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EDITORIAL VIEWPOINT

Interaction of the heart and its close and distant neighbours: report of the Meeting of the ESC Working Groups Myocardial Function and Cellular Biology

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1. Introduction

Intercellular and inter-organ communication is required to maintain homoeostasis in multicellular organisms. Given its life-supporting function, the heart is at the centre of a heavily studied network of interactions. Despite the long-recognized ability to receive and relay inputs to and from far and close neighbours, surprising new networks of interactions as well as new communication factors and mechanisms are currently emerging. The Myocardial Function and the Cellular Biology of the Heart Working groups of the European Society of Cardiology convened during 2–5 May 2013 to discuss emerging data and ongoing studies on intercellular and inter-organ communication, in health and disease.

2. New emerging communication factors

Cells interact through either direct contact or secreted agonists. The signal transduction machinery inside cells eventually translates interactions with limited numbers of agonists into a cellular response. This process of scanty molecules being pinpointed by high-affinity receptors is now flanked by other emerging mechanisms of communication that involve highly abundant messengers binding to relatively low-affinity detectors. Two novel intercellular communication paths are currently arising: the flooding of cells with secreted vesicles, the so-called exosomes, with various but information-rich contents, and the sensing of different species of circulating RNA, such as miRNAs that, once captured, elicit post-transcriptional regulation of gene expression, and the extracellular RNAs (eRNA) that, from the outside of cells, trigger membrane enzyme activation and generation of classical receptor agonists.

2.1 Exosomes and miRNA

An exciting new concept in the field of intercellular and inter-organ communication is the idea that microscopic vesicles may ferry proteins and RNA through interstitial fluid and blood. Building on this concept, miRNA and proteins from plasma microvesicles and exosomes may offer a great potential as biomarkers for cardiovascular risk and cellular injury. Specific miRNAs act as master regulators of angiogenesis, cardiomyocyte proliferation, and regulation of cell survival. In particular, the importance of miR-199, which seems to regulate a critical switch between cardiac atrophy and proliferation, was highlighted[1]. However, the miR-199 and the miR-21 genes also raise new questions on the specificity of miRNA approaches because in both cases the same gene produces two different miRNAs from complementary strands: miR-199a-3p and miR-199a-5p as well as miR-21-5p and its antisense miR-21-3p, both of which are active with completely different target sequences and probably also with different biological roles. This finding implies that the classical over-expression or gene targeting experiments using over-expression of the pre-miRNAs or deletion of the complete miRNA gene by genetic knockouts lack specificity and suggest to one to always control results by specifically inhibiting each of the two complementary microRNAs. Antagomir-mediated silencing provides such a level of specificity. An example of select silencing of single or even dual miRNA products such as miR-199a-5p and/or miR-214–3p was presented as an unpublished result. Both miRNA products dose-dependently target one isoform of peroxisome proliferator-activating receptor (PPAR-beta) in the failing heart, and combinatorial silencing of miR-199a-5p and miR-214-3p de-repressed PPAR-beta, yielding more energetic advantage in mouse hearts in the setting of sustained pressure overload.

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2.2 New ways intact and damaged cells talk to each other

Nucleic acids (RNA and DNA) have been classically known as key molecules for intracellular communications between the nucleus and the cytoplasm in delivering information that determines single cell fate. However, RNA can also work as a potent communicator between cells and its real potential is only starting to emerge. eRNA is released from inflammatory cells upon stress, possibly as a consequence of the activation of classical signal transduction pathways, such as those leading to PI3K triggering. Once discharged, eRNA activates TNF-alpha release from monocytes through the shedding of pre-TNF via activation of TNF-alpha converting enzyme; this amplifies the inflammatory reaction in stressed tissue. Furthermore, eRNA accumulates in atherosclerotic lesions of dyslipidaemic mice, and systemic infusion of RNase1 reduces the size of these lesions *in vivo*. RNase1 is highly expressed in the endothelium and it is not clear whether this RNase1 may degrade all circulating RNA species, including specific miRNAs. Interestingly, eRNA is also released after ischaemia/reperfusion, thereby amplifying inflammation and oxidant stress; of note, ischaemic pre-conditioning increases endothelial release of RNase1, potentially participating in protection against the pro-inflammatory effects of eRNA.

3. Novel and revisited networks: intra-organ/intercellular communication

Besides emerging new concepts in cell-to-cell communication, highlights of the meeting were the meticulous characterization of provider/responder networks of different cell types that control multiple cardiac reactions in health and disease. While the cardiomyocytes still appear at centre stage, input and dialogue with other close neighbours, such as neurons, fibroblasts, and inflammatory and endothelial cells, are becoming a major focus with a high potential for drug target discovery (*Figure 1*).

3.1 Neuron/cardiomyocytes

It is well known that cardiomyocytes respond to catecholamines, but how these agonists are delivered to the heart is still not clearly understood. Innervation of the outer layer of the myocardium by sympathetic terminations is one of the important intercellular networks that is receiving renewed interest.[2] Close apposition to autonomic nerves confines the trophic effect of beta2-AR signalling to a specific layer of cardiomyocytes. As a consequence, innervated cells in the outer layer of the myocardium are larger and decrease in size, following a gradient across the ventricular wall. Denervation of the heart has profound effects on myocyte size, and this might have consequences in the recovery after cardiac transplantation.

The effects of beta2-AR signalling inside cardiomyocytes that implement the trophic response need to be integrated with the control of contractility and rhythm. In these processes, juxtaposition of neuronal terminals to specific cardiomyocyte membrane domains might help to compartmentalize signalling events. In these myocytic structures, scaffold proteins are coming to centre stage because of their ability to organize functionally interacting proteins in large multimeric complexes. PI3K signalling is known to control cell size, but it is becoming increasingly clear that it also influences the cAMP/PKA axis, impinging on inotropy and chronotropy. An emerging concept is the ability of PI3K-gamma to work as a scaffold protein for PKA,[3] thus integrating different aspects of beta2-AR-elicited cardiac responses.[4] The involvement of multiprotein signalling hubs appears a common feature not only for the signalling compartmentalization of beta-ARs, but also in alpha-ARs. Downstream alpha-AR receptors, the interaction between the two scaffold proteins AKAP-Lbc and PKN, act to spatially restrict MAPK activation, ultimately leading to p38 activation and cardiac hypertrophy.[5] Proof-of-concept studies with peptides disrupting complexes in adrenergic signalling are providing new promising avenues for treatment of heart failure. Targeting protein–protein interactions is a daunting task even for modern medicinal chemistry, but it is expected that future drugs may successfully modulate these interactions.

3.2 Fibroblasts/cardiomyocytes and vice versa

Another example of the versatility of alpha- and beta-adrenergic crosstalk is illustrated by the phenotype of mice with cardiac-specific over-expression of the third isotype of beta-adrenoceptors, beta3-AR; activation of this receptor (the expression of which is increased in human cardiomyopathies) attenuates hypertrophic remodelling *in vivo* and decreases the alpha-adrenergic increase in cell size and protein synthesis in cardiomyocytes; dissection of the signalling showed coupling of the beta3-AR to both nitric oxide synthase and AMP-activated protein kinase, with downstream activation of autophagy. Activation of beta3-AR in cardiac myocytes also attenuates fibrosis *in vivo*, as well as in superfusion experiments with fibroblasts *in vitro*; gel-free proteomic analysis of the secretome of alpha-AR-stimulated cardiac myocytes identified connective tissue growth factor (CTGF) as the main pro-fibrotic paracrine factor which is decreased under beta3-AR stimulation.

In another model of pulmonary hypertension, the TNF receptor superfamily member, fibroblast growth factor-inducible molecule 14, or Fn14, was identified as a key mediator of right ventricular fibrosis, which is attenuated in $Fn14^{-/-}$ mice; activation of Fn14 induced myofibroblast differentiation and collagen production. Notably, endothelin-1 released from pulmonary endothelial cells induced Fn14 expression in fibroblasts and may similarly regulate myocardial fibrosis in the left ventricle.

3.3 Endothelium and cardiomyocytes

Endothelial cells and cardiomyocytes actively interact to maximize oxygen and nutrient availability. Rising interest focuses on how physical exercise can tweak this crosstalk to improve muscle contractile function, increase endurance, and increase resistance to various diseases, including peripheral artery disease and cachexia resulting from heart failure or cancer. Dr Z. Arany summarized the role of the transcriptional co-activators PGC-1alpha and beta in multiple aspects of muscle metabolism and showed how both co-activators powerfully activate mitochondrial biogenesis and fatty acid oxidation. These factors are important regulators for the crosstalk between muscle and vascularization, thereby co-ordinating fuel consumption and metabolic demands with fuel delivery.[6] How PGC-1alpha acts in blood vessels seems to depend on highly cell type-specific co-factors and still require further investigation. However, a key feature in this system is the ability of PGC-1alpha to induce VEGF expression in muscle independent of hypoxia.[7] Although these actions are, thus, mediated by classical growth factors, unexpected communication factors between muscles and endothelium are emerging. For example, the GATA2 transcription

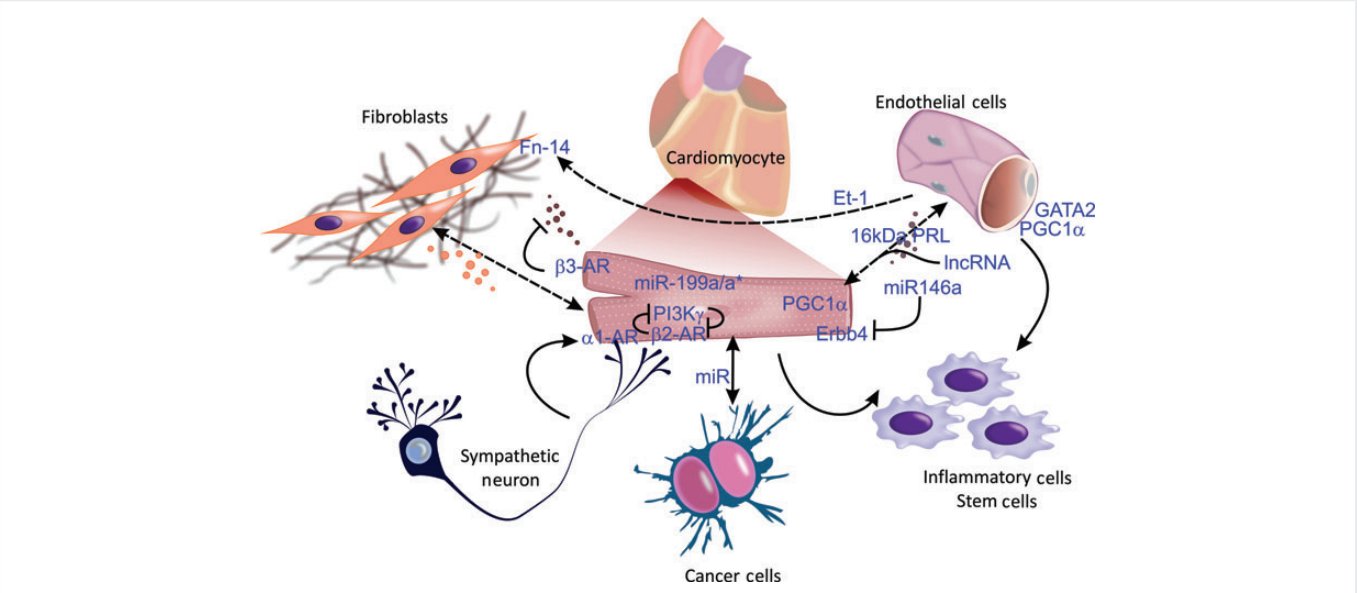

Figure 1 A selection of local and distal endocrine–paracrine signalling among the diverse cell types in or to the heart. Cardiac myocytes, fibroblasts, and endothelial and inflammatory or progenitor cells respond to local/systemic signals and exchange messenger molecules. In particular, microRNAs and non-coding long RNA molecules are packaged in exosomes, exported in the extracellular space and/or peripheral circulation, and then taken up by recipient cells. For example, exosomes enriched in miR-21* are exchanged between fibroblasts and cardiac myocytes and influence the hypertrophic response; exosomes loaded with miR-146a are released by endothelial cells activated by the 16 kDa fragment of prolactin and target the erbB4 pathway in cardiac myocytes, resulting in myocyte loss and cardiomyopathy; other miRNAs also drive the re-programming of progenitor or differentiated cardiac myocytes to promote regeneration. Other extracellular RNA (eRNA) species, including long non-coding RNA, may be released by inflammatory or endothelial cells upon activation of specific transcription factors (e.g. GATA2) or an ischaemic insult and modulate specific pathways in distant cells, such as pro-inflammatory TNF-alpha or p38-MAPK in cardiac myocytes. Some of these eRNA (and miRNAs?) may be amenable to degradation by RNAse 1 abundantly produced by endothelial cells, e.g. during ischaemic pre-conditioning. More classical endocrine or neurotransmitter molecules also modulate tissue remodelling through paracrine signals, e.g. catecholamines activate alpha1-AR in cardiac myocytes to produce the pro-fibrotic cytokine CTGF, a pathway negatively modulated by beta3-AR; norepinephrine released in the sympathetic post-ganglionic synaptic cleft produces trophic effects on cardiac myocytes through beta2-AR; and pulmonary endothelial cells release endothelin-1 that distally activates fibroblast growth factor-inducible molecule 14 (Fn14) expression driving right (and perhaps left) ventricular fibrosis. See text for more details.

factor can promote the expression and release of non-coding long RNAs from endothelial cells. These molecules are packaged in exosomes that can be taken up by cardiac myocytes leading to inactivation of stress-sensing kinases (J. Heineke, unpublished).

3.4 Inflammatory cells and cardiomyocytes

Inflammation has long been debated as a critical event in heart failure. Clearly, recruitment of leucocytes to failing hearts is a major cause of fibrosis and functional deterioration. However, how this inflammatory response is elicited in 'sterile' conditions is just starting to emerge. In an elegant study,[8] multiple genetically modified mouse models were used to demonstrate that mitochondrial DNA escaping from autophagy and DNAse II-mediated digestion (i) leads to Toll-like receptor 9-mediated inflammatory responses in cardiomyocytes, (ii) triggers the recruitment of inflammatory cells, and (iii) induces myocarditis and (iv) dilated cardiomyopathy. This supports the novel concept that mitochondrial DNA can act as an alarming signal and fits the idea that extracellular nucleic acids play major roles in shaping intercellular communication in inflammatory responses, particularly involving cells of the innate immune system. Another paracrine signalling pathway amplifying inflammation is by oncostatin M, released from monocytes/neutrophils.[9] This cytokine can exert multiple roles including the unexpected ability to induce a metabolic shift in cardiomyocytes from oxidative phosphorylation to glycolysis. However, it can also induce cardiomyocyte de-differentiation and expression/secretion of Reg 3beta. This poorly characterized cytokine, in turn, recruits macrophages and amplifies innate immune responses. On the other hand, cells of the adaptive immune system also participate in the control of cardiac remodelling, especially in response to an ischaemic insult. In myocardial infarction, a delicate balance of inflammatory responses is critically needed to favour the formation of a solid scar without exacerbating maladaptive remodelling. In this balance, the function of Treg cells has captured attention as a new crucial player. As Treg cells are known to dampen immune responses by instructing neighbouring cells to limit excessive inflammatory reactions, these new hints might revitalize a dropping interest in the therapeutic use of anti-inflammatory treatments in heart failure.

4. Novel and revisited networks: inter-organ tweeting

4.1 Distal preconditioning/communication

The endogenous cardioprotective phenomenon of remote ischaemic conditioning is the exemplary paradigm of inter-organ communication. In remote ischaemic conditioning, the application of one or more brief cycles of non-lethal ischaemia and reperfusion to an organ or tissue

renders the heart resistant to a sustained episode of lethal acute ischaemia–reperfusion injury (reviewed by the WGs;[10,11]).

The crucial discovery that limb ischaemia could be used to provide a remote pre-conditioning stimulus to protect the heart from acute myocardial infarction has greatly facilitated the translation of this therapeutic strategy of inter-organ protection into the clinical setting. Using a cuff placed on the upper arm or thigh to induce brief cycles of ischaemia and reperfusion has been reported to protect the heart in several different clinical scenarios of acute ischaemia–reperfusion injury. However, the mechanistic pathways linking the remote ischaemic conditioning-treated limb to the heart remains unclear and has been attributed to a neural–hormonal pathway as outlined by Andrew Redington during the meeting.[12] In this regard, several novel blood-borne factors were discussed as potential candidates for the circulating factor that mediates remote ischaemic conditioning-induced cardioprotection, including microparticles and/or exosomes (Sean Davidson, UK), miRNAs (possibly miR-144), and eRNAse (Klaus Preissner, Germany).

Of note is the opposing concept that factors released from tissues undergoing prolonged periods (>7 days) of ischaemia can negatively affect the heart. For example, rats with ischaemic limbs show impaired recovery of cardiac function after MI, potentially through the release by skeletal muscles of peptides such as dermcidin (Schiapparella, unpublished). *In vivo* down-regulation of dermcidin in rats protects the heart from an ischaemic insult, implying that this small peptide may act as a negative regulator of heart function in inter-organ communication

4.2 Crosstalk between solid tumours and the heart

Cachexia is a potent predictor of morbidity and mortality in patients with heart failure and advanced cancer. Pathological mechanisms in cachectic conditions in both disease types may include inflammation and peripheral organ dysfunction, which correlate with disease severity and clinical outcome and lead to cardiac atrophy. The importance of the systemic effects of tumour disease on the heart is understudied and most experimental models testing cardiotoxic effects of anti-cancer drugs have been performed in healthy mice. Denise Hilfiker-Kleiner presented data on how tumour cells affect the molecular phenotype of the heart for promoting sarcomere protein degradation.[13] Her own unpublished data emphasize that paracrine effects of tumour cells directly alter cardiac signalling pathways, which is in part responsible for cardiac atrophy. Down-regulation of miR-199a-5p, which leads to enhanced activity of the ubiquitin proteosomal system, seems to play a key role here.[14]

5. Can we manipulate communications to change the fate of cardiac diseases?

The ability of miRNAs to travel in the extracellular medium via exosomes and microvesicles represents a novel intercellular paracrine communication mechanism, impacting on multiple cellular functions. Owing to their ability to target multiple transcripts in a co-ordinated manner, miRNA, in fact, can profoundly affect cell fate.

One striking example was the report by M. Giacca's laboratory on the ability of miR-590 and miR-199a to re-program adult cardiomyocytes to proliferate, paving the way for a potential mechanism of cardiac regeneration.[15] Using an unbiased high-throughput functional screening of a human genomic miRNA library, a number of miRNAs that promoted neonatal cardiomyocyte proliferation were selected. When these miRNAs are stably over-expressed *in vivo* by AAV9 transduction, mouse hearts are significantly enlarged due to cardiomyocyte hyperplasia. Cardiomyocyte numbers are increased but neither hypertrophy nor increased levels of ANP can be detected. More interestingly, AAV9-driven expression of miR-590 or miR-199a in the heart after infarction exerts a marked beneficial effect in reducing infarct size and in improving cardiac function. Thus, manipulating miRNA expression can represent a powerful and innovative approach to triggering cardiac regeneration.

miRNA-based intercellular communication, however, can also be the basis of pathological cardiac malfunction, as reported by Ricke-Hoch from Hilfiker-Kleiner's laboratory. The 16 kDa N-terminal peptide of prolactin is a critical factor inducing peripartum cardiomyopathy, a life-threatening heart disease. The 16 kDa prolactin peptide induces increased expression of miR-146a in human endothelial cells, impairing their survival and angiogenic potential. At the same time, endothelial cells stimulated with the prolactin peptide release exosomes loaded with miR-146a that can deliver their cargo to cardiomyocytes. In these cells, miR-146a targets ErbB4, Notch1, and IRAK1 transcripts, blunting ErbB4 protective signalling and reducing cardiomyocyte metabolic activity. Importantly, blocking miR-146a with specific antagomirs significantly attenuates peripartum cardiomyopathy in a mouse model, thus suggesting that manipulation of intercellular communication represents a promising approach to changing the outcome of cardiac diseases.[16] Another example of miRNA-based communication in diet-induced hypercholesterolaemia is the down-regulation of miR-25 in the myocardium, which leads to oxidative stress and cardiac dysfunction via up-regulation of NOX4.[17]

6. Emerging questions and future directions

While presentations at the meeting illustrated the exciting new insights into pathways for intercellular communication and new mechanisms of intracellular response, they also raised a number of critical issues and questions to be addressed in the future.

For example, what is the limit of signal complexity? If cells can dispatch vesicles with complex contents, can cells dispatch messages inside their fully developed organelles? An intriguing paracrine signalling mechanism is the formation of tunnelling nanotubes from 'stem' cells to adult cardiomyocytes, with exchange of entire organelles such as mitochondria (Rodriguez, unpublished). This appears to lead to cardiomyocyte 're-programming' and protection from stress-induced apoptosis. Of note, no re-programming occurs if rho-0 stem cells are used, indicating dependence on functional mitochondria. Further experimental work is clearly needed to better assess the biological relevance of this way of communication.

Similarly, the emerging concept of exosome-mediated communication appears to require better methodological standardization. General protocols are needed to help in defining the specificity and selectivity of these processes. The mechanisms of selective recruitment of molecules into exosomes as well as target cell selection are still poorly understood. In fact, while recruitment of miRNAs in exosomes seems to be a selective process,[18] it remains to be defined how this is achieved in a specific cell. At the same time, whether and how exocytic vesicles can travel in the extracellular fluid and reach their cellular target is still poorly understood. Moreover, half-life and degradation processes of

circulating exosomes are not known. Understanding these mechanisms is of great importance for the development of novel targeted drug delivery approaches.

Cell communication is a well-established target for therapeutic intervention but signal transduction modulators often fail to make it to the clinic because of unpredicted effects in non-target organs. In fact, cell communication often relies on common mechanisms of intracellular response. However, how can these common signalling pathways elicit opposing responses in different cell types? Several of the reports (by Ricke-Hoch, Arany, Diviani, and Hirsch) suggest the presence of co-factors and signalling modules that vary depending on the cell type (e.g. PGC1α, NFkappaB, and PI3K-gamma). Understanding how these processes work at the molecular level might help to rationalize the frequently unpredictable side effects of global signal transduction pathway blockade and thus to obtain better drugs in the future.

In summary, this report has highlighted findings of an exciting meeting that set the stage for further exploration of a rapidly growing field with great translational potential. A more in-depth review of several of the topics will be presented in a Spotlight Issue on 'Heterocellular signalling and crosstalk in the heart in ischaemia and heart failure' to be published in May 2014.

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