Intercellular communication in the heart – therapeutic opportunities for cardiac ischemia

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Abstract

The maintenance of tissue, organ and organism homeostasis relies on an intricate network of players and mechanisms that assist in the different forms of cell-cell communication. Myocardial infarction, following heart ischemia and reperfusion is associated with profound changes in key processes of intercellular communication, involving gap junctions, extracellular vesicles and tunneling nanotubes, some of which have been implicated in communication defects associated with cardiac injury, namely arrhythmogenesis and progression into heart failure. Therefore, intercellular communication players emerged as attractive powerful therapeutic targets aiming at preserving a fine-tuned crosstalk between the different cardiac cells in order to prevent or repair some of harmful consequences of heart ischemia and reperfusion, reestablishing myocardial function.

Keywords: intercellular communication, acute myocardial infarction, gap junctions, extracellular vesicles, remote ischemic conditioning, cardioprotection

Intercellular communication: general concepts

A fine-tuned communication between the various cell types that compose the heart is vital for the maintenance of myocardial homeostasis. The human heart is a highly specialized tissue containing billions of individual cardiomyocytes, whose contractile activity is supported by fibroblasts, endothelial and smooth muscle cells (SMCs), immune cells, sympathetic and parasympathetic neurons [1,2]. Intercellular communication in the heart can either occur directly, by the establishment of cell-cell contacts, including gap junctions (GJs) and tunneling nanotubes, or at longer distances involving the release of soluble chemokines, cytokines, growth factors and vesicle-enclosed mediators (**Key Figure**) [2].

In stress conditions, such as acute myocardial infarction (AMI), an orchestrated crosstalk between cardiac cells assumes particular importance to sustain efficient responses in wound healing and extracellular matrix remodeling [3]. AMI is a common clinical presentation of ischemic heart disease, mostly secondary to **atherothrombotic coronary artery disease** (see Glossary) that restricts the supply of oxygen and nutrients to the tissue, ultimately resulting in cell death [4]. In clinical practice, blood flow can be reestablished following primary percutaneous coronary intervention (PCI), which is an important therapeutic strategy aiming to reduce infarct size and preserve heart function, but that often induces additional damage to cardiomyocytes due to oxidative stress, calcium overload and inflammation (so-called myocardial reperfusion injury) [4]. Novel exciting evidence have revealed intercellular communication as a druggable target, both in

cell-based therapies, to enable successful cell integration and coupling to a new environment, and in non-cellular approaches mediated by paracrine factors, such as extracellular vesicles (**BOX 1**), to improve endogenous recovery. In this review, we provide a comprehensive and critical overview of the communication strategies used by cardiac cells under physiological and pathological conditions, discussing novel therapeutic approaches for targeting cell-cell communication in AMI.

Intercellular communication mediated by connexin channels in the heart

The importance of gap junctions for cardiac function

GJ-mediated intercellular communication (GJIC) constitutes the most direct pathway to synchronize responses in multicellular organisms, participating both in rapid physiological processes, such as action potential propagation, and slower processes, including development and differentiation (reviewed in [2,5]). GJs are formed after docking of two hexameric structures, called hemichannels, composed of transmembrane proteins called connexins (Cx). GJs can be densely packed into large plaques containing up to thousands of channels, which connect the cytoplasm of neighboring cells, allowing the transfer of small molecules, including ions, metabolites, secondary messengers, microRNAs (miRNAs) and linear peptides [2]. GJIC can be regulated at different levels, including connexin synthesis, trafficking and degradation, as well as channel composition and gating, with connexin **post-translational modifications** (PTMs) playing a vital regulatory role.

Of the 21 connexin genes encoded in the human genome, three main isoforms are expressed in the adult heart: Cx40, Cx43 and Cx45, with distinct regional expression patterns, where they contribute to maintain the electrophysiological properties of different myocardial regions (reviewed in [6]). Cx43 (also known as GJA1) is the predominant isoform in the heart, being expressed in the atria, ventricles and ventricular conduction system [6]. Cx43 is mainly localized at the **intercalated discs** (IDs)**,** where GJs ensure the rapid anisotropic impulse propagation underlying synchronized heart beating [2] (see Clinician's Corner). Evidence has furthermore pointed to an active role for Cx43 in cardiac conduction via non-**electrotonic mechanisms.** First regarded as a compensatory mechanism to dysfunctional GJIC, **ephaptic transmission** emerged as a crucial part of a "mixed mode" conduction mechanism in the heart [7]. Accordingly, the presence of Cx43 implicates the **perinexus** in ephaptic conduction, where the membranes of adjacent cardiomyocytes form a narrow gap (<30 nm), with a local enrichment in electrical and mechanical junction proteins, including voltage-gated Na^+ channels (Na_v) [8,9]. Importantly, Cx43

is required for proper localization of $Na_v1.5$ channels, contributing to modulate sodium current density at the IDs [10].

The importance of GJIC in the heart is not restricted to cardiomyocytes. In the vasculature, GJ coupling mediated mainly by Cx37, Cx40, Cx43 and Cx45 regulates endothelial stiffness, vasomotor tone and arterial blood pressure [11]. In contrast with the well-established roles of GJs in **homocellular interactions**, their contribution and relevance to heterocellular contacts remains elusive. Fibroblast-cardiomyocyte GJs ensure cardiomyocyte electrical synchrony [12], while Cx43-GJs between cardiac macrophages and cardiomyocytes contribute to electric impulse propagation through the distal atrioventricular node [13]. More recently, GJ-mediated electro-metabolic signaling between ventricular myocytes and microvascular endothelial cells was demonstrated, likely regulating coronary arterial blood flow [14].

The role of connexin-based hemichannels in cardiomyocytes

Besides GJs, recent findings suggest that undocked hemichannels (HCs) play a role under disease conditions. Forward trafficking creates a continuous stream of sarcolemmal HCs underway to the IDs, accumulating in the perinexus before becoming incorporated into GJ plaques [5,15]. Sarcolemmal undocked HCs are normally closed and are supposed to only open upon GJ formation. Accumulating evidence suggests that Cx43-based HCs open in response to several conditions including metabolic inhibition, ischemia [16,17], arrhythmogenic cardiomyopathy in plakophilin-2 deficient mice [18] and Duchenne Muscular Dystrophy mice [19]. Cx43 HC opening can be activated by electrical stimulation to positive membrane potentials (≥+40 mV) [20], or by chemical activation of ryanodine receptors (RyR) at negative diastolic potentials [21]. Given their high single-channel conductance (~220 pS), Cx43 HCs-mediated passage of ions, like Na^+, K^+ and Ca^{2+} may contribute to pathologic signaling, through increased sarcolemmal electrical current, excess ionic flow, osmotic disturbances and increased Ca^{2+} entry [18,22].

The impact of ischemia and reperfusion on gap junction intercellular communication

Electrical uncoupling and arrhythmogenesis at the onset of ischemia are associated with perturbed GJIC between cardiomyocytes. Intracellular acidosis and Cx43 PTMs impact on HC opening and GJ closure, with concomitant removal of Cx43 from the IDs that can be redistributed to the lateral sarcolemma or targeted for degradation (**BOX 2**) [2,5]. Besides its importance for **lymphangiogenesis**, Cx43-mediated intercellular communication was recently reported to improve cardiac lymphatic function, reducing **interstitial edema** and preserving cardiac function after acute myocardial infarction (AMI) [23].

Paradoxically, GJ-mediated metabolic coupling is often associated with the propagation of harmful metabolites, contributing to extend the myocardium injured area [2,5]. Accordingly, restriction of GJIC in myocardial ischemia/reperfusion injury (IRI) models reduces infarct size, likely by limiting cell-to-cell spread of necrosis [24]. In addition, Cx43 HC-dependent ATP release [22] and opening of Cx43-HCs in subsarcolemmal mitochondria may worsen IRI [25]. On the other hand, opening of mitochondrial Cx43-HCs has also been implicated in **ischemic preconditioning** (IPC)-mediated cardioprotection [26].

In contrast with transient and reversible channel activity changes, long-term level of GJIC control involves Cx43 degradation. During AMI, Cx43 is "tagged" with ubiquitin, signaling its internalization and lysosomal degradation in cardiomyocytes, which relies on AMPK-mediated autophagy in early periods of ischemia and Beclin-1 during reperfusion [27]. Increased Cx43 degradation was also observed in the absence of the internally translated Cx43-20 kDa (Cx43-20k) isoform, correlating with abnormal cardiac electrical excitation [28]. Furthermore, loss of function of the **long non-coding RNA** (lncRNA) CCRR was implicated in dysfunctional **endocytosis** and Cx43 degradation, contributing to arrhythmogenesis in failing hearts [29].

The impact of ischemia on post-transcriptional control of Cx43 is an emerging concept. In accordance, overexpression of miRNA-1 correlates with repressed *GJA1* translation and arrhythmogenesis [30], while high levels of the RNA-binding protein CUGBP Elav-like family member (CELF)-1 cause *GJA1* mRNA degradation, impacting cardiac contractile dysfunction in a pre-clinical model of myocardial infarction [31].

Besides cardiomyocytes, other cardiac cell types may be differentially affected by ischemia and contribute to post-infarct remodeling. In agreement, upregulation of Cx43 in ischemic cardiac fibroblasts, results in enhanced GJ electrical coupling with cardiomyocytes across the scar, associated with a high incidence of arrhythmias [32,33]. Within **myoendothelial junctions**, the cardioprotective role of hypoxic preconditioning involves an upregulation of Cx40 and Cx43-GJs that contributes to preserve vasorelaxation [34], while endothelial Cx40 limits neutrophil infiltration at the onset of IRI [35]. In contrast, ATP release by Cx43-HCs in cardiac fibroblasts and myofibroblasts impacts on fibrosis and inflammation during ischemia [11].

Intercellular communication mediated by extracellular vesicles in the heart

Extracellular vesicles (EVs) represent a heterogeneous population of bilayered nanosized vesicles, composed of a large variety of active macromolecules, including proteins, metabolites, lipids and nucleic acids that retain functional activity after delivery to target cells [3,36,37]. Since EVs can mirror the pathophysiological state of producing cells, being ubiquitously found in easily

accessible biological fluids, including saliva, blood and urine, EVs have been considered as clinically relevant disease biomarkers [38,39] and therapeutic targets [3,40]. In healthy individuals, circulating EVs are mostly derived from platelets and erythrocytes and, to a lesser extent from leucocytes and endothelial cells, being recognized as critical regulators of inter-organ communication, maintenance of immune and vascular homeostasis [41]. It is also conceivable that EV-mediated crosstalk between different tissues and organs is essential to maintain organisms' viability.

Despite their presumed importance in maintaining heart homeostasis, most of the studies focused on the role of EVs in pathological or stressed conditions [37,42]. Consistently, cardiomyocyte-derived EVs induce gene expression changes in fibroblasts [43], while fibroblast EVs can trigger cardiomyocyte hypertrophy [44]. Besides affecting gene transcription and *de novo* protein synthesis on target cells, cardiomyocyte EVs transfer functional glucose transporters and glycolytic enzymes into endothelial cells, providing metabolic coupling under stress conditions [36]. EV-mediated communication is also important to balance pro- and anti-atherogenic mechanisms, modulating cardiovascular risk. In fact, EVs secreted by macrophage foam cells promote migration and adhesion of SMCs [45], while endothelial EVs induce an atheroprotective phenotype in SMCs under physiological shear stress [46].

The role of extracellular vesicle-mediated communication in ischemic wound healing

The importance of EV-mediated communication in the regulation of inflammation during AMI has long been documented. In fact, ischemia induces the accumulation of cardiomyocyte and endothelial cell-derived EVs in the myocardial interstitial space, which can be subsequently taken up by immune cells [47]. Moreover, circulating EVs from AMI mice downregulate C-X-C chemokine receptor type 4 (CXCR4) expression in bone marrow cells, contributing to mobilize progenitor cells, which represents a critical step in the systemic response to ischemia that can be therapeutically exploited [48]. Interestingly, while cardiomyocyte EVs modulate steady state macrophage activity that ensure heart homeostasis, this crosstalk is strongly affected during myocardial ischemia, likely contributing to adverse cardiac remodeling [42]. On the other hand, ischemic EVs released by macrophages induce an upregulation of adhesion molecules in endothelial cells, and decrease fibroblast proliferation by the transfer of miRNA-155 [49], ultimately enhancing vascular and tissue inflammation [50]. In the opposite direction, transfer of miRNA-208a by cardiomyocyte-derived EVs into fibroblasts contribute to sustain fibrosis in IRI [51].

EV-mediated communication can also be involved in compensatory pathways triggered during ischemia. For example, ischemic cardiomyocyte EVs activate endothelial cell angiogenesis, mainly by the transfer of miRNA-222 and miRNA-143 [37], whereas EVs derived from endothelial cells subjected to IPC [52] or from fibroblasts during hypoxia/reperfusion [53] restrain cardiomyocyte injury. Studies showing that hypoxia-induced release of cardiomyocyte-derived EVs enriched in tumor necrosis factor (TNF)-α [54] and miRNA-30a [55] modulate apoptosis and autophagy in recipient cardiomyocytes respectively, demonstrate that EVs also mediate autocrine and homocellular paracrine signaling in the ischemic heart.

Circulating EV numbers, protein and RNA content constitute powerful markers of both cardiovascular risk and injury [3]. Blood endothelial microparticles are associated with higher cardiometabolic risk [39], whereas increased levels of platelet and endothelial-derived vesicles associate with ST-segment elevated myocardial infarction [56]. Additionally, high levels of serum circulating miRNA-1, miRNA-499, miRNA-21, miRNA-133a and miRNA-208a were detected in **acute coronary syndrome** (ACS) patients and AMI mice, likely within EVs released by the infarcted myocardium [51,57,58]. Levels of miRNA-192, miRNA-194 and miRNA-34a in EVs from patients' sera can also constitute predictive indicators of heart failure post-AMI [59].

Intercellular communication via tunneling nanotubes in heart pathophysiology

Tunneling nanotubes (TNTs) are actin-based membrane protrusions that can extend up to 100 µm, enabling the exchange of proteins, RNAs, organelles and virus between connected cells [2]. The presence of TNT-like bridges between capillary endothelial cells and myocytes was reported during both cardiac and skeletal muscle development [60]. Additionally, TNT-mediated communication between cardiomyocytes and endothelial progenitors [61] or cardiac fibroblasts [62] accounts for the transfer of functional mitochondria and Ca^{2+} propagation. Importantly, studies carried out in cultured cells, as well as rat and human heart tissues, recently demonstrated that the number of TNTs formed between cardiomyocytes and fibroblasts increases during ischemia, likely impacting on arrhythmogenesis, fibrosis and injury resistance, representing an emerging therapeutic tool [63]. In agreement, formation of TNT-like bridges between MSCs and cardiomyoblasts following *in vitro* IRI correlates with decreased cell death [64], whereas TNT-mediated mitochondrial transfer from MSCs to cardiomyocytes mediates cardioprotection in doxorubicin-induced injury models [65] and reprograms adult cardiomyocytes towards a progenitor-like state [66]. Nonetheless, more compelling evidence is required to demonstrate to which extent TNT-driven mitochondrial transfer contribute to the observed phenotypes.

The study of TNT-mediated communication has been hampered by technical constraints related to their fragile and transitory nature and the lack of specific molecular markers. However, several strategies have enabled the characterization of these structures *in vitro*, mainly by optical fluorescence and electron microscopy, resorting to the labeling of cytoskeletal proteins and the use of fluorescently labelled lysosomes and mitochondria to track TNT-mediated organelle transfer [63].

Experimental models to study the impact of intercellular communication in cardiac health and disease

Despite the importance of *in vitro* approaches to elucidate basic mechanisms underlying cell-cell communication (**BOX 3**), these strategies fail to reflect the biological complexity of organisms and the actual implications of intercellular communication derailment to cardiac diseases. Therefore, more comprehensive approaches should be considered to tackle this problem.

Animal models

Studies aiming at assessing *in situ* levels of cardiac GJIC, important to identify arrhythmia trigger sites in ischemic hearts, have resorted to optical mapping techniques, using voltage- or Ca^{2+}-sensitive dyes [67,68]. Moreover, double-transgenic mice expressing voltage-sensitive fluorescent proteins were used to assess electrical coupling between cardiomyocytes and non-myocytes in the scar border zone of injured hearts, which was suggested to involve TNTs [69].

Multiple studies have focused on the development of labelling strategies to track exogenous EV biodistribution by *in vivo* bioluminescence, fluorescence-mediated tomography, magnetic resonance imaging (MRI), single-photon emission computed tomography (SPECT) and positron emission tomography (PET) (reviewed in [70]). More recently, Luo et al. have developed a mouse model that enables spatiotemporal tracking of endogenous cardiomyocyte-derived EVs, using a nano-luciferase reporter fused with the EV surface marker CD63, which will be crucial to explore inter-organ communication and clinical applications of EVs [71].

Human heart

Although intercellular crosstalk in the human heart has been characterized *in vitro* (**BOX 3**), using induced pluripotent stem cells (iPSCs) and liquid biopsies obtained from patients, noninvasive approaches to study *in vivo* communication networks still represent a major challenge [47,72]. Mechanistic links between ion channel function, including GJIC, and action potential propagation and arrhythmogenesis in the human heart can be inferred by electrocardiographic imaging

combined with tagged MRI that enables the mapping of electrical excitation and mechanical contraction [73]. These techniques are not only important for clinical diagnosis, but can also be incorporated in computational models to facilitate therapy guidance [74]. In addition, mathematical models built on micro-computed tomography 3D-reconstructions of the human cardiac conduction system may be useful to characterize the interplay between disease-associated morphological remodelling and arrhythmogenesis [75]. More recently, single-cell transcriptome profiling of the human heart enabled the identification of communication hubs between different cardiac cell subsets, based on putative ligand-receptor interactions [76]. It is conceivable that similar approaches could be applied to unveil GJ-, TNT- or EV-mediated cell-cell communication networks.

Computational Models

Computational simulation has contributed to elucidate the interplay between GJ-mediated and ephaptic coupling [7], to predict the impact of GJ conductance changes in ischemic areas [77], as well as to study the impact of fibroblast-myocyte interactions in arrhythmogenesis [78]. Additionally, mathematical modeling was used to distinguish the effects of heterocellular contacts and paracrine signaling on cardiac contractility and arrhythmogenicity in mesenchymal stromal cells (MSCs)-based therapeutics [79]. Systems biology may also identify potential targets for future experimental validation. In agreement, a microarray data-driven computational modeling tool detects covarying miRNAs in hypoxic cardiac-derived progenitor cells (CPCs)-derived EVs and predicts functional outcomes on cardiac function and repair [80]. *In silico* identification of gene-disease associations between EV biogenesis, secretion and myocardial IRI, might also be extended to other forms of intercellular communication, including GJs and TNTs [81].

Potential therapeutic approaches targeting intercellular communication

Therapeutic strategies targeting connexin-based channels

Given the growing evidence for HC involvement in IRI and arrhythmia, the corner stone of therapeutically targeting cardiac connexin channels has bifurcated into a twofold aim: preventing GJs to close and HCs from opening, which has been mainly achieved with small peptides (**Figure 1, BOX 4**). Additionally, pharmacological and genetic tools aiming to improve delivery of Cx43 to the IDs or to prevent GJ remodeling have been tested in pre-clinical AMI models. In agreement, microtubule stabilizers, such as taxol [82], preserve ID localization of Cx43, with a concomitant decrease in ventricular arrhythmogenesis. More compelling evidence arose from systemic adeno-

associated virus serotype 9 (AAV9)–mediated gene transfer of Cx43-20k, which stabilized Cx43 at the IDs in a mouse AMI model [15].

Parallel studies demonstrated that heart-specific knockout of CELF1 increased Cx43 levels, which ameliorated contractile dysfunction after chronic myocardial infarction [31]. Moreover, transplantation of human skeletal muscle-derived stem/progenitor cells overexpressing Cx43 prevents arrhythmogenesis in infarcted rat hearts [84], while intramyocardial lentiviral delivery of Cx43 promotes the formation of functional GJ in (myo)fibroblasts and CD45+ cells in the infarcted myocardial scar, reducing arrhythmia incidence [85]. Light-induced depolarization of macrophages expressing photoactivatable channelrhodopsin 2 (ChR2) improved atrioventricular conduction via GJIC with cardiomyocytes, uncovering the potential of **optogenetics**-based therapeutic tools [13].

Pharmacological inhibition of c-Src kinase has been reported to enhance Cx43 phosphorylation, preventing downregulation of Cx43 and restoring electrical conduction following permanent coronary artery ligation in mice [86]. Nevertheless, divergent functional outcomes have been observed following the targeting of Cx43 phosphorylation, likely resulting from differences in the nature of the stimuli, the animal model and/or the specific phosphorylation sites involved.

Therapeutic strategies based on extracellular vesicles

EVs secreted from different cell types have been extensively demonstrated as useful therapeutic strategies for AMI (**Figure 1**). Noteworthy, intracardiac injection of ischemic cardiomyocyte EVs elicits the formation of new functional blood vessels in AMI mice [37]. Nonetheless, currently technical limitations have hampered the isolation of *in vivo*-derived specific EVs. In a more advanced stage for therapeutic use are EVs secreted by different progenitor cells *in vitro*, whose administration induces endothelial cell proliferation and angiogenesis [87,88], mediate immune responses via macrophages, T-cells [89] and B-cells [90], reduce fibrosis [91] and cardiomyocyte apoptosis [92], ultimately improving cardiac function [93]. Although many targets and mediators were suggested, including PAPP-A protein [94], EMMPRIN [95], numerous miRNAs [37,49,92] and the long non-coding RNA MALAT1 [88], additional effort is still needed to fully understand their protective effects. Interestingly, intramyocardial injection of CPC-EVs prevents adverse remodeling and preserves cardiac function in porcine models of myocardial infarction [91]. Moreover, fusion of an ischemic myocardium-targeting peptide enhanced the specificity of therapeutic MSC-EVs, significantly reducing infarct size and preserving cardiac function [96].

Although phase I clinical trials have attested its safety, standardized EV isolation techniques [97], a better knowledge of pharmacokinetics and the development of controlled-delivery systems [98,99] are still required to improve EV-based therapeutics.

Endogenous extracellular vesicles as cardioprotective strategies

Endogenous EVs can mediate the effects of innate cardioprotective strategies, such as remote ischemic conditioning (RIC; **Figure 1**) [100–102]. In RIC, cycles of brief non-lethal ischemia and reperfusion applied to the limb can be cardioprotective following a lethal episode of acute ischemia/reperfusion injury (IRI). It has been postulated that EVs generated in the limb following RIC enter the circulation and convey cardioprotective signals to cardiomyocytes in the heart [103,104].

Initial studies implicating cardioprotection transfer via endogenously produced EVs showed that direct IPC (3x5-min alternating episodes of global ischemia and reperfusion) of isolated perfused *ex vivo* rat hearts increased the number of EVs in the coronary effluent. Importantly, perfusion of naïve isolated rat hearts with IPC effluent limited infarct size, but failed to do so when EVs were depleted from the conditioned effluent [101]. In a subsequent study, EVs derived from rats and human healthy volunteers reduced cardiomyocyte death and limited infarct size *in vitro*, *ex vivo* and *in vivo* injured rodent hearts, likely via activation of the toll-like receptor 4-Erk1/2-p38MAPK-HSP27 signaling pathway in cardiomyocytes [100]. EVs isolated from diabetic rats failed to activate this pathway and to protect non-diabetic cardiomyocytes against simulated IRI, the reasons for which remain unclear [105]. Conversely, plasma EVs from non-diabetic rats protect diabetic cardiomyocytes against simulated IRI, suggesting that the diabetic heart is still amenable to cardioprotection by ‘healthy’ EVs [105]. Although hind-limb RIC in rats increases plasma EVs numbers, RIC-EVs did not confer greater cardioprotection than those isolated from control animals [100]. However, other studies reported that plasma EVs produced during limb RIC are cardioprotective, likely due to their miRNA-24 content [102]. Consistently, in a rat model of limb RIC (4x5-min episodes of ischemia and reperfusion), the levels of circulating EVs and vesicle-enclosed miRNA-24 were increased, compared to sham control. These EVs were shown to counteract hydrogen peroxide-induced apoptotic cell death in H9c2 cells through the downregulation of Bim [102]. Furthermore, intramyocardial injection of RIC-EVs reduced infarct size and preserved cardiac function, which was abrogated with miRNA-24 antagomirs [102]. However, no evidence was provided to show that plasma EVs containing miRNA-24 produced by limb RIC were actually taken up by the ischemic heart, nor that vesicle-enclosed miRNA-24 actually contributed to the infarct-limiting effects of RIC [102]. Interestingly, repeated episodes of

limb RIC initiated 4 weeks post-infarction and applied daily for 28 days, ameliorate cardiac remodeling, with a concomitant increased expression of miRNA-29a in plasma EVs and in the infarcted heart [106]. Limb RIC can also generate plasma EVs containing miRNA-21, which protect the kidneys against sepsis-mediated acute injury by suppressing NF-kB activation and triggering PI3K-Akt signaling [107].

Concluding Remarks

Both GJ- and EV-mediated cell-cell communication are essential to maintain cardiac contractile function. A dramatic GJ remodeling during ischemia and reperfusion has been associated with intercellular communication derailment, implicated in fatal arrhythmia and heart failure. Notwithstanding, therapies aiming at regulating Cx43 channel activity, either via HCs or GJs, have been developed and tested. Despite the promising results of the Cx43-mimetic peptide αCT1 in dermal wound healing (NCT04331080[i]) [108–110], phase II clinical trials with danegaptide in AMI were disappointing, likely reflecting the dual function of Cx43 channels as mediators of metabolic and electrical coupling. Moreover, this extends the relevance of exploring emerging cell-cell communication mechanisms, such as TNTs and EVs, as promising therapeutic targets for AMI. On the other hand, approaches acting directly on Cx43 synthesis, such as antisense oligodeoxynucleotides [111] may constitute potential alternative approaches for AMI treatment.

Therapeutic strategies based on administration of unmodified EVs have demonstrated great potential in preserving cardiac function following AMI. However, engineered EVs can also be envisioned as promising clinical tools for targeted drug delivery [96]. As long-distance communication conveyers, circulating EVs have been correlated with AMI severity and heart failure progression, posing as suitable disease biomarkers. Furthermore, endogenous EVs play cardioprotective roles against IRI, including in the context of RIC.

In conclusion, the different forms of intercellular communication in the heart constitute promising targetable approaches for patients presenting with AMI. To succeed with this strategy, it is crucial to resolve the underlying mechanisms, to envisage means to preserve proper electrical conductance, preventing arrhythmia, to block dissemination of harmful signals throughout the heart and to stimulate long-distance cardioprotective strategies.

Figure Legends

Key Figure: Intercellular communication networks in the cardiovascular system. Communication between the various cell populations that compose the heart can occur directly, by the formation of gap junctions (GJs) and tunneling nanotubes (TNTs), or at longer distances via extracellular vesicles (EVs). By the exchange of small molecules and ions between neighbor cells, GJ enable electrical and metabolic coupling, contributing to synchronized heart beating and vascular homeostasis. Connected cells can transfer organelles via TNTs, participating upon cardiac development and cardioprotection. EVs can fuse with the plasma membrane of target cells or transfer their cargo via Cx43-based channels, impacting on cardiovascular homeostasis and repair after injury.

Figure 1: Therapeutic strategies targeting intercellular communication in acute myocardial infarction. Acute myocardial infarction is associated with intercellular communication derailment, which contributes to arrhythmogenesis and exacerbates cell injury. While primary percutaneous coronary intervention (PCI) is still the gold standard treatment, therapeutic targeting of the various forms of intercellular communication has been successfully implemented in pre-clinical models of AMI. Targeting of Cx43-based channels, both gap junctions (GJs) and hemichannels (HCs), with small peptides or using gene therapy tools have also been shown to decrease infarct size. Exogenous stem cell-derived extracellular vesicles (EVs) and isolated miRNAs have been demonstrated to increase angiogenesis, decrease fibrosis and contribute to immunomodulation in the ischemic heart, ultimately increasing cardiac repair. The cardioprotective effects of limb remote ischemic conditioning (RIC) were reported to involve the action of endogenous EVs. In addition, several vesicle-enclosed miRNAs have been proposed as useful circulating biomarkers in AMI patients and animal models.

Figure I: Peptide-based strategies targeting Cx43-based channels. Topology and sequence of Cx43 illustrating various peptides affecting channel function. AAP10 is a peptide enhancing GJ coupling. Gap26 and Gap27 mimic well conserved sequences on the extracellular loops of the connexin protein that first inhibit HCs and with some delay also GJs; due to their conserved nature, they also inhibit other connexin channels. L2 is a Cx-mimetic peptide composed of a sequence on the cytoplasmic loop of Cx43 that restrains molecular interactions between the cytoplasmic loop and the C-terminal tail; it inhibits Cx43 HCs and prevents GJ closure. RRNY is a non-mimetic peptide based on the pharmacophore of the L2 binding site. Gap19 is nonapeptide sequence within the L2 domain inhibiting Cx43 HCs. RyRHCIp is a mimetic peptide of a type-2

ryanodine receptor sequence that interacts with the green Cx43 sequence on the C-terminal tail *in silico*. H1, H2: α-helical structures. Relevant phosphorylation sites discussed are also shown. SH3 is an interaction and signaling hub, containing MAPK phosphorylation sites.

Clinician's Corner

- Given its importance for proper embryonic heart development, *GJA1* mutations are rarely implicated in long-term cardiac diseases and have only been found in few cases of sudden infant death syndrome, as well as in congenital heart disease patients, where it related with ventricular septal defects. *GJA1* mutations were also identified in 2 familial cases of oculodentodigital dysplasia (ODDD), which presented recurrent ventricular tachycardia. More frequently, cardiac disorders, namely those characterized by conduction defects and arrhythmogenesis, including heart failure and AMI, are associated with GJIC impairment, usually due to GJ remodeling, including subcellular redistribution, PTMs and degradation, rather than Cx43 mutations.

- One of the main pitfalls of cellular therapies based on the administration of pluripotent stem cell-derived cardiomyocytes is the establishment of efficient electrical connections, required for proper cell engraftment and functional synchronization. Based on preclinical work, cellular paracrine effects have been proposed and foreseen as strategies for reparative actions. Nonetheless, the potential risk of tumor formation following administration of stem cell-derived EVs should be carefully addressed before clinical application.

- Small peptides, including TAT-L2 and RRNY allow the combined therapeutic targeting of Cx43-GJs and HCs, which need further scrutiny of their therapeutic potential. Moreover, Gap19/TAT-Gap19 proves to be a crucial tool to investigate Cx43 HC involvement in disease states, including the role of altered Ca^{2+} homeostasis in arrhythmogenic cardiomyopathies and atrial fibrillation.

- Therapeutic strategies targeting intercellular communication should be tailored according to the stage of the cardiac lesion (acute ischemia *versus* chronic remodeling) and, consequently, the molecular players involved. For example, pharmacological inhibition of GJ degradation should target AMPK during ischemia or Beclin-1 during reperfusion. In addition, therapies targeting GJIC should aim to preserve electrical coupling but restrain detrimental metabolic coupling, which is closely associated with phosphorylation-mediated channel gating.

- The levels of Cx43 in EVs can constitute a new potential therapeutic target, being beneficial to enhance the release of vesicle content into recipient cells in the context of AMI and IRI. Moreover, changes in Cx43 levels of circulating EVs, considered as an additional feature of Cx43 remodeling, can constitute a new marker of heart damage.

- In the setting of cardioprotection conferred by limb RIC, plasma EVs have been shown to play a key role in mediating the cardioprotective signal from the limb to the heart. Potential mechanisms include the transfer of cytoprotective miRNAs to the heart.

Glossary

Acute coronary syndrome: term used to describe the spectrum of clinical manifestations associated with a sudden reduction in coronary blood flow, including ST-segment elevation myocardial infarction (STEMI), non–ST-segment elevation myocardial infarction (NSTEMI) and unstable angina.

Atherothrombotic coronary artery disease: atherosclerotic plaque disruption with subsequent thrombus formation, which constitutes the major cause of acute coronary syndrome.

Electrotonic mechanisms: passive spread of charge between electrical-excitable cells (e.g. via GJ). Electrotonic potentials can lead to membrane depolarization above the threshold, triggering action potentials.

Endocytosis: process whereby cells internalize proteins, microorganisms or other macromolecules from the external environment, involving membrane invagination.

Ephaptic transmission involves the generation of electrical fields through the extracellular space, altering the excitability of neighboring cells.

Fluorescence recovery after photobleaching: microscopy-based method to determine the diffusion kinetics of a fluorescent molecule. After bleaching a defined region of interest, using high laser power, the movement of non-bleached fluorescent molecules into the photobleached area is monitored by time-lapse imaging.

Homocellular interactions: Interactions established by cells of the same type.

Intercalated discs: specialized region at the cell ends of cardiomyocytes that ensures mechanical and electrical coupling. IDs are composed by three main structures: desmosomes, adherens junctions and GJ.

Interstitial edema: accumulation of excess fluid in the extracellular space due to water leakage from damaged capillaries.

Ischemic preconditioning: characterized by several brief episodes of ischemia (or hypoxia) prior to a longer period of detrimental ischemia.

long non-coding RNA: type of non-coding RNA with over 200 nucleotides, associated with regulation of chromatin structure, alternative splicing and protein complex scaffolding.

Lymphangiogenesis: formation of new lymphatic vessels, usually from pre-existing vessels, which can occur during heart development or in response to injury.

Myoendothelial junctions: plasma membrane juxtapositions that serve as a conduit for GJ-mediated transfer of molecules between endothelial and vascular smooth muscle cells.

Optogenetics: technique that involves the use of light to control genetically modified cells or organisms, engineered to express light-sensitive ion channels.

Organoid: self-organized 3D multicellular *in vitro* tissue construct, derived from stem cells, which can recapitulate organ architecture with high fidelity.

Perinexus: membrane microdomain at the periphery of the GJ plaque that plays important roles on GJ formation and facilitate ephaptic coupling.

Post-translational modifications: generally, refers to the enzymatic modification of proteins, which can occur as part of their biosynthetic pathway (after protein translation) or during the normal protein lifecycle. Phosphorylation and ubiquitination are two of the most common.

Scrape-loading/dye transfer: technique that relies on the loading of a membrane-impermeable and GJ-permeable fluorescent dye (e.g. Lucifer Yellow) by scraping a cell monolayer. Dye diffusion into neighboring cells connected by functional GJ can be monitored by microscopy.

Shear stress: tangential stress generated by the blood flowing on the endothelial surface of the arterial wall.

Text Boxes

BOX 1

Extracellular vesicle-mediated communication

EVs can be classified in intracellular formed exosomes (50-200 nm), secreted after fusion of multivesicular bodies (MVBs) with the cell surface, microvesicles (100-1000 nm), formed by outward budding of the plasma membrane, and apoptotic bodies (100-5000 nm), mostly associated with cellular clearance and immunomodulation [2,3]. Due to the lack of specific markers and techniques to unequivocally purify each subset of vesicles, the indiscriminate use of the term EVs is currently recommended [112].

The nature and relative content of EV cargo varies according to the producing cell type and its physiological state, but do not necessarily reflect the molecular composition of their cells of origin [37,113]. Although initially considered as a rather random process, ample evidence now indicates that the EV-mediated flow of information is highly specific and regulated, with the sorting of molecules being selectively determined during EV biogenesis.

Once reaching their destination, EVs have different ways to interact with target cells and/or to unload the vesicular content. EVs can mediate juxtacrine signaling, eliciting a signal transduction cascade at the cell surface, or release its intraluminal cargo into the cytoplasm of acceptor cells after fusion or endocytosis [2]. More recently, the discovery that Cx43 can also be present in EVs suggested an alternative mode of EV-target cell communication [114,115]. In the proposed model, Cx43 HC at the EV surface can dock with unopposed HC at the plasma membrane of target cells, forming a GJ-like structure that enable the transfer of small molecules [114,116].

Although the study of EV-mediated communication remains technically challenging, mainly due to their small size, multiple strategies have been employed to visualize EV secretion and uptake *in vitro*. For example, labeling of EV membranes, using lipophilic fluorescent dyes, such as those from the PKH, DiR and DiD families, intraluminal cargo, with carboxyfluorescein succinimidyl ester (CFSE) and calcein-acetoxymethyl ester, or nucleic-acid selective fluorescent compounds are used to track EV-target cell interactions [114]. Moreover, transmission electron microscopy (TEM) has revealed the detailed ultrastructure of EVs isolated from cultured cells and biological fluids, which when coupled with immunogold-labeling, can be used to characterize EV protein content.

BOX 2

Molecular mechanisms underlying impaired trafficking and degradation of Cx43 during ischemia

Changes on the phosphorylation profile of Cx43 impact both GJ channel function and Cx43 trafficking at the onset of ischemia (**Figure 1**) [2,5]. Accordingly, ischemia-induced dephosphorylation of S325/S328/S330 on ID-localized Cx43 is associated with reduced GJ assembly [2]. Recent *in vitro* experiments suggest that Cx43-S279 and S282 are targets of protein phosphatase 2A (PP2A) during ischemia/reperfusion injury (IRI), which correlates with the generation of abnormal Ca^{2+} transients and cardiomyocyte apoptosis [117]. While Cx43 dephosphorylation in response to IRI has been closely related with impaired electrical coupling and increased susceptibility to arrhythmia, concomitant phosphorylation of other Cx43 residues may protect the myocardium from IRI. In agreement, rapid loss of phosphorylated Cx43-S365 (the GJIC 'gatekeeper'), with a consequent protein kinase C (PKC)-mediated phosphorylation of S368 is observed following ischemia [2]. Although phosphorylated Cx43-S368 can remain at the IDs, contributing to a significant reduction in unitary electrical conductance, a paradoxical increase in selective GJ permeability was observed [2,118]. Therefore, Cx43 phosphorylation may contribute to cardioprotection, by impairing GJ-mediated metabolic coupling and limiting tissue damage and infarct size [119]. Moreover, hyperphosphorylation of Cx43-S373 during myocardial ischemia can trigger subsequent Cx43-S368 phosphorylation and ubiquitination, required for internalization of GJ channels [120,121].

The presence of Cx43 at the lateral cardiomyocyte membranes has been extensively reported in animal models of AMI, as early as 30 minutes post-coronary occlusion, where it associates with dysfunctional GJIC, likely acting as an arrhythmogenic substrate [72,122]. Ischemia-induced accumulation of Cx43 at the perinexus and/or lateral membranes may also facilitate the establishment of GJ between cardiomyocytes and fibroblasts or macrophages, further exacerbating tissue damage [13]. The molecular mechanisms underlying pathological redistribution of Cx43-channels are currently under debate, which may uncover relevant therapeutic targets. In agreement, accumulation of lateralized Cx43 in hearts from patients with end-stage ischemic cardiomyopathy, may result from impaired microtubule dynamics [123]. In line with this concept, cardiac-specific overexpression of Cx43-20k isoform enhanced the delivery of Cx43 HC to cardiac IDs, preserving GJ coupling during myocardial ischemia [15]. Other studies have suggested that loss of zonula occludens-1 (ZO-1)-mediated scaffolding enables Cx43-channel diffusion from the IDs to the lateral membranes during acute ischemia [122]. On the other hand, ubiquitination of Cx43, mediated by the E3 ligase Nedd4 also contributes for Cx43 remodeling in the ischemic heart [121]. In fact, phosphorylation and ubiquitination of Cx43 promote the interaction with Eps15 homology domain-containing protein 1 (EHD1), ultimately responsible to drive lateralization of Cx43, via an endocytic recycling-like mechanism [72].

BOX 3

Cultured cell-based approaches to study cardiac intercellular communication

Cell type-specific studies of intercellular communication mechanisms have resorted to isolated primary cardiomyocytes, endothelial cells and fibroblasts from neonatal rodent hearts, and to the few cell lines available - the mouse atrial cardiomyocyte HL-1 [72], the rat ventricular myoblast H9c2 [42] and the mouse cardiac endothelial cell line (MCEC) [37]. Particularly, these models are useful to investigate GJ-mediated metabolic coupling, mainly through dye diffusion-based microscopy techniques, including **fluorescence recovery after photobleaching** (FRAP) and dye transfer (DT) following **scrape-loading** (SL/DT) or microinjection.

Major differences in energy metabolism and injury resistance are observed among models and culture conditions, which have important implications on cell-cell communication outcomes. For example, the metabolic signature of EVs derived from three-dimensional (3D)-like bioreactors is significantly different from those obtained from conventional 2D cultures, whereas HL-1 and H9c2 cells display distinct mitochondrial function and IRI resistance [124]. Therefore, disease-associated phenotype changes are more accurately reflected by primary cells derived from genetically altered animals, surgical disease models or iPSCs from patients with inherited mutations on cardiac proteins. Regarding primary cells, the sex and age of the animals should also be considered. Although technically demanding, terminally differentiated rod-shaped adult cardiomyocytes present the most similar sarcomeric architecture and behavior as those in intact hearts, being ideal for injury-induced GJ remodeling and electrophysiology studies [72].

Stem cell-derived 3D multicellular cultures and heart-on-chips show greater potential for precision medicine applications. **Organoid**-based studies provided important mechanistic hints on vascular development and cardiomyocyte stress resistance, as well as high-throughput drug screening, enabling the identification of cardiomyocyte proliferation modulators [125]. These models have also been used to investigate network integrated responses. For example, secretion of vascular endothelial growth factor (VEGF) by non-myocytes was shown to increase Cx43 expression and contractile function in cardiomyocytes [126], while Cx43-mediated communication between cardiomyocytes and fibroblasts was reported to enhance maturation of microtissues [127]. Disease modeling can be achieved by manipulating the mechanical properties of the biomaterials employed, including extracellular matrix hydrogels [128] or synthetic fibers, which impact on cell attachment, alignment and stiffness [129]. Moreover, cardiac cells can be integrated in microfluidic devices that recapitulate *in vivo* microenvironment complexity, including electrical stimulation, cyclic stretch, fluid flow and chemical gradients generation [129]. These platforms

have been also used to demonstrate TNT-mediated mitochondrial transfer from stem cells to myocytes [61], and for high-throughput dye diffusion studies to assess GJIC [130]. Recently, a heart-on-chip model was designed to study the effects of ischemia on cardiac function, providing accurate single-cell recordings of beat frequency and action potential [129].

BOX4

Peptide-based strategies targeting Cx43-based channels

Pharmacological approaches aiming at preventing Cx43-GJs closure were first achieved with molecules derived from the anti-arrhythmic peptide (AAP) family that promote synchronized beating of *in vitro* cardiomyocytes, by affecting Cx43 phosphorylation [22]. Subsequently, several derived molecules including AAP10 peptide, rotigaptide (L-amino acid reversed sequence version of AAP10, aka ZP123 [131,132]) and the dipeptide danegaptide (aka Gap-134, ZP1609 [133]) were developed, both of which prevent GJ closure in ischemia and reduce myocardial IRI in large animal models (**Figure I**). Disappointingly, a phase II clinical trial with danegaptide could not confirm beneficial effects on cardiac IRI outcomes in humans (NCT01977755[ii]) [134].

Another class called 'Cx-mimetic peptides' have been developed based on connexin protein sequences and their molecular interaction with other domains within the connexin protein, including Gap26 and Gap27 (reviewed in [22]). Gap26/27 rapidly inhibit HC opening, but also inhibit GJs with some delay [22,135]. Although Gap26/27 were shown to reduce infarct size in *in vivo* and *ex vivo* cardiac IRI small animal models, their inhibitory effect on GJs is an unacceptable constraint [16,17]. L2 and RRNY peptides prevent GJ closure upon acidification and Cx43 HC opening, making them interesting molecules for therapeutically targeting both channel types (**Figure I**) [25,136,137]. RRNY is a non-mimetic pharmacophore-based sequence that also inhibits mitochondrial Cx43 HCs [25]. Gap19 is a nonapeptide stretch within the L2 sequence; it has intrinsic membrane permeability that is further improved by fusion to a membrane translocation sequence (e.g. TAT). Both TAT-Gap19 and RRNY protect against IRI, but RRNY does so more potently, probably as a result of its combined GJ enhancing and HC inhibiting effects [25,138]. More recently, a RyR-based peptide RyRHCIp designed to interfere with RyR/Cx43 interaction, was demonstrated to block cardiomyocyte Cx43 HC opening induced by RyR activation with caffeine (**Figure I**) [21].

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Resources

[i] https://clinicaltrials.gov/ct2/show/NCT04331080

[ii] https://clinicaltrials.gov/ct2/show/NCT01977755

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