) and in mice (identified by a "*Gj*" prefix) (**Table 1**; note that two connexins, connexin 25 and 59, are human only, whereas two, connexin 23 and 33, are mouse only). All protein products of connexin genes are thought to share the same topology, with cytoplasmic N– and C–termini (NT, CT, respectively), four transmembrane domains (TM1-4) connected by two extracellular loops (referred to either as EL1 or EC1 and EL2 or EC2, respectively) and a cytoplasmic loop (CL) [1]. Connexin protomers are post–translationally oligomerized to form hexameric assemblies, termed connexons, prior to membrane insertion either within the endoplasmic reticulum or in the trans–Golgi network [2-6]. Each connexon delineates a pore

in the plasma membrane, and the coaxial arrangement of two docked connexons from adjacent plasma membranes establishes a communication pathway, termed intercellular gap junction channel (IGJC) between the cytoplasm of the two adjoined cells [7-12] (**Figure 1**). Connexons may be homomeric (composed of six identical connexin subunits) or heteromeric (composed of more than one species of connexins). IGJCs may be homotypic (if connexons are identical) or heterotypic (if the two connexons are different) [13]. IGJCs are thought to cluster into plaques via long-range protein aggregation driven from interparticle interactions as well as lateral pressures between the junction and the surrounding glycocalyx [14, 15].

2. Importance and role of connexin hemichannels

Unpaired connexons can establish a communication pathway between cell cytoplasm and the extracellular milieu by operating as regular plasma membrane channels, referred to as hemichannels (HCs), as detailed hereafter (see also refs. [16-20]). Thus, in a seminal article DeVries and Schwartz reported that a reduced extracellular concentration of ionized calcium ($[Ca^{2+}]_{ex}$) produced a current in solitary horizontal cells isolated from catfish retinas, which could be suppressed by dopamine. Lucifer Yellow CH (LY) entered solitary horizontal cells bathed in a medium containing a reduced $[Ca^{2+}]_{ex}$. Adding dopamine to the extracellular solution, or increasing the $[Ca^{2+}]_{ex}$, blocked the influx of the dye. The authors concluded that ions and dye flowed through "hemi-gap-junction channels expressed in the surface membrane" [21].

The study of connexin HCs activity in the cell plasma membrane gained momentum from a concurrent article by Paul *et al*. reporting that (a) Cx46 induced nonselective channels in the oolemma that were voltage dependent and opened by large depolarizations and (b) oocytes expressing Cx46 were permeable to LY but not FITC-labelled BSA, indicating the presence of selective membrane permeabilities [22]. Trexler *et al*. showed that HCs comprised of rat Cx46 exhibited complex voltage gating, had a unitary conductance of ~300 pS and induced substantial ion fluxes in cells expressing Cx46 [23]. Li *et al*. investigated the putative opening of Cx43 HCs in Novikoff, NRK, and HeLa cells by uptake of dye (5(6)-carboxyfluorescein, 7-hydroxycoumarin-3-carboxylic acid and fura-2) from the culture medium when $[Ca^{2+}]_{ex}$ was reduced, leading to "a hypothesis that a physical interaction [between Ca^{2+} and HCs] was involved in the process, instead of a metabolic mechanism" [24]. The pioneering studies mentioned above spurred the nowadays widespread use of dye uptake as a standard assay of HC function [25].

Years later, Muller *et al*. used atomic force microscopy (AFM) and revealed that the pore at the extracellular mouth of Cx26 HCs changed diameter from 13 ± 3 Å, in 0 mM $[Ca^{2+}]_{ex}$ to 5 ± 3 Å in 0.5 mM $[Ca^{2+}]_{ex}$ [26]. Thimm *et al.* reported a similar effect for Cx43 HCs [27]. Currently, a vast body of data currently supports the notion that HCs remain predominantly closed at negative membrane potential and/or in the presence of mM levels of extracellular divalent cations (Ca^{2+} and Mg^{2+}) [24, 28-32]. However, numerous factors modulate the open probability of HCs, including mechanical strain, changes in pH, transmembrane voltage, post translational modifications (and in particular protein phosphorylation) and cellular redox state, metabolic inhibition, nitric oxide (NO) and linoleic acid [33-42]. In particular (as mentioned above) lowering the $[Ca^{2+}]_{ex}$ favors the opening of HCs formed by several different types of Cxs [43]. In contrast, the open probability of HCs shows a more complex, bell-shaped dependence on cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) with a peak around 500 nM [44-46]. A pivotal role for calmodulin has been proposed also for this incompletely characterized, yet fundamental control mechanism [47, 48].

When open, HCs mediate the diffusive release of paracrine messengers, most importantly ATP [49-55], but also glutamate and prostaglandins, NAD(+) or glutathione [56]. Solute permeation through HCs has been scrutinized in numerous studies by experimental methods (see e.g. [20, 57-61]) as well as molecular dynamics simulations [62-67]. Based on

pore size estimates, the list of permeating molecules could potentially cover >35,000 members of the Human Metabolome Database [1] [68]. In several instances, molecules released through open HCs contribute to paracrine and autocrine signalling pathways during inflammation and cell death [69-72].

3. Anti-HC antibodies: an ongoing search with a remarkable pedigree

Polyclonal antibodies are generated by the immune response to an antigen involving the activation of multiple B-cells all of which target a specific epitope on that antigen; as a result, a large number of antibodies are produced with different specificities and epitope affinities [73]. As antibodies usually bind extracellular domains of proteins, the two extracellular loops of the connexin proteins (EL1/EC1 and EL2/EC2), which are exposed in unpaired HCs, became natural targets for the generation of a variety of antibodies (**Figure 2**).

The search started with Meyer *et al.*, who selected Novikoff hepatoma cells (that are readily dissociated without the use of proteases and, when reaggregated, form functional junctions over a time course of minutes [74]) to test (the first) affinity-purified rabbit polyclonal antibodies (EL-46, EL-186, Cx32-164-189) against a connexin 43 (Cx43) EL1 peptide (a.a. 46-76, GDEQSAFRCNTQQPGCENVCYDKSFPISHVR), a Cx43 EL2 peptide (a.a. 186-206, TCKRDPCPHQVDCFLSRPTEK) and a Cx32 EL2 peptide (a.a. 164-189, RLVKCDVYPCPNTVDCFVSRPTEKTV), respectively. Cells were dissociated in EDTA, allowed to recover and reaggregate for 60 min in media containing Fab fragments of the above mentioned antibodies, resulting in no cell-cell dye transfer 4 min after microinjection in 90% of the treated cell pairs (in control experiments, cell-cell dye transfer was detected within 30 s). In addition, no gap junctions were detected by freeze-fracture electron microscopy (EM) and no adherens junctions by thin section EM between cells treated with the Fab fragments that blocked cell-cell dye transfer [75].

Following suit, Hofer and Dermietzel immunized rabbits against a Cx43 EL1 peptide (aa 42–54: ESAWGDEQSAFRC) and a EL2 peptide (aa: 186–206, TCKRDPCPHQVDCFLSRPTEK), aiming to generate antibodies for labelling and blocking HCs in vital cells. Of the two retrieved polyclonal antibodies, respectively named aEL1-42 and aEL2-186, only the latter (which was not specific as it also recognized bands at positions 30 kDa and 32 kDA in western blots of brain homogenates) was retained for further analyses. Whole antibody as well as Fab_2 fragments of aEL2-186, applied to unpermeabilized cultured astrocytes, produced staining patterns that were most prominent in subconfluent cultures at membrane processes and filopodia, suggesting that HCs accumulate at the site of growing tips of noncontiguous cells. Incubation with either Fab_2 or complete EL2-186 antibodies led to a significant reduction of LY-labelled RGCN cells (an immortalized astrocytic cell line) in in 0 mM $[Ca^{2+}]_{ex}$, leading to the conclusion that (a) dye uptake operates through HCs in the plasma membrane and (b) HCs are accessible for blocking by external loop antibodies. In addition, LY as well as Ca^{2+} transfer between adjacent cultured astrocytes were significantly reduced after exposure to aEL2-186 for a time interval exceeding by a factor of four the Cx43 half-life measured by pulse-chase assays [76].

Together, the experiments performed by Hofer and Dermietzel and Meyer *et al.*, shaped the notion that, by interfering with the docking of unopposed connexons in adjacent cells, antibodies targeting the extracellular domain of connexins may act as cell adhesion inhibitors, eventually impeding the formation of new gap junction channels [77]. Then, Siller-Jackson *et al*. immunized rabbits against a Cx43 EL2 peptide (a.a. 185-206, YTCKRDPCPHQVDCFLSRPTEK) to generate yet another anti-Cx43 polyclonal antibody, referred to either as Cx43(E2) or Cx43-E2. HCs opening and associated release of prostaglandin E2 from MLO-Y4 cells (an osteocyte-like cell line) in response to fluid shear

[1] http://www.hmdb.ca/structures/search/metabolites/mass

stress were significantly inhibited by this antibody, which recognized the extracellular domain of Cx43, did not block pannexin or $P2X_7$ channels and also failed to detect other connexins including Cx32, Cx26, Cx50, and Cx46 (note, however, that data to support this latter claim were not shown). Scrape-loading LY transfer assays showed gap junction function was not affected by incubating MLO-Y4 cells with increasing concentrations (28, 56, and 100 μg/ml) of Cx43(E2) antibody for 4 h [78]. Subsequently, Kar *et al.* used the Cx43(E2) antibody to study H_2O_2-induced cell death of primary osteocytes and MLO-Y4 cells. There was a dose-dependent increase of HCs function by H_2O_2, and the antibody completely blocked dye uptake induced by H_2O_2, exacerbating further H_2O_2-induced cell death [79]. Zhou *et al.* showed that the opening of osteocyte Cx43 hemichannels mediated by mechanical stimulation caused the release of ATP, which exerted an inhibitory effect on breast cancer cells. This inhibition was attenuated by the Cx43(E2) antibody, suggesting that Cx43 hemichannels in osteoblast bone cells may provide an intrinsic self-defence mechanism against breast cancer metastasis [80]. Finally, Xu H. *et al.* studied mechanical sensing by osteocyte Cx43 by simulating unloading caused by weightlessness with a random position machine (RPM). Dye uptake assay showed that HCs opening increased after RPM for 2 h, leading to increased prostaglandin E2 release. This increase was blocked by the Cx43(E2) antibody [81]. Together, these studies highlighted the usefulness of the Cx43(E2) polyclonal antibody as a research tool in diverse contexts.

Based on the work of Meyer *et al*. [75] and Hofer and Dermietzel [76], Clair *et al.* selected an eleven amino-acid peptide in human connexin 26 (Cx26) EL1 (a.a. 42–53:VWGDEQADFVC) and another fourteen amino-acid peptide in EL2 (a.a. 172–184: AWPCPNTVDCFVSR) to immunize rabbits [82]. In HeLa cells expressing Cx26, connexin 32 (Cx32) or Cx43, staining with Connexin Extracellular Loop 1 Antibody (CELAb1, an antibody raised against the EL1 peptide) labelled surface structures putatively identified as HCs. A similar labelling was also obtained with Connexin Extracellular Loop 2 Antibody (CELAb2, an antibody raised against the EL2 peptide; note, however, that also in this case data to support this second claim were not shown). The lack of specificity exhibited by both polyclonal antibodies was explained based on a.a. sequence alignment for the ELs of Cx26, Cx32 and Cx43 (see also **Supplementary Table S1**) [82]. Anselmi *et al*. and Majumder *et al*. used CELAbs to label ATP-releasing Cx26 and Cx30 HCs on the endolymphatic surface of non-sensory cells in the mouse organ of Corti [54, 83].

When tested functionally in low $[Ca^{2+}]_{ex}$, both CELAbs inhibited equally well LY uptake in HeLa cells expressing Cx26 or Cx32, whereas inhibition was less efficient in cells expressing Cx43, suggesting unequal relationship between binding and blocking for these different connexin isoforms. No inhibition of dye transfer could be observed when monolayers of Cx26 expressing cells were microinjected with LY in the presence of CELAbs, indicating that the antibodies preferentially inhibited HCs but had little effects on connexin-mediated intercellular communication [82]. CELAbs demonstrated also the importance of HC signalling since they inhibited dye uptake at low $[Ca^{2+}]_{ex}$, and at physiological $[Ca^{2+}]_{ex}$ during invasion of intestinal epithelial cells by Shigella [82], the causative agent of bacillary dysentery [84, 85].

Summarizing, the experiments performed with Cx43(E2) and CELAbs showed targeting the extracellular domain of connexins with these polyclonal antibodies could label/inhibit HCs without interfering with IGJC function.

4. Addressing unmet medical needs with anti-HCs monoclonal antibodies (mAbs)

Although the body's natural reaction to antigens is inherently polyclonal, mAbs can bind an antigen with a high degree of affinity and specificity and, due to this remarkable property, "have made a striking transformation from scientific tools to powerful human therapeutics" [86]. Selection of mAbs from combinatorial phage-display libraries [87-91] is a

key technology that has earned the 2018 Nobel prize for Chemistry.[2] As unique protein product of well-defined DNA sequences, mAbs can be generated in numerous way by highly reproducible, scalable processes [92] that can meet the good manufacturing practice (GMP) standards required for pharmaceutical products [93]. The spectrum of potentiality of antibodies has been further expanded by the discovery of naturally occurring heavy chain antibodies (hcAbs, two polypeptide chains, 75 kD) containing a highly stable and soluble single antigen-binding V-domain designated VHH or nanobody (15 kDa). Compared with full length antibodies, nanobodies have a smaller size, higher solubility, higher stability, and increased capability of tissue penetration *in vivo* [94]. Together, these properties are leading mAbs to become the largest and fastest growing class of therapeutic proteins [95, 96]. A recent review by Caufriez *et al*. lists no fewer than 18 diseases linked to the opening of (connexin) HCs [97] (**Table 2**). In the following, we dwell in particular with two disease classes for which anti-HC mAbs with excellent therapeutic potential are available.

4.1 Cancer

Cx43 is a major gap junction protein in astrocytes, and its expression is enhanced significantly in glioma-associated astrocytes, especially at the peri-tumoral region, where it is thought to promote glioma invasion [98-101]. It mediates also gap-junctional coupling between collectively invading breast cancer cells and, via HCs, adenosine nucleotide/nucleoside release into the extracellular space [102]. Thus, autocrine purinergic signalling through Cx43 HC is a critical pathway in leader cell function and collective invasion [102].

Aiming to fight cancer, Baklaushev *et al.* generated a mAb targeting an epitope in Cx43 EL2 (aa 173-208, QWYIYGFSLSAVYTCKRDPCPHQVDCFLSRPTEKTI) and used it to label extracellular structures on GFAP-positive reactive astrocytes in brain sections of a rat with experimental C6 glioma [103]. The antibody was subsequently referred to as MAbE2Cx43, and shown to accumulate *in vivo* at the peritumoural invasion zone after conjugation either with radio-isotope or a fluorescent dye [104]. Deceleration of glioma growth in rats receiving intravenous administration of MabE2Cx43 was accompanied by a significant prolongation of lifespan and complete recovery without delayed relapses in ~20% of the animals, suggesting that "the mechanism of tumour-suppressing effects of antibodies can be related to inhibition of specific functions of Cx43 in glioma cells in the peritumoural zone" [105]. Forty-eight hours after *in vivo* delivery of PEGylated immunoliposomes based on MabE2Cx43 antibodies, fluorescent-labeled liposomal nanocontainers were detected at the periphery of the glioma [106]. Treatment with MabE2Cx43 in combination with radiotherapy was associated with mutual boosting of the therapeutic efficiencies, leading to a significant inhibition of tumour development and prolongation of rat survival median to 60 days, "presumably due to the increase in blood-brain barrier permeability for antibodies after irradiation of the brain and to additional inhibitory effect of antibodies towards radioresistant migrating glioma cells" [107]. Intravenous administration of cisplatin-loaded nanogels conjugated with MabE2Cx43 in the experimental model of glioma 101/8, ensued in a 27 day median extension the rat lifespan [108]. Together, these studies (a) convincingly demonstrated that antibodies against the extracellular domain of Cx43 can be used to directly limit glioma growth, as well as for targeted transport of diagnostic and therapeutic drugs; (b) support to the notion that cancer phenotype can be altered as a direct consequence of experimental targeting of connexins (reviewed in refs. [101, 109]).

In this vein, two human-mouse chimeric mAbs, named HMAb1 and HMAb2, that bind to Cx43 HCs, were generated by Jiang *et al.* according to Patent Application Publication No. US 2019/0359696 A1 (pub. Date: Nov. 28, 2019) [110]. Complementarity determining

[2] https://www.nobelprize.org/prizes/chemistry/2018/winter/facts/

regions (CDRs) of these mAbs are provided in Table 1 and Table 2 on page 14 of the patent document. HMAb1, a Cx43 HC inhibitor, falls in line with the other antibodies described above. In contrast, HM2Ab2 was found to *activate* Cx43 HCs. In addition, HM2Ab2 inhibited osteolytic tumour growth, at odds with studies of Cx43 role in glioma (as discussed above), but in accord with the finding that osteolytic tumour growth was augmented in osteocyte-specific Cx43 knockout mice (as reported in the patent). Of note, the patent claims HMAb1 and HMAb2 can be used for treating or preventing not only breast cancer, prostate cancer, osteosarcoma or a metastasis, but also osteoporosis, osteopenia, or, osteoarthritis, multiple sclerosis, Alzheimer's disease or Parkinson's disease, spinal cord injury (SCI), traumatic brain injury (TBI), or stroke [110].

Involvement of Cx43 in these disabling human pathologies has been reviewed extensively [72, 101, 109, 111-116]. Future will tell whether HMAb1 and HMAb2 are indeed endowed with such "magic bullet" properties. Clearly, therapeutic efficacy in just one of these unmet medical needs would be a major step forward for mankind.

As a closing remark for this section, we note that recent results have widened significantly the therapeutic potential of gap junctions. In particular, Gadok *et al*. engineered cell-derived drug-loaded lipid vesicles termed "connectosomes", containing functional HCs and a displaying a single-domain camelid antibody on the surface. Newly formed IGJC between vesicle and target cells expressing a surface antigen recognized by the antibody, promoted the selective delivery of the chemotherapeutic doxorubicin to a target cell population. Based on the outcome of these experiments, the authors suggested that this innovative targeted targeting strategy could be used to deliver a range of membrane impermeable drugs and reagents, such as siRNA peptides, and other macromolecules, to diverse populations of target cells [117].

4.2 Monogenic diseases

At least 29 dominant mutations in 3 human Cx genes (*GJA1, GJA3, GJA5, GJA5, GJA8, GJB1, GJB2, GJB3, GJB6*) generate HCs with augmented activity that have been implicated in 11 monogenic diseases (**Table 3** and **Figure 3**), mostly rare**,** affecting facial appearance, dentition (small and carious teeth), eyes (microphthalmia, microcornea), fingers (syndactyly), inner ear (deafness), skin (various disorders), lens (cataracts), nervous system (myelination defects) and heart (atrial fibrillation). In several instances, aberrant connexin HCs cause an unbalance of Ca^{2+} signalling and/or augmented release of ATP which, acting as deregulated paracrine factor, compromises tissue homeostasis [118-121]. A limited number of mouse models, which carry some of these mutations, have been generated by different laboratories using different targeting strategies and genetic backgrounds (**Table 3**).

Aiming to address unmet medical need in the field of monogenic diseases, Xu L. *et al.* used a bait peptide, named pepEC1.1 (biotin-KEVWGDEQADFVCNTL, corresponding to a.a. 41-56 of Cx26 EL1), to select human antibody fragments by "panning" a vast library expressed in phage. The selected fragments comprised a heavy chain variable domain (V_H) and a light chain variable domain (V_L) connected by a 7 a.a. flexible spacer to create a single-chain fragment variable (scFv) complex, whose structure was solved by X-ray crystallography (Protein Data Bank accession code 5WYM). The gene encoding the pepEC1.1-binding scFv complex, preceded by 60 nucleotides encoding a self-cleaving interleukin 2 secretion signal peptide [122], was inserted into a cloning plasmid to generate a scFv-Fc fusion protein, comprising constant hinge, CH_2 and CH_3 domains of human secreted IgG1 (UniProtKB – P01857). A chimeric version with constant hinge, CH_2 and CH_3 domains of mouse secreted IgG1 (UniProtKB – P01868) was also generated. A FreeStyle™ 293-F cell line was stably transfected with the expression vector of either scFv-Fc polypeptide. Both protein products harvested from the cell culture supernatant formed homodimers (named abEC1.1 and abEC1.1m, respectively) with a MW of ~103 kDa through a diabody interaction

between V_H and V_L [123] and disulfide bonds in the hinge region [124]. In low extracellular Ca^{2+} conditions (0.2 mM), patch clamp recordings from HeLa cells expressing Cx26 HCs yielded half maximal inhibitory concentration (IC_{50}) of ~40 nM for abEC1.1. The inhibitory effect was incomplete, leaving a residual conductance of ~18 at saturating concentrations (< 1 μM). The dose-inhibition response curve vs. abEC1.1 concentration was fitted with a modified Hill function $y= \alpha\ [1+(x/\gamma)^n]^{-1} + \beta$, where x is antibody concentration (in nM), α = 0.832, $\beta = (1-\alpha) = 0.168$, γ = 36.926 nM and $n = 2$, suggesting inhibition required binding of two abEC1.1 homodimers to a single HC. Experiments with intact epithelia in organotypic cochlear cultures, showed abEC1.1 did not interfere with IGJC function [125].

Then, Ziraldo *et al*. demonstrated abEC1.1 inhibited equally well Cx26, Cx30 and Cx32 HCs, when applied at < 1 μM concentration, but was ineffective on pannexin 1 channels [126]. By performing *in silico* analysis of HC-abEC1.1 interaction, critical amino acids (N54, T55, L56, Q57, P58, P175 and N176) that are conserved in the extracellular domain of Cx26, Cx30 and Cx32 were identified. Remarkably, even a single a.a. difference in the above a.a. list reduced drastically the inhibitory effects of abEC1.1 on all other tested Cx HCs (Cx30.2/31.3, Cx30.3, Cx31, Cx31.1, Cx37, Cx43 and Cx45) [126], most of which are expressed in the skin and its appendages [127-132].

Finally, Kuang *et al.* used abEC1.1 and abEC1.1m to treat $Cx30^{A88V/A88V}$ adult mutant mice, the only available animal model of Clouston syndrome (OMIM #129500), a rare orphan disease caused by Cx30 p.A88V leaky HCs (**Table 3**). Two weeks of antibody treatment sufficed to repress cell hyperproliferation in skin and reduce hypertrophic sebaceous glands (SGs) to wild type (wt) levels. These effects were obtained whether mutant mice were treated topically, by application of an abEC1.1 cream formulation, or systemically, by intraperitoneal injection of abEC1.1m. *In vitro* experiments with mouse primary keratinocytes and HaCaT cells revealed abEC1.1 blocked Ca^{2+} influx and diminished ATP release through leaky Cx30 p.A88V HCs, suggesting these scFv-Fc mAbs gained control over leaky HCs and contributed to restoring epidermal homeostasis [133]; see also **Figure 4**.

The phenotype of $Cx30^{A88V/A88V}$ mutant mice does not overlap perfectly with that human carriers of the Cx30 p.A88V mutation [134-138]. Nonetheless, these mice exhibit hyperproliferation in the skin and its appendages [139], a feature shared by most Cx-related genodermatoses [119, 132, 140, 141]. Also $Cx26^{G45E/+}$ mice, a model of keratitis-ichthyosis-deafness (KID) syndrome (OMIM #148210), have hyperkeratosis, scaling, skin folds, hyperplasia, acanthosis, papillomatosis and increased cell size, as well as increased HC currents in transgenic keratinocytes [142]. In addition, $Cx26^{S17F/+}$ mice, another model for KID syndrome [143], show altered epidermal lipid processing and Ca^{2+} distribution. Xu *et al*. proved abEC1.1 was effective *in vitro* against Cx26 p.G45E and p.D50N KID-related hyperactive HCs. In addition, at least three Cx32 mutations (S85C, D178Y, F235C) causing *Charcot-Marie-Tooth disease, X-linked dominant 1* (CMT1X, OMIM #302800), a demyelinating peripheral neuropathy, produce HCs with augmented activity (**Table 3**) and, possibly, deregulated ATP release [144]. The results summarized above suggest abEC1.1 might be effective in both contexts.

5. Concluding remarks

All known antibodies that modulate connexin HCs target the two small extracellular loops of the connexin proteins. Recent advances are paving the way to engineering stable antibodies against intracellular epitopes [145], however such antibodies would unlikely be able to discriminate between fully formed junctional channels and undocked HCs, and thus may produce unwanted side effects. The extracellular region of different connexins is highly conserved, especially the first extracellular loop (**Figure 2A** and **Supplementary Table S1**). This, combined with the fact that only few residues of each connexins are exposed (**Figure 5**), suggests that it will be difficult to generate an antibody that specifically recognizes (and

selectively modulates the function of) a given homomeric HC. Based on the literature reviewed in this article, designing/finding an antibody for each Cx subfamily appears a more feasible task.

The molecular mechanism of action of the antibodies and the HCs is unknown in most of the cases. A model based on molecular dynamics simulation suggests abEC1.1 sterically obstructs the HC preventing the passage of most ions and possibly all other molecules [125, 126]. The same model also shows that a VH-VL pair is large enough to recognize the extracellular region of three connexins at the same time, implying that each HC can interact with two different VH-VL pair. It is reasonable to think that other antibody HC blockers share the same molecular mechanism and stoichiometry of abEC1.1, however the existence of activating antibodies such as HM2Ab2 suggest different binding modes are also possible.

Most of the antibody-development to date efforts have targeted Cx43 (**Table 4**; the only antibody explicitly developed for other HCs is abEC1.1). However, Cx46 is also important as a glioblastoma cancer stem cell regulator that is required for stem cell maintenance [146]. Furthermore, Cx46 is contained in extracellular vesicles (EVs) released from breast cancer cells overexpressing Cx46 (EVs-Cx46) that facilitate the interaction between EVs and the recipient cell, resulting in an increase in their migration and invasion properties [147]. Combinatorial antibody libraries with their huge amount of affinity binders [148] coupled with phage display technology [87] are rich sources of modulators for cell surface receptors or membrane channels. The search for new antibodies may develop and unprecedented potential for therapeutic applications, as it may benefit tremendously from novel whole-cell screening platforms that permit *in situ* selection of antibodies against membrane proteins in native state [149, 150]. The demonstrated efficacy of mAbs in reaching and modulating HCs *in vivo*, together with their relative specificity for connexins overlapping EL epitopes, should hopefully stimulate an interest for widening the scope of anti-HC mAbs.

Summarizing, there is no shortage of currently incurable diseases linked to the opening of HCs [97] (**Table 2**). For all of them, therapeutic intervention may in principle benefit from anti-HC mAbs capable of modulating HC function selectively and specifically. Monoclonal antibodies are particularly promising since they have the potential to overcome the lack of specificity of small molecule alternatives and have superior *in vivo* stability compared with connexin mimetic peptides, some of which also are effective modulators of HC function [151].

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Author Contributions

Conceptualization, F.M., F.Z.; Funding acquisition, F.M., F.Z.; Project administration, F.M., F.Z.; Resources, F.Z.; Investigation, D.B., V.D.; Visualization, D.B., V.D.; Writing – original draft, F.M.; Writing – review & editing, D.B., V.D., F.Z., F.M..

Declaration of Competing Interests

Drs. F. Zonta and F. Mammano report a patent: “Fully human antibody specifically inhibiting connexin 26”, Inventors: Qu Z, Yang G, Mammano F, Zonta F, International application number: PCT/CN2016/109847, pending to ShanghaiTech University; and a patent: “Composition and Methods to treat Ectodermal Dysplasia 2, Clouston Type”, Inventors: Mammano F, Yang G, Zonta F, International Application No.: PCT/CN2019/088689, International Filing Date: 2019-05-28, pending to ShanghaiTech University. All other Authors have nothing to declare.

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Table 1 – connexin genes and proteins

	Homo sapiens				***Mus musculus***			
#	**Gene symbol**	**Protein name**	**OMIM ID**	**NCBI CCDS database ID**	**Gene symbol**	**Protein name**	**MGI ID**	**NCBI CCDS database ID**
1	*GJA1*	CX43	121014	CCDS5125.1	*Gja1*	Cx43	95713	CCDS23851.1
2	*GJA3*	CX46	121015	CCDS9289.1	*Gja3*	Cx46	95714	CCDS27152.1
3	*GJA4*	CX37	121012	CCDS30669.1	*Gja4*	Cx37	95715	CCDS18667.1
4	*GJA5*	CX40	121013	CCDS929.1	*Gja5*	Cx40	95716	CCDS17652.1
5	–	–	–	–	*Gja6*	Cx33	95717	CCDS30506.1
6	*GJA8*	CX50	600897	CCDS30834.1	*Gja8*	Cx50	99953	CCDS17651.1
7	*GJA9*	CX59	611923	CCDS432.1	–	–	–	–
8	*GJA10*	CX62	611924	CCDS5025.1	*Gja10*	Cx57	1339969	CCDS18015.1
9	*GJB1*	CX32	304040	CCDS14408.1	*Gjb1*	Cx32	95719	CCDS30314.1
10	*GJB2*	CX26	121011	CCDS9290.1	*Gjb2*	Cx26	95720	CCDS27153.1
11	*GJB3*	CX31	603324	CCDS384.1	*Gjb3*	Cx31	95721	CCDS18668.1
12	*GJB4*	CX30.3	605425	CCDS383.1	*Gjb4*	Cx30.3	95722	CCDS18669.1

13	*GJB5*	CX31.1	604493	CCDS382.1	*Gjb5*	Cx31.1	95723	CCDS18670.1
14	*GJB6*	CX30	604418	CCDS9291.1	*Gjb6*	Cx30	107588	CCDS27154.1
15	*GJB7*	CX25	611921	CCDS5008.1	–	–	–	–
16	*GJC1*	CX45	608655	CCDS11487.1	*Gjc1*	Cx45	95718	CCDS25504.1
17	*GJC2*	CX47	608803	CCDS1569.1	*Gjc2*	Cx47	2153060	CCDS24760.1
18	*GJC3*	CX29/30.2/31.3	611925	CCDS34697.1	*Gjc3*	Cx29	2153041	CCDS19782.1
19	*GJD2*	CX36	607058	CCDS10040.1	*Gjd2*	Cx36	1334209	CCDS16563.1
20	*GJD3*	CX31.9	607425	CCDS58547.1	*Gjd3*	Cx30.2	2384150	CCDS25369.1
21	*GJD4*	CX40.1	611922	CCDS7191.1	*Gjd4*	Cx39	2444990	CCDS29049.1
22	–	–	–	–	*Gje1*	Cx23	1923993	CCDS48505.1

Note: 40 unique genes (20 human + 20 mouse) in total. Click on the above CCDS links to access nucleotide and amino acid sequences.

Table 2 – Human diseases associated with the opening of wild type connexin HC

Disease	**Connexin**	**Reference**
Inflammation	Cx43	[152-154]
Atherosclerosis	Cx37/Cx43	[155, 156]
Diabetes	Cx43	[157, 158]
Ischemia/stroke	Cx43	[159, 160]
Myocardial ischemia/reperfusion	Cx43	[161]
Cardiomyopathy	Cx43/Cx45	[162]
Chronic kidney disease	Cx43	[163]
Microbial gut infection	Cx43	[164]
HIV infection of astrocytes	Cx43	[165]
Acute liver failure	Cx32/Cx43	[166]
Liver fibrosis and cirrhosis	Cx43	[167]
Nonalcoholic steatohepathitis	Cx32/Cx43	[168]
Alzheimer's disease	Cx43	[169]
Epilepsy	Cx36/Cx43	[170, 171]

Table 3 – Human connexin variants potentially targeted by inhibitory anti-HCs antibodies, and corresponding mouse models of disease

Affected organ(s)/tiss	**Type of disease / OMIM #**	**Gene**	**Cx**	**Mutation**	**Reference /Mouse model** (if
Various organs	Oculodentodigital dysplasia (ODDD) /	*GJA1*	Cx43	G138R	[172] / MGI:3776013

	#164200			I31M	EM:02386 (Note 1)
				G143S	
				G60S (Note 2)	[173]/ MGI:2682016
Heart	Atrial fibrillation, familial, 11 (ATFB11) / #614049	*GJA5*	Cx40	G38D	[174]
				V85I	[175, 176]
				L221I	
Skin *:*	Palmoplantar keratoderma and congenital alopecia 1 (PPKCA1)	*GJA 1*	CX43	G8V	[177, 178]
	Erythrokeratodermia variabilis et progressiva (EKVP3) / #617525			A44V (Note 3)	[178-180]
				E227D (Note 3)	[178, 180]
	Erythrokeratodermia variabilis et progressiva 1 / #133200	*GJB 3*	Cx31	R42P (Note 4)	[181]
	Ectodermal dysplasia 2, Clouston type / #129500	*GJB 6*	Cx30	G11R	[135] MGI:5607781
				A88V	[135] MGI:5607781 EM:07626)
Inner ear and skin	Keratitis-ichthyosis-deafness (KID) syndrome / #148210	*GJB 2*	Cx26	G12R	[182]
				N14K	
				D50N	
				I30N	[183]
				D50Y	
				N14Y	[184]
				S17F	[143, 185, 186] / MGI:4867482 EM:05215
				A40V	[187]
				G45E	[188, 189] / MGI:5635524
				D50A	[190]
				A88V	
Lens	Cataract 14, multiple types (CTRCT14) / #601885	*GJA 3*	Cx46	G143R	[191]

	Cataract 1, multiple types (CRTCT1) / #116200	*GJA8*	Cx50	G46V	[192]
Nervous system	Charcot–Marie–Tooth neuropathy, X-linked dominant 1 (CMTX1) / #302800	*GJB1*	Cx32	S85C	[193]
				D178Y	[32]
				F235C	[194]

Note 1: MGI, Mouse Genome Informatics; EM, INFRAFRONTIER/European Mouse Mutant archive.

Note 2: G60S is not listed among human variants of Cx43 (*GJA1*) in the UniProt database, https://www.uniprot.org/uniprot/P17302; see also ref. [195]. It is, however, a mouse model for Oculodentodigital dysplasia.

Note 3: In tissue sections of human affected skin, the E227D and A44V mutants of Cx43 (*GJA1*) do not localize to intercellular junctions and accumulate in a subcellular compartment. In addition, cells transfected with either mutant demonstrate aggregation of Cx43 in the Golgi [180].

Note 4: According to Tattersall *et al*. "Previous studies suggest that Cx-associated cell death may be related to abnormal 'leaky' HCs but we produced direct evidence against that being the major mechanism. Additionally, our immunocytochemistry showed upregulation of components of the unfolded protein response (UPR) in cells expressing the EKV-associated Cx31 mutants but not (WT)Cx31 or (66delD)Cx31" [196].

Table 4 – anti-HCs antibodies mentioned in this article

Name	Type	Target	Organism	Ref.	Major properties
EL-46, EL-186, Cx32-164-189	Polyclonal	Cx43 and/or Cx32	Rabbit	[75]	Blocked cell-cell dye transfer and prevent formation of gap-junction channels
aEL2-186	Polyclonal	Cx43	Rabbit	[76]	Significantly reduced Ca^{2+} and LY transfer through IGJCs
Cx43-E2	Polyclonal	Cx43	Rabbit	[78-81]	Significantly inhibited HC opening
CELAb1, CELAb2	Polyclonal	Cx26, Cx32, Cx43	Rabbit	[54, 82, 83]	Efficiently inhibited LY uptake of Cx26 and Cx32 HCs, less efficient for Cx43 HCs
MAbE2Cx43	Monoclonal	Cx43	Mouse	[103-108]	Effective *in vivo* in animal models of glioma.
HMAb1	Monoclonal	Cx43	Humanized	[110]	HC inhibitor

HMAb2	Monoclonal	Cx43	Humanized	[110]	HC activator
abEC1.1	Monoclonal	Cx26, Cx30, Cx32	Human	[125, 126, 133]	Efficiently inhibited Cx26, Cx30 and Cx32 HCs. Did not interfere with fully formed gap junction channels in organotypic cochlear cultures. Effective *in vivo* in Cx30A88V mutant mice.

Figure captions

Figure 1. Structure of gap junction channels. Gap junction channels arrange in a hexagonal pattern to form a junctional plaque. A) Side view. Two connexins have been removed from one of the channels depicted in panel A. The connexin in the internal part of the pore has been coloured with brighter colours to promote visual perception of depth. B) View from the cytoplasm of one of the two cells (adapted from ref. [1]).

Figure 2. Conservation of Extracellular loops in connexin family. A) Sequence alignment (top) and sequence logo (bottom) of the first and second extracellular loop of the human connexin family. Amino acids are coloured according to the "Rasmol color scheme". B) Position of the alpha carbons of the residues that are conserved in at least 90% of the family (numeration is based on position in the Cx26 sequence). The two sequences are largely conserved in the family, especially the three cysteine pairs that create disulfide bridges between the two loops, and the prolines responsible for the sharp apical turns.

Figure 3. Point mutations associated with hyperactive connexin HCs). Two opposing connexin of a hemichannel are shown. Mutation leading to natural variant associated with hyperactive hemichannels are represented by spheres in either of the two represented connexin protein. The position of a given residue in the protein was determined using Cx26 as a template, and taking into account the sequence alignment of **Figure 2** and **Supplementary Table S1**. The cytoplasmic loop and the C-terminus are represented as dotted lines, due to the large differences in length and sequence of these two regions across the connexin family. Amino acids are coloured according their topology in the protein (NT: red, TM1: orange, EC1: yellow, TM2: green, ICL: tan, TM3: purple, EC2: blue; TM4: cyan, CT: pink).

Figure 4. Schematic representation of putative antibody mechanism of action. The abEC1.1 antibody (inset) comprises a Cx-binding scFv complex (VH-linker-VL) fused to a fragment constant (Fc) composed of hinge (h), CH2 and CH3 domains of secreted IgG1. Two scFv-Fc homodimers bind the outer vestibule of the connexin hemichannel (CxHC) blocking ATP release and Ca^{2+} influx. The ATP released by open/unblocked CxHCs diffuses in the extracellular milieu and activates G-protein coupled P2Y receptors (P2YR), triggering a canonical signal transduction cascade that leads to Ca^{2+} release from the endoplasmic reticulum via phospholipase C (PLC), PIP2 (not shown) and IP3. At the same time, Ca^{2+} flows into the cell through ATP-gated P2X ionotropic receptors (P2XR) and open/unblocked

CxHCs. Diffusion of Ca^{2+} in the nucleus promotes transition of chromatin structure from fibrous to globular, chromosome condensation, mitosis and cell proliferation (adapted from ref. [133]).

Figure 5. Plausible epitopes for antibody recognition. Side view (A) and top view (B) of a HC embedded in the plasma membrane. A single connexin protein in shown in cartoon representation; the rest of the HC (blue) and the plasma membrane (carbon atoms in cyan, oxygen atoms in red, phosphate atoms in blue) are represented in terms of volume occupancy. Segments of the extracellular loops that are accessible and can be recognized by an antibody are shown as pink spheres corresponding to the position of their alpha carbon in (A) and (B). They comprise two short fragments in EC1 (corresponding to residues 54 – 59 in Cx26) and the second half of EC2 (corresponding to resides 166 – 178 in Cx26). Every other part of the protein is masked either by the plasma membrane or by adjacent connexins. The segments of the EC loops facing the pore are as likely to be inaccessible to an antibody, due to steric hindrance. C) and D) show how the putative accessible residues are arranged in the whole HC (side and top view respectively. Despite being two short fragment in a single connexin, they produce a large surface once the proteins are arranged in the hexamer. E) shows the putative mode of interaction of two abEC1.1 antibodies, each one of which is a scFv-Fc homodimer, with the extracellular domain of the HC. In particular, yellow and purple molecules are coupled in one diabody, while green and orange in the second one. Connexon and membrane are shown with the same color code of panel A and B. Panel (F) is a top view of the antibody-hemichannel complex showing the complete obstruction of the channel pore (the membrane is not show for clarity).

Declaration of competing interests

☐ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☒The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Drs. F. Zonta and F. Mammano report a patent: "Fully human antibody specifically inhibiting connexin 26", Inventors: Qu Z, Yang G, Mammano F, Zonta F, International application number: PCT/CN2016/109847, pending to ShanghaiTech University; and a patent: "Composition and Methods to treat Ectodermal Dysplasia 2, Clouston Type", Inventors: Mammano F, Yang G, Zonta F, International Application No.: PCT/CN2019/088689, International Filing Date: 2019-05-28, pending to ShanghaiTech University. All other Authors have nothing to declare.

Highlights

- Connexin hemichannels are implicated in several human diseases
- It is possible to develop antibodies specifically modulating connexin hemichannels
- The efficacy of anti connexin hemichannel antibodies has been proven in mice