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SYMPOSIUM REVIEW

Endothelial cell–cardiomyocyte crosstalk in heart development and disease

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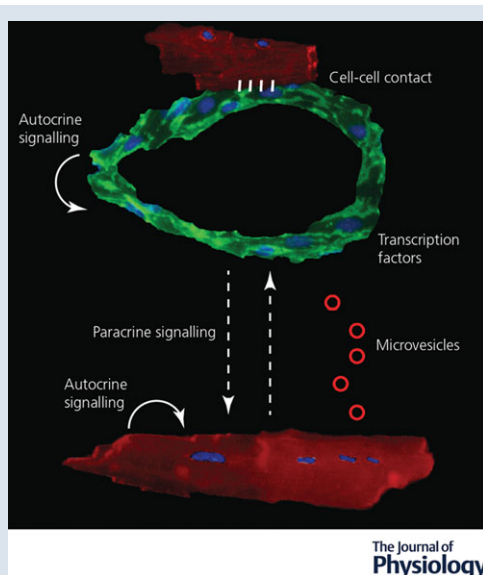
Abstract The crosstalk between endothelial cells and cardiomyocytes has emerged as a requisite for normal cardiac development, but also a key pathogenic player during the onset and progression of cardiac disease. Endothelial cells and cardiomyocytes are in close proximity and communicate through the secretion of paracrine signals, as well as through direct cell-to-cell contact. Here, we provide an overview of the endothelial cell–cardiomyocyte interactions controlling heart

Serena Zacchigna and Mauro Giacca serve as Professors of Molecular Biology at the University of Trieste, Italy and Principle Investigators of the Cardiovascular Biology and Molecular Medicine laboratories of the International Centre for Genetic Engineering and Biotechnology (ICGEB) in Trieste, Italy, respectively. Mauro Giacca also serves as the Director-General of the ICGEB. Andrea Colliva and Luca Braga are post-docs in the same laboratories. The main research interests of Serena Zacchigna focus around the mechanisms leading to new blood vessel formation in ischaemic muscles and hearts, whereas Mauro Giacca is a leader in the field of cardiac regeneration, having particular interest in understanding why mammalian cardiomyocytes permanently exit from the cell cycle at birth and how this can be overcome therapeutically. The two laboratories have been extensively collaborating over the last few years to explore the intercellular crosstalk between cardiomyocytes and cardiac endothelial cells.
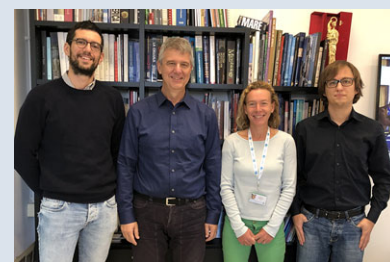
development and the main processes affecting the heart in normal and pathological conditions, including ischaemia, remodelling and metabolic dysfunction. We also discuss the possible role of these interactions in cardiac regeneration and encourage the further improvement of *in vitro* models able to reproduce the complex environment of the cardiac tissue, in order to better define the mechanisms by which endothelial cells and cardiomyocytes interact with a final aim of developing novel therapeutic opportunities.

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Abstract figure legend Cardiomyocytes (labelled in red by anti-α-actinin antibodies) and endothelial cells (labelled in green by anti-CD31 antibodies) crosstalk in multiple ways, including paracrine communication (dashed arrows) through either secreted molecules or vesicles (red circles), direct cell–cell contact (hinges) and autocrine signalling (curved arrows). This crosstalk plays an important role during embryonic development, normal post-natal life and several pathological conditions, thus representing a novel target for the treatment of cardiovascular disorders.

Endothelial cell–cardiomyocyte crosstalk during development

During early embryonic development, oxygen efficiently diffuses from the blood into the cardiac lumen to the thin ventricular walls. As the ventricular mass increases, oxygen diffusion is no longer sufficient to meet the requirement of the developing myocardium, and coronary vessels start to emerge and infiltrate the whole heart ($\sim$E10.5). Recent evidence has identified three possible origins for coronary endothelial cells (ECs), the proepicardium, the endocardium and the sinus venosus, although the relative contribution of each source still remains a matter of debate (Red-Horse *et al.* 2010; Wu *et al.* 2012; Chen *et al.* 2014). Whatever their origin, the ongoing molecular crosstalk between ECs and cardiomyocytes (CMs) in the developing myocardium is exciting in light of its possible relevance for cardiac regeneration, as the ultimate goal of cardiac regeneration is to rebuild a myocardial architecture with a fully integrated vascular network. Indeed, ECs contribute to the physiological and structural maturation of CMs, as shown by studies in engineered cardiac tissue that recapitulate heart development (described later). In this section, we will discuss the communications between CMs and ECs during embryonic development that might also be relevant for heart regeneration.

Cell-specific gene inactivation strategies have revealed multiple bidirectional signals occurring in the developing heart (Fig. 1).

On the one hand, CMs secrete a few growth factors able to modulate the growth of blood vessels. Vascular endothelial growth factor (VEGF)-A, herein referred to as VEGF, is a key controller of angiogenesis. The deletion of the gene encoding this factor, specifically in CMs, determines reduced number of coronary microvessels, thinned ventricular walls, depressed basal contractile function, induction of hypoxia-responsive genes

involved in energy metabolism and abnormal response to β-adrenergic stimulation (Giordano *et al.* 2001). Despite the fact that CMs constitute less than one-third of the total number of cells in the heart, the CM-specific deletion of the VEGF gene decreases total mRNA levels by more than 85%, pointing to cardiac myocytes as the major source of this growth factor (Giordano *et al.* 2001). Of note, the structure of the coronary arteries is not affected in this condition, indicating that different mechanisms support the development of both the cardiac microvasculature and the major epicardial coronary arteries. Beside stimulating angiogenesis, CM-derived VEGF also controls endocardial-to-mesenchymal transformation during the formation of cardiac cushions (i.e. the endocardium-derived structures that build the heart septa and valves). This requires endocardial calcineurin/nuclear factor of activated T cells (NFAT) signalling to repress VEGF expression in the myocardium underlying the site of prospective valve formation ($\sim$E9). This repression of VEGF is essential for endocardial cells to transform into mesenchymal cells (Chang *et al.* 2004). Soon after the onset of endocardial cushion formation, VEGF is specifically upregulated in the atrio-ventricular (AV) field of the heart tube, where it negatively regulates endocardial-to-mesenchymal transformation and terminates the formation of endocardial cushions (Dor *et al.* 2001). CM-derived Angiopoetin-1 (Ang1) also plays a major role in cardiac vessel development. Transgenic mice expressing Ang1 in CMs in an inducible manner die at $\sim$E12.5 because of major cardiac malformations, including dilated atria, significant thinning of the myocardial wall, outflow tract collapse and absence of coronary arteries (Ward *et al.* 2004).

On the other hand, developing ECs send multiple signals that contribute to myocardial development. For instance, EC-specific deletion of the platelet-derived growth factor (PDGF)-B gene causes major cardiac

abnormalities, including myocardial wall thinning, chamber dilation, septal defects and hypertrabeculation (Bjarnegard *et al.* 2004). Similar developmental defects are observed in mice with endothelium-restricted deletion of the tumour-suppressor gene neurofibromin (*NF1*), which results in dilated endocardial cushions, septal abnormalities and double-outlet right ventricle (Gitler *et al.* 2003). Of note, these defects also occur in *NF1* knock-out mice, but not in mice missing NF1 in CMs, pointing to ECs as the most relevant source of NF1 for proper cardiac development (Gitler *et al.* 2003). Yet, the pathway linking NF1 expression in ECs to proper CM organization and cardiac tissue formation during development is largely unknown.

Finally, perhaps the most characterized pathway of EC–CM crosstalk during development is neuregulin (NRG) signalling. ECs in the developing endocardium secrete abundant levels of NRG-1, which binds the ErbB2 and ErbB4 receptors expressed by CMs, essentially contributing to the formation of both endocardial cushions and myocardial trabeculae (Meyer & Birchmeier, 1995). This essential role of NRG-1 in promoting trabeculation is consistent with its capacity to induce the proliferation, survival and hypertrophy of neonatal cardiomyocytes (Zhao *et al.* 1998).

Endothelial cell–cardiomyocyte crosstalk during normal post-natal life

In the adult heart, capillaries are densely distributed throughout the myocardium, with the number of ECs being approximately 3 times higher than that of CMs. Yet, the large CM mass exceeds that of the smaller ECs by 25 times (Brutsaert, 2003). While ECs within the coronary arteries functions as in any other arterial vascular system in the body, essentially providing blood supply to the myocardium, capillary and endocardial ECs are in close proximity to CMs and provide multiple autocrine and paracrine signals controlling cardiac function (Fig. 2).

In the normal adult heart, ECs release various factors controlling CM contractility. A first player is nitric oxide (NO), which is synthetized by the NO synthase class of enzymes. Three isoforms of NO synthase exist, including the neuronal (NOS1), the inducible (NOS2)
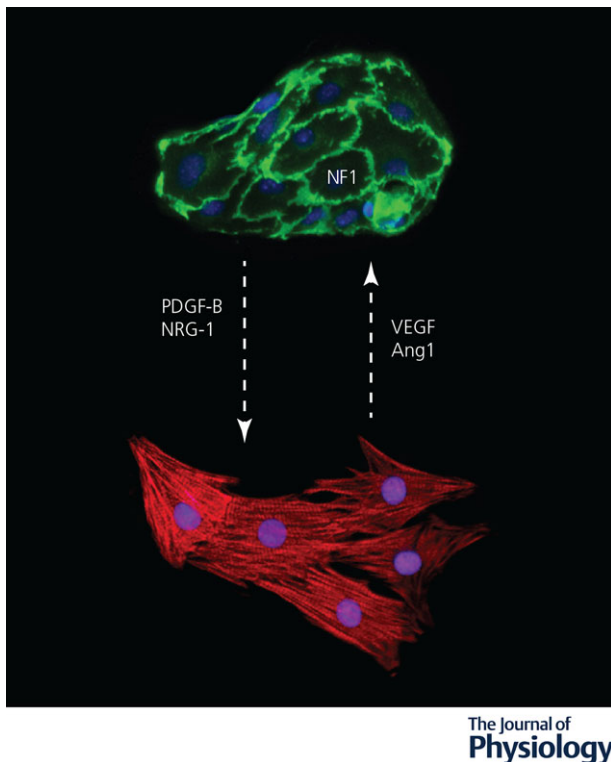

Figure 1. Endothelial cell–cardiomyocyte crosstalk during development
Secreted molecules and transcription factors mediate a bidirectional signalling between developing endothelial cells (labelled by anti-CD31 antibodies in green) and cardiomyocytes (labelled by anti-α-actinin antibodies in red). The dashed arrows indicate the direction of the signals. Nuclei are stained in blue by 4′,6-diamidino-2-phenylindole (DAPI). Ang1, angiopoietin-1; NF1, neurofibromin-1; NRG-1, neuregulin-1; PDGF, platelet-derived growth factor; VEGF, vascular endothelial growth factor.
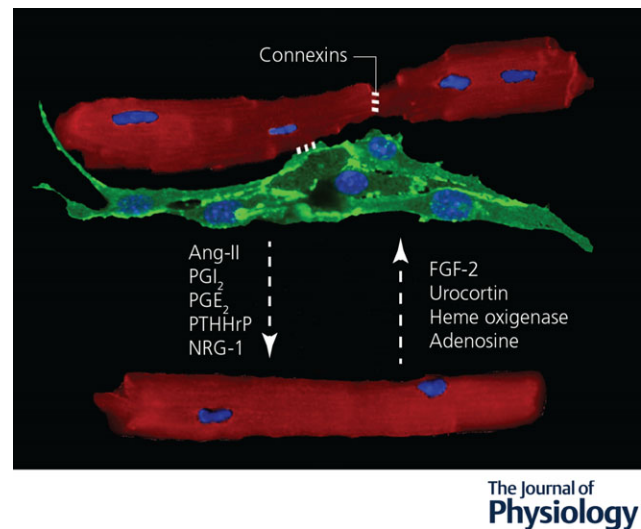

Figure 2. Endothelial cell–cardiomyocyte crosstalk during post-natal life
Secreted molecules and gap junctions mediate a bidirectional signalling between adult endothelial cells (labelled by anti-CD31 antibodies in green) and cardiomyocytes (labelled by anti-α-actinin antibodies in red). Nuclei are stained in blue by DAPI. The dashed arrows indicate the direction of the signals. Molecules secreted by endothelial cells are indicated on the left, those secreted by cardiomyocytes on the right, while those secreted by both cell types are in the middle. Ang-II, angiotensin-II; ET-1, endothelin-1; FGF2, fibroblast growth factor 2; NO, nitric oxide; NRG-1, neuregulin-1; PGE_2, prostaglandin E_2; PGI_2, prostacyclin; PTHrP, parathyroid hormone-related peptide.

and the endothelial (NOS3) isoforms. NOS1 and NOS3 are constitutively active and produce NO in response to calcium, whereas NOS2 is inducible and increases its production of NO upon adequate stimulation (i.e. cytokines) and independently from calcium activation. In the adult heart, NO is mainly produced by ECs and, consistent with its activity on vascular smooth muscle cells, controls the onset of ventricular relaxation (positive lusitropy), thus optimizing pump function at every cardiac contraction (Hsieh *et al.* 2006*b*). This effect has been attributed to cGMP- and protein kinase G (PKG)-mediated phosphorylation of troponin I, leading to myofilament calcium desensitization, relaxation hastening and improved distensibility (Layland *et al.* 2002). In contrast, the inotropic effect exerted by low concentrations of NO relies on mechanisms independent of cGMP and possibly involving the activation of calcium and potassium channels through either G protein-mediated response (Layland *et al.* 2002), formation of peroxynitrite (Malan *et al.* 2003) or direct channel *S*-nitrosylation by NO (Kohr *et al.* 2011). Generation of peroxynitrite is maximal in conditions of oxidative stress, when high levels of super-oxide anion react with NO. At the same time, ECs are able to protect CM from oxidative stress by modulating several buffering systems at different levels, such as ATP-binding cassette transporter subfamily G member 2 (ABCG2) for the secretion of glutathione (Higashikuni *et al.* 2012), NRG-1 (Kuramochi *et al.* 2004) (see also below), tetrahydrobiopterin (BH4) (Leucker *et al.* 2013) and thioredoxin (Trx) (Subramani *et al.* 2016). While most of these buffering systems have been discovered in conditions of cardiac stress (i.e. ischaemia–reperfusion injury and pressure overload), it is plausible that they play a major role in maintaining cardiac homeostasis.

Besides ECs, CMs also express the different endothelial NO synthases, although at lower levels compared to ECs. This supports a possible autocrine NO signalling in CMs, warranting further studies using transgenic animals with EC- and CM-specific deletion of NO signalling.

Cardiac ECs also produce abundant levels of endothelin-1 (ET-1), a potent vasoactive peptide. In the adult heart, ET-1 can bind either ET_B receptors expressed by ECs or ET_A receptors expressed by smooth muscle cells and CMs. The activation of ET_B receptors stimulates the release of nitric oxide and prostanoids, also preventing apoptosis (Niwa *et al.* 2000). In contrast, stimulated ET_A receptors induce cell contraction by increasing intra-cellular calcium in both smooth muscle cells and CMs (Rich & McLaughlin, 2003). Yet, CM-specific ET_A receptor knockout mice have normal cardiac function and physio-logically respond to either angiotensin II (Ang-II) or iso-proterenol, indicating a minor role of this pathway in the homeostasis of the adult heart. While the ET_B receptor is traditionally considered to be mainly expressed by ECs and its mRNA was not detected in human CMs (Modesti

et al. 1999), recent evidence indicates that CM-specific ET_B receptor knockout mice are particularly resistant to extreme hypoxia, consistent with previous reports showing the presence of ET_B on CM nuclear membrane and its role controlling nuclear calcium signalling in an intracrine manner (Merlen *et al.* 2013; Stobdan *et al.* 2018).

EC Ang-II, prostacyclin (PGI_2) and prostaglandin $(PG)E_2$ additionally contribute to cardiac contraction control in physiological conditions, with different responses observed dependent on dose, species and experimental condition (*in vivo versus* isolated hearts/CMs) (Brutsaert, 2003).

Beside producing small molecules and peptides, ECs also secrete numerous proteins, including growth factors and *matricellular proteins*, able to modulate CM structure and function (Frangogiannis, 2012). For instance, cardiac ECs express and secrete parathyroid hormone-related peptide (PTHrP), which exerts positive inotropic, chronotropic and lusitropic effects in adult ventricular CMs (Jansen *et al.* 2003). Over the last decades, numerous publications have described the cardioprotective role of a series of factors endogenously produced in the heart, including periostin, tenascin-C, thrombospondins, frizzled-related protein 3, insulin-like growth factor 1, connective tissue growth factor, Dickkopf-3, bone morphogenetic protein-2 and -4, interleukin-1β and -6, placental growth factor (PLGF), leukemia inhibitory factor, Wnt1-induced secreted protein, midkine and adrenomedullin, without investigating their cellular source. Considering the abundance and location of cardiac ECs, it is reasonable that these cells represent an important source of most of these cardioprotective proteins, able to modulate cardiac contractility and CM remodelling under stress conditions (Segers *et al.* 2018).

In addition to its major role during development, NRG-1 is also essential for normal function of the adult heart, where it continues to be expressed by microvascular ECs, but not by those laying large vessels. In accordance, both the ErbB2 and ErbB4 receptors are expressed by adult CMs. The first evidence for a role of the NRG-1 signalling pathway in cardiac homeostasis came from clinical studies in patients with metastatic mammary carcinoma treated with an anthracycline in combination with trastuzumab (Herceptin), a monoclonal antibody against ErbB2. This treatment is associated with a particularly high risk of cardiotoxic cardiomyopathy and heart failure (Slamon *et al.* 2001), consistent with the experimental observation that postnatal disruption of NRG-1/ErbB signalling by gene targeting in mice leads to dilated cardiomyopathy (Crone *et al.* 2002) .

Much less is known about the signals produced by CMs and able to regulate EC biology. Besides NO, additional CM-secreted molecules, including ET1, fibroblast growth factor (FGF)-2, urocortin, haem oxygenase and adenosine, are known to control the vascular tone,

although the mechanism of their action and in particular whether they mainly target ECs, smooth muscle cells or both, remain not fully understood (Tirziu *et al.* 2010). Recent data indicate that the release of pro-angiogenic molecules by CMs in the adult heart is constitutively inhibited by a mechanism requiring the presence of the transcription factor RBPJ (recombination signal binding protein for immunoglobulin *κ* J region) and inhibition of hypoxia-inducible factors (HIFs), but independent from Notch1. Deletion of *rbpj* in cardiomyocytes results in VEGF up-regulation, increased capillary density and cardioprotection from hypoxia (Diaz-Trelles *et al.* 2016). Thus, CMs can produce both pro-angiogenic and angiostatic factors, the activity of which has to be finely tuned in normal and stress conditions.

In addition to soluble factors, intercellular communications can occur via extracellular vesicles (Colombo *et al.* 2014). These vesicles shuttle a wide range of functional lipids, proteins, mRNAs, microRNAs and long non-coding RNAs. In particular, the relevance of exosomal transfer in the communication between CMs and ECs has so far been studied *in vitro* and in response to pathological stimuli, such as diabetes or ischaemia, and is therefore discussed in the next sections.

Although largely unexplored, direct cell–cell contact might occur between ECs and CMs. Both cell types express connexins, proteins that form gap junctions in the plasma membrane, allowing the movement of ions, second messengers and metabolites. The most abundant connexins (Cx) in the heart are Cx43, Cx40 and Cx45, which are variably expressed by CMs throughout the different cardiac regions and are responsible for their coupling during normal cardiac rhythm. ECs also express various connexins, including Cx37, Cx40 and Cx43. The first evidence for the existence of CM–EC gap junctions was provided in 2004 by an *in vitro* study showing that Cx43 junctions were associated to improved CM survival and structural organization (Narmoneva *et al.* 2004). Whilst the functional relevance of this heterocellular gap junction has never been documented *in vivo*, impaired EC–CM coupling has been proposed as one of the mechanisms leading to arrhythmia and heart failure in patients with hyperhomocysteinaemia. In these patients, the elevated levels of homocysteine determine Cx43 degradation and disruption of the gap junctions, together with peri-vascular fibrosis, both of which could reasonably contribute to the high risk for sudden cardiac death that characterizes this condition (Givvimani *et al.* 2011).

Endothelial cell–cardiomyocyte crosstalk in diabetes and metabolic dysfunction

The adult heart is composed of cells that are markedly heterogeneous in their metabolism. CMs are the most energy and oxygen demanding cells in the heart due to their continuous contraction. In healthy conditions, CMs produce most of their ATP through oxidative phosphorylation (OxPhos), using mainly fatty acids (70%), but also other substrates (glucose, ketones and amino acids) as carbon sources (Kolwicz *et al.* 2013). ECs instead produce 85% of their ATP anaerobically through glycolysis, limiting fatty acid oxidation (FAO) and OxPhos to favour the biosynthesis of nucleotides and other metabolites required for replication and angiogenesis (Potente & Carmeliet, 2017).

Alterations in cardiac metabolism during metabolic syndrome and diabetes are driving forces leading to heart failure, as underscored by the 50–80% of deaths for cardio-vascular diseases in patients with diabetes (http://www.who.int/healthinfo/global_burden_disease/estimates/en/). Although most of these cases are due to atherosclerosis, 'diabetic cardiomyopathy', i.e. myocardial dysfunction in the absence of general cardiovascular risk factors, is emerging as a significant cause of heart failure in diabetic patients.

In type 2 diabetes, which often involves not only hyperglycaemia but also increased levels of circulating triglycerides, both CMs and ECs undergo massive changes in their metabolism. In particular CMs reduce the expression of GLUT4, resulting in decreased glucose consumption, together with increased fatty acid uptake and oxidation (An & Rodrigues, 2006) through the activation of either AMP-activated protein kinase (acute diabetes) or peroxisome proliferator-activated receptor *α* (chronic diabetes) (Finck *et al.* 2002; Kewalramani *et al.* 2007). This leads to excessive production of ATP, with consequent down-regulation of FAO-associated genes and accumulation of lipids, leading to cardiac 'lipotoxicity' and diastolic dysfunction. Moreover, the low level of glucose oxidation increases the metabolic flux toward accessory pathways, such as the polyol and hexosamine biosynthetic pathway, leading to accumulation of advanced end glycation products (AGE) and increased protein *O*-GlcNAcylation (Fulop *et al.* 2007).

On the other hand, ECs exposed to hyperglycaemia experience massive oxidative stress, due to their incapacity to metabolize the excess of glucose in the blood (Potente & Carmeliet, 2017). In response to oxidative stress, polyADP-ribose polymerase 1 inactivates glyceraldehyde phosphate dehydrogenase, diverting the glucose flux from glycolysis to accessory pathways and leading to AGE accumulation, similar to what is observed in CMs, in addition to increased superoxide levels (Du *et al.* 2003). The increased reactive oxygen species (ROS) levels induce eNOS uncoupling and generation of peroxynitrite, leading to decreased NO availability and vasoconstriction (Sasaki *et al.* 2008). These metabolic shifts lead to an overall increase in oxygen consumption, vascular dysfunction and toxic lipid accumulation sustained by oxygen shortfall and consequent FAO inhibition.

Clarifying the intercellular crosstalk between CMs and ECs in modulating their respective metabolism could suggest innovative approaches to prevent heart failure in diabetic patients (Fig. 3). Most of the signals from CMs to ECs modulate the 'gatekeeper' function of the endothelium in controlling the uptake of fatty acids from the bloodstream. Fatty acids circulate inside lipoproteins and have to be hydrolysed to free fatty acids by lipoprotein lipase (LPL), anchored to the luminal side of the EC (Olivecrona & Olivecrona, 2010). In the heart, CMs are the major source of LPL, which is secreted and anchored to the heparan sulfate proteoglycans associated with the myocyte membrane. ECs secrete heparanase to release LPLs, which are internalized through glycosylphosphatidylinositol-anchored high density lipoprotein binding protein 1 (GPIHBP1) and eventually translocated into the luminal side (Camps *et al.* 1990). High glucose levels induce the re-localization of endothelial heparanase from the perinuclear compartment to the plasma membrane and its secretion by coronary ECs, consistent with the increased LPL translocation into the vessel lumen observed in mouse models of diabetes (Wang *et al.* 2009).
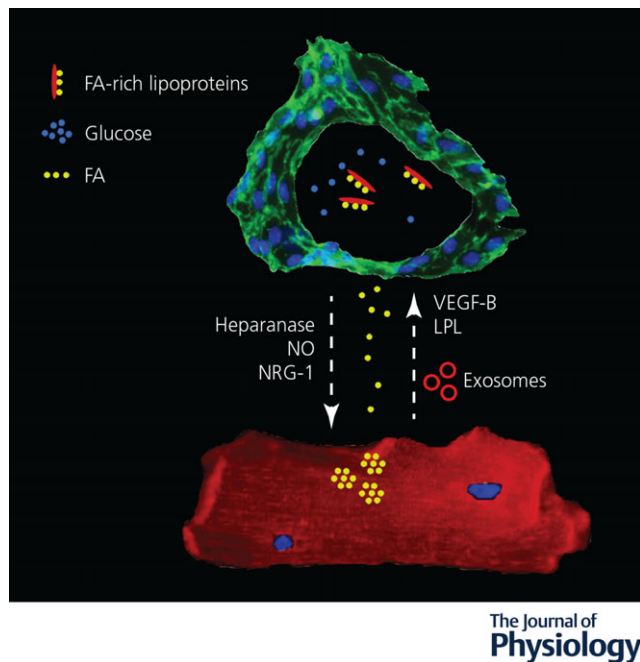

Figure 3. Endothelial cell–cardiomyocyte crosstalk in diabetes and metabolic dysfunction
Secreted molecules and exosomes (red circles) mediate a bidirectional signalling between endothelial cells (labelled by anti-CD31 antibodies in green) and cardiomyocytes (labelled by anti-α-actinin antibodies in red) upon exposure to high levels of glucose (blue dots) and fatty acids (yellow dots). FA, fatty acids; LPL, lipoprotein lipase; NO, nitric oxide; NRG-1, neuregulin-1; VEGF-B, vascular endothelial growth factor-B.

VEGF-B is emerging as one of the key regulator of FA uptake in the diseased heart. This factor is abundantly expressed by adult CMs and binds the VEGFR-1 and neuropilin-1 (NRP-1) receptors, expressed by ECs. VEGF-B$^{-/-}$ mice show decreased expression of fatty acid transporter (FATP)-3 and -4 in ECs and reduced lipid droplets in CMs. Consistently, the exogenous administration of VEGF-B enhances FA uptake in cultured ECs by biding to VEGFR-1 and NRP-1. When fed a high fat diet or crossed with diabetic mouse lines, VEGF-B$^{-/-}$ mice maintain an increased insulin sensitivity, with improved blood lipid and glucose profiles (Hagberg *et al.* 2012). However, the CM-specific overexpression of VEGF-B does not alter FA uptake and lipid accumulation, but rather leads to a metabolic shift from FAO to glucose oxidation, as well as to an expanded coronary tree through the activation of VEGFR-2 in cardiac ECs (Kivela *et al.* 2014). Remarkably, VEGF-B expression is decreased in different human cardiomyopathies, consistent with its major role in controlling both CM and EC metabolism in both healthy and diseased heart (Bry *et al.* 2014).

In addition to secreted proteins, exosomes produced by CMs have been shown to regulate the metabolism of ECs, particularly in response to altered glucose levels. Exosomes derived from glucose-starved CMs increase the proliferation and tube formation capacity of human umbilical vein ECs (HUVECs) and stimulate both glucose uptake and glycolysis in cardiac microvascular ECs *in vitro* (Garcia *et al.* 2016). Furthermore, CMs isolated from the Goto–Kakizaki rat, a model of type 2 diabetes, inhibit the angiogenic properties of cardiac ECs through the exosomal transfer of miR-320 (Wang *et al.* 2014).

Do ECs regulate CM metabolism? Although very little is known in this respect, one possible player is NRG-1, which is normally secreted by ECs and upregulated upon ischaemia, as mentioned earlier. This up-regulation appears inhibited in type 1 diabetes, as diabetic rats fail to overexpress NRG-1 and exhibit impaired membrane localization of ErbB2 and ErbB4 receptors in CMs after myocardial infarction. Of note, NRG-1 increases glucose uptake in cultured CMs via ErbB2–ErbB4 heterodimers, phosphoinositide 3-kinase α and Akt.

Another likely player in the process is NO, which is abundantly secreted by ECs and stimulates glucose uptake by CMs via a cGMP-dependent mechanism (Tada *et al.* 2000). Whether this crosstalk is relevant in disease conditions characterized by poor NO availability, including diabetes and hypercholesterolaemia, is still unknown.

Endothelial cell–cardiomyocyte crosstalk during cardiac remodelling

Despite its highly structured architecture, the heart is an organ capable of extensive remodelling. Immediately after

birth, the heart faces a pressure overload, which physiologically induces CM hypertrophy, resulting in the gross enlargement of the organ. In adult life, the same happens to the mother's heart during pregnancy as well as to the hearts of athletes as a result of extreme and/or repetitive exercise. Physiological hypertrophy is reversible and considered an adaptive process aimed at decreasing the ventricular wall stress by increasing its thickness. Morphologically, the increase in CM area is accompanied by enhanced angiogenesis in the absence of fibrosis. In contrast, pathological hypertrophy is irreversible and is typically associated with loss of myocytes, fibrotic replacement, decreased capillary density, cardiac dysfunction, and increased risk of heart failure and sudden death. It usually represents the evolution of a first phase of compensatory hypertrophy, sustained by a prolonged wall stress and caused by various disorders, including systemic or pulmonary hypertension, ischaemic, genetic or metabolic cardiomyopathy.

As physiological hypertrophy normally occurs during the first weeks after birth supporting the post-natal growth of the heart, it is not surprising that the same molecules mediating EC–CM crosstalk during development also play a major role in this type of cardiac remodelling (Fig. 4). On the one hand, CMs undergoing a first phase

Figure 4. Endothelial cell–cardiomyocyte crosstalk during cardiac remodelling
Secreted molecules mediate a bidirectional signalling between adult endothelial cells (labelled by anti-CD31 antibodies in green) and cardiomyocytes (labelled by anti-α-actinin antibodies in red) during pathological remodelling. Nuclei are stained in blue by DAPI. The dashed arrows indicate the direction of the signals. Molecules acting in autocrine manner are indicated by curved arrows. APLN, apelin; ET-1, endothelin-1; IL-6, interleukin-6; NO, nitric oxide; NRG-1, neuregulin-1; PLGF, placental growth factor; VEGF, vascular endothelial growth factor.

of compensatory hypertrophy produce pro-angiogenic factors (i.e. VEGF) (Zheng *et al.* 2001; Zentilin *et al.* 2010) able to stimulate blood vessel growth to supply enough nutrients to the growing myocardial mass. On the other hand, enhanced coronary angiogenesis *per se*, as can be achieved by cardiac-specific overexpression of angiogenic factors, leads to cardiac hypertrophy in the absence of any additional pro-hypertrophic stimulation (Tirziu *et al.* 2007). The precise mechanism by which increased angiogenesis induces CM hypertrophy still needs to be fully elucidated, but it reasonably involves the secretion of EC-derived pro-hypertrophic factors.

In pathological conditions, ECs control and promote cardiac remodelling by the production of secreted factors with both paracrine and autocrine effects. To limit the degree of complexity, here we keep the focus on signals secreted by cardiac ECs exerting a direct effect on CMs (Fig. 4).

Besides its role in controlling cardiac homeostasis, as discussed above, NO blunts the hypertrophic response of cultured CMs exposed to potent pro-hypertrophic agents, such as noradrenaline and Ang-II. Accordingly, $NOS3^{-/-}$ mice spontaneously develop cardiac hypertrophy and exhibit exaggerated hypertrophy after both myocardial infarction and chronic pressure overload compared to wild-type mice (Scherrer-Crosbie *et al.* 2001).

As mentioned before, NO stimulates the production of cGMP by activating soluble guanylyl cyclase. Increased levels of cGMP activate PKG, which is a negative regulator of the pro-hypertrophic transcriptional regulator NFAT (Fiedler *et al.* 2002). This axis is emerging as a key player in the development of HF with preserved ejection fraction (HFpEF), a syndromic condition characterized by the impairment of proper left ventricular filling during diastole due to increased wall stiffness. In the context of HFpEF, various comorbidities, especially obesity, elicit a systemic pro-inflammatory state, which is sensed by ECs that respond by producing ROS, which in turn limit NO availability, as mentioned before. Limited NO availability results in low cGMP levels and impaired PKG activity, leading to hypo-phosphorylation of titin (Fiedler *et al.* 2002) and subsequent increase in CM stiffness.

Release of inflammatory cytokines, including tumour necrosis factor-α, interleukin (IL)-1, IL-6 and IL-11, represent an interesting example of multicellular crosstalk during pathological cardiac remodelling. These molecules can be produced by immune cells but also by other cell types including ECs. Besides the aforementioned effect of inflammatory mediators on NO availability, some of these cytokines play a more prominent role during pathological cardiac remodelling. For instance, IL-6 is emerging as a pro-hypertrophic and pro-fibrotic cytokine, as IL-6 knockout mice undergo attenuated cardiac remodelling in response to both pressure overload (Zhao *et al.* 2016) and administration of Ang-II, a potent mediator of CM
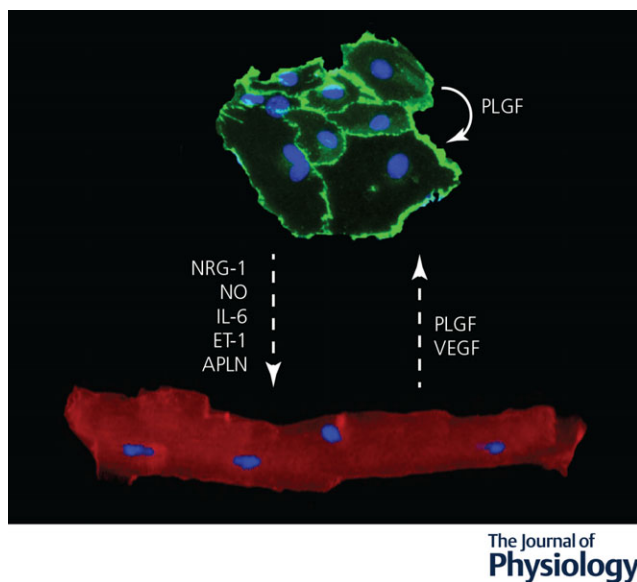
hypertrophy (Gonzalez *et al.* 2015). While the relevant cellular source of IL-6 exerting these effects is still undefined, interesting insights into the molecular mechanisms involved have been shed by studying the Noonan syndrome, which is determined by mutations in the Ras–extracellular signal-regulated kinase (ERK) pathway. Patients in which the disease is caused by kinase-activating *RAF1* alleles typically develop pathological left ventricular hypertrophy, which is well reproduced in knock-in mice harbouring the *Raf1*(L613V/+) mutation. Interestingly, the EC-specific expression of the mutant protein is sufficient to drive cardiac hypertrophy and the secretion of IL-6 by *Raf1*(L613V/+)-expressing ECs is essential in promoting CM hypertrophy, clearly pointing to this interleukin as a key mediator of the EC–CM crosstalk during cardiac remodelling (Yin *et al.* 2017).

As mentioned earlier, ET-1 is a potent vasoconstrictive peptide secreted by ECs, acting on ECs and CMs, which both express ET receptors. Evidence that it may act as a paracrine regulator of cardiac hypertrophy first came from studies on cultured neonatal rat ventricular CMs, which responded to ET-1 by increasing protein synthesis and cell size, as well as by reactivating the fetal gene programme (Ito *et al.* 1991). In final stages of pressure overload-induced cardiac hypertrophy, ET-1 is upregulated and is likely responsible for the enhanced vasoconstriction and compromised endothelial NO function, which induces oxidative stress and eventually leads to both CM loss and vascular degeneration (Tsai *et al.* 2017). Yet, the EC-specific deletion of ET-1 worsens the degree of hypertrophy and cardiac function in the same animal model, indicating that either excessive or insufficient ET-1 signalling is detrimental in this context and that adequate levels of ET-1 are required for a proper cardiac adaptation of the heart to the increased pressure (Heiden *et al.* 2014). Furthermore, ET-1 is up-regulated by ECs in response to Ang-II through a mechanism involving the receptor-mediated mobilization of intracellular calcium and activation of protein kinase C (Emori *et al.* 1991). Finally, ECs increase the production of ET-1 in response to mechanical stretch, which invariably occurs during pressure overload (Hasdai *et al.* 1997). Collectively, these data indicate that ECs sense various signals associated to increased blood pressure, i.e. Ang-II during renovascular hypertension, or more generally mechanical stretch, and respond by releasing ET-1, which is able to induce CM hypertrophy (van Wamel *et al.* 2001). Despite these multiple indications of the involvement of the ET-1 system in the pathogenesis of cardiac dysfunction, results with ET receptor antagonists in congestive heart failure have been disappointing. While some earlier studies have suggested benefit (Luscher *et al.* 2002), larger studies have been neutral, with indication of possible worsening of the patient's conditions (O'Connor

et al. 2003), similar to what has been more recently observed in patients with HFpEF and pulmonary hypertension (Koller *et al.* 2017).

NRG-1 secreted by ECs also induces CM hypertrophic growth, consistent with the observation that mice with cardiac specific deletion of the ErbB2 receptor develop dilated cardiomyopathy. Inhibition of the NRG-1/ErbB signalling during pregnancy results in left ventricle dilation and decreased cardiac output, associated with decreased survival to pregnancy (Lemmens *et al.* 2011). Thus, EC–CM crosstalk via NRG-1/ErbB signalling also plays a crucial role during cardiac adaptation to stress. During both pregnancy and pressure overload, levels of PLGF are increased upon production by both CMs and ECs. This induces capillary growth and fibroblast proliferation, which secondarily supports cardiac hypertrophy through intermediate paracrine growth factors, including IL-6 (Yao *et al.* 2005; Accornero *et al.* 2011).

Finally, a major player in the EC–CM crosstalk during cardiac remodelling is Apelin (APLN), a secreted protein binding to the G-protein coupled receptor APJ. In the heart, APLN is mainly produced by endocardial cells and ECs, while its APJ receptor is expressed by CMs, ECs and smooth muscle cells (Kleinz *et al.* 2005). Consistent with the broad expression of the receptor, the acute administration of APLN reduces the vasomotor tone with positive chronotropic and inotropic effect, as well as a trend towards an increase in lusitropy (Ashley *et al.* 2005). While initial biochemical studies suggested a beneficial effect of APLN during cardiac remodelling based on the ability of APJ to activate Gαi, thus activating cardio-protective pathways (Rockman *et al.* 2002), genetic models have recently revealed a more complex scenario. While APJ knockout mice are partially protected from pressure-overload cardiac hypertrophy and transition to heart failure, APLN knockout mice develop a normal hypertrophic response. This apparent discrepancy suggests that the APJ receptor may be activated also by APLN-independent mechanisms. In fact, the APJ receptor can also act as a mechanosensor, able to transduce pro-hypertrophic stimuli in a G-protein-independent, but β-arrestin-dependent manner, upon cell membrane stretch (Scimia *et al.* 2012). Recently, the generation of both CM- and EC-specific APJ knockout animals has revealed that its role as ligand-independent stretch sensor controlling cardiac hypertrophy is essentially exerted on CMs, with minimal, if not null, role of ECs in this process (Parikh *et al.* 2018).

Endothelial cell–cardiomyocyte crosstalk during ischaemia

Cardiac ischaemia is a clinical condition in which the importance of CM-derived factors influencing the

vasculature is crucial. Increasing evidence indicates that a major, if not the only, mechanism by which cell therapy can be beneficial after myocardial infarction is by replenishing cytokines and growth factors that can no longer be produced by the dead CMs and that act on endothelial cells inducing angiogenesis. At the same time, CMs also convey anti-angiogenic signals to ECs. As mentioned before, RBPJ in CMs antagonizes HIFs and inhibits the secretion of pro-angiogenic molecules, thereby desensitizing the angiogenic response to hypoxia (Diaz-Trelles *et al.* 2016).

Whether ischaemic ECs convey protective or toxic signals (or both) to CMs is controversial. On the one hand, multiple evidence supports the idea that ECs can improve CM survival after ischaemia. The generation of ROS, including H_2O_2, in response to ischaemia, stimulates the release of NRG-1 from ECs and the activation of ErbB4 signalling in CMs, with their protection from apoptotic cell death induced by ischaemia–reperfusion (Kuramochi *et al.* 2004). Accordingly, NRG-1 heterozygous knockout mice are hypersensitive to doxorubicin-induced cardio-toxicity (Liu *et al.* 2005), which is known to be sustained by the generation of ROS, similar to ischaemia–reperfusion injury. On the other hand, under hypoxic conditions cardiac ECs release transforming growth factor β, which in turn promotes endothelial to mesenchymal transition (EndoMT) in an autocrine loop, but also induces apoptosis in CMs (Sniegon *et al.* 2017). While several studies have reported that EndoMT contributes to the development of cardiac fibrosis after ischaemia or in other pathological settings (Xu *et al.* 2016), recent progresses in genetic lineage tracing has challenged its role *in vivo* after cardiac injury (Li *et al.* 2018), warranting further studies to confirm the extent and function of EndoMT after cardiac ischaemia.

Multiple secreted factors have been implicated in the response to cardiac ischaemia, often exerting pleiotropic functions, including maintenance and expansion of the coronary vasculature, remodelling of the extracellular matrix and protection of the CMs that have survived in the peri-infarct region. One example is follistatin-like 1 (Fstl1), which is highly expressed by ischaemic CMs and acts in an autocrine manner to promote myocyte survival by binding to the disco-interacting protein 2 homolog A (DIP2A) receptor. This receptor was shown to be expressed and induce Akt activation in ECs (Ouchi *et al.* 2010). However, its actual expression by cardiac ECs and whether it could mediate post-infarction coronary angiogenesis still remain open questions.

An additional, well documented, cardioprotective factor is Ang1. Besides its role in cardiac development, which was mentioned before, Ang1 promotes CM survival via integrin-β1-mediated ERK activation and preserves cardiac function upon ischaemia–reperfusion in mice (Lee *et al.* 2011). At the same time, Ang1 also acts on cardiac ECs by regulating VE-cadherin phosphorylation and preventing vascular leakage in different models of cardiac ischaemia (Takahashi *et al.* 2003).

Finally, PDGF is an important signal mediating EC–CM crosstalk in ischaemic conditions. PDGF is a dimeric glycoprotein that can be composed of two A subunits (PDGF-AA), two B subunits (PDGF-BB), or one of each (PDGF-AB). Primary cardiac micro-vascular ECs constitutively express PDGF-A, but not PDGF-B. Their co-culture with cardiac myocytes induces the expression of PDGF-B, resulting in the secretion of PDGF-AB. PDGF-AB in turn acts in an autocrine manner to up-regulate the expression of von Willebrand factor, VEGF and VEGFR-2, thus enhancing the haemostatic and angiogenic properties of ECs (Edelberg *et al.* 1998). While the mechanism by which ECs sense the presence of CMs and start producing PDGF-B are completely unknown, it is interesting to note that aged ECs lose this response. Consistently, treatment of the heart with PDGF-AB restored the angiogenic capacity of the aged myocardium in a rat model of cardiac ischaemia, limiting the extent of myocardial infarction (Edelberg *et al.* 2002). PDGF-BB also activates Akt phosphorylation in CMs in a time- and dose-dependent manner, thus preventing apoptosis after myocardial infarction (Hsieh *et al.* 2006*a*).

As already introduced before, intercellular communications can also occur via extracellular vesicles, including exosomes. Over the last few years, several studies have reported that exosomes modulate angiogenesis in various organs, including the heart, and that, in particular, exosomes produced by ischaemic CMs can stimulate cardiac angiogenesis, at least in part through the delivery of pro-angiogenic miRNAs (i.e. miR-222 and miR-143) (Ribeiro-Rodrigues *et al.* 2017). An overall view of the EC–CM crosstalk in the ischaemic heart is provided in Fig. 5.

Endothelial cell–cardiomyocyte crosstalk in cardiac regeneration

Different from the adult mammalian heart, which has a very limited regenerative potential and heals any injury through scarring, the heart of some lower vertebrates, such as fish and amphibians, is able to regenerate new myocardium after damage. In both zebrafish and axolotls the regenerative process starts with the formation of a blood clot, followed by CM cell cycle re-entry and proliferation (Flink, 2002; Poss, 2007). Neonatal rodents can also regenerate their heart, producing new CMs after either the resection of the cardiac apex or the induction of a myocardial infarction, while they lose this regenerative capacity after the first week of life (Porrello *et al.* 2011; Sadek *et al.* 2014). Furthermore, anecdotal evidence indicates that the human heart might also possess some regenerative potential during early post-natal life (Haubner *et al.* 2016).

Several studies have shown that the epicardium, once properly activated, can give rise to fibroblasts, smooth muscle cells, endothelial cells and, to a lesser extent, also cardiomyocytes, able to colonize the damaged myocardium promoting cardiac repair. The role of the epicardium in cardiac regeneration has been extensively discussed elsewhere (Cao & Poss, 2018; Simões & Riley, 2018) and does not fall into the focus of this review.

While the major focus in cardiac regeneration has been on the formation of new CMs, it is obvious that the regenerated area has to be properly vascularized. This occurs through the proliferation and migration of ECs residing in the vessels of the surrounding cardiac tissue (Marin-Juez *et al.* 2016). Less obvious is the observation that in both zebrafish and neonatal mice, ECs invade the damaged area well before CMs start proliferating, indicating that angiogenesis invariably precedes heart

Figure 5. Endothelial cell-cardiomyocyte crosstalk during ischaemia
Secreted molecules mediate a bidirectional signalling between adult endothelial cells (labelled by anti-CD31 antibodies in green) and cardiomyocytes (labelled by anti-α-actinin antibodies in red) upon ischaemia. Nuclei are stained in blue by DAPI. The dashed arrows indicate the direction of the signals. Molecules secreted by endothelial cells are indicated on the left, while those secreted by both cell types are in the middle. Molecules acting in autocrine manner are indicated by curved arrows. Ang1, angiopoietin-1; Fstl1, follistatin-like 1; NRG-1, neuregulin-1; PDGF, platelet-derived growth factor; TGF-β, transforming growth factor β.

regeneration (Marin-Juez *et al.* 2016). This also suggests that the new vessels not only provide oxygen and nutrients to proliferating CMs, but likely regulate the regenerative process, similar to what occurs in the liver, in the lung and in the pancreas (Ding *et al.* 2011, 2014; Kao *et al.* 2015).

The identification of the molecular mechanisms by which ECs and CMs communicate in these models stands as a crucial step toward the development of innovative and effective strategies promoting cardiac regeneration.

In vitro models to study the crosstalk between endothelial cells and cardiomyocytes

Most of the current knowledge on the molecular mechanisms controlling heart development and disease has been obtained using *in vivo* models. Yet, animal models are expensive, have a low-throughput power and do not allow one to dissect the intercellular crosstalk in detail. *In vitro* models instead can be manipulated to control different variables, are cheaper and suitable for automation for big data analysis. Although the complex architecture of the cardiac muscle and its mechanical function are very difficult to reproduce 'in a dish', artificial cardiac tissue has been produced over the years using a variety of approaches, which have been very useful in investigating the EC–CM crosstalk.

Adult CMs isolated from the murine heart maintain a structured sarcomeric organization, mature electrical properties and beat. Unfortunately, these cells are difficult to isolate and maintain in culture, rapidly lose their mature phenotype in the presence of serum and do not easily integrate into three-dimensional *in vitro* models. Neonatal murine ventricular CMs, together with CMs derived from either induced pluripotent stem cells (iPSC) or human embryonic stem cells (hESC) are the most common sources of myocytes for *in vitro* studies, due to the possibility of obtaining large numbers of cells and also of introducing genetic modifications to mimic pathological conditions. Yet, these cells have an immature phenotype, characterized by small size, immature cytoskeletal and sarcomeric organization and abnormal electrical properties.

ECs are easier to isolate, but their organ source and the age of donor are highly variable in the various models used so far. Several studies have relied on the use of cells derived from extra-cardiac tissues, such as HUVECs or murine ECs derived from the aorta. Alternatively, commonly employed ECs are human or murine cardiac microvasculature ECs, human coronary ECs, iPSC- and hESC-derived ECs.

The EC–CM *in vitro* crosstalk has been analysed using either classical co-culture systems or more sophisticated three-dimensional engineered tissues. The easiest set-up is the two-dimensional co-culture of CMs and ECs. This is an
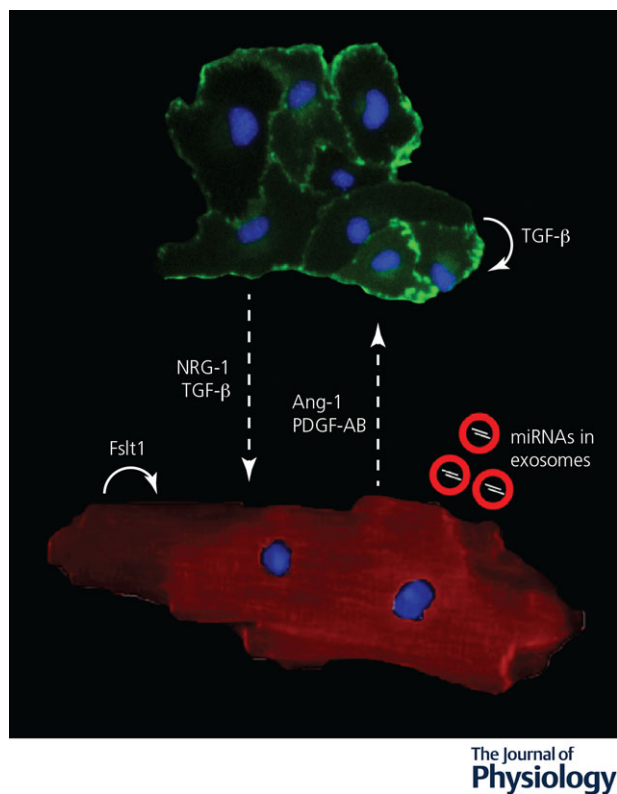
easy and inexpensive approach, only requiring a medium that allows preservation of both cell types and is the only platform suitable to study the crosstalk between adult CMs and other cells. More specifically, the existence of soluble signals acting in a paracrine manner between ECs and CMs has been defined using transwell systems, using both neonatal (Zhang *et al.* 2015) and adult (Chiu *et al.* 2016) cardiac cells.

Since two-dimensional cultures fail to recapitulate many features of the native tissue, including multiple cell–cell interaction, cell shape and the stiffness of the substrate used for cell adhesion (Baker & Chen, 2012), multiple approaches of three-dimensional tissue engineering have been developed in an attempt to create functional cardiac tissues or organoids.

Different scaffolds have been considered, composed of a variety of bio-materials, including chitosan (Hussain *et al.* 2013), hydrogels (Narmoneva *et al.* 2004) and porous sponges of poly-L-lactic and polylactic-glycolic acid (Caspi *et al.* 2007), on which CMs and ECs can be seeded or encapsulated at different ratios. Alternatively, CMs can be seeded on a pre-formed vascular network (Narmoneva *et al.* 2004). Two- and three-dimensional miniaturized chips have also been developed for cell micro-patterning and anisotropic cultures to achieve controlled cell–cell interaction and proper myofibril orientation. However, these systems are still poorly exploited for the study of EC–CM crosstalk (Kofron & Mende, 2017). A promising approach stems from the cell sheet engineering technology, which allows generation of dense and multi-stratified tissue patches suitable for both drug screening and tissue transplant studies. ECs co-cultured in CM sheets are able to spontaneously create a microvasculature network within the cardiac tissue and to connect with the capillaries of the host myocardium upon transplantation (Sekine *et al.* 2008; Stevens *et al.* 2009).

Beside scaffold-based cultures or controlled cell layering, many three-dimensional systems exploit the capacity of cells to self-aggregate forming cardiac organoids. For instance, embryoid bodies seeded on an EC feeder layer show enhanced differentiation towards the CM phenotype, with faster and synchronized beating (Pasquier *et al.* 2017). Stem cell-derived CMs self-organize into a spherical cardiac microtissue when cultured in either hanging drops or spherical-bottom low adhesion plates (Fennema *et al.* 2013). These CM organoids display enhanced contractility and functional maturation when primary coronary ECs are incorporated into the micro-tissue. Interestingly, the same effect is not achieved if ECs have extra-cardiac origin, suggesting that organotypic signals are required to promote hESC-derived CM maturation (Ravenscroft *et al.* 2016). Instead of mixing the two cell types, hESCs could be induced to differentiate toward a cardiac mesodermal fate, in order to generate simultaneously both ECs and CMs (Giacomelli *et al.* 2017).

Overall, these models support a major role for cardiac ECs in promoting CM structural and functional maturation.

To further reproduce cardiac microenvironment cues, several studies have introduced cyclic stretch or electrical stimuli on both two- and three-dimensional co-culture systems to mimic the rhythmic beating of the heart. Mechanical stretch induces several changes in *in vitro* cardiac tissues, such as hypertrophy, metabolic shift, increased contractility and stem cell differentiation (Tulloch *et al.* 2011). However, a standardized method for cyclic stretch conditioning of engineered cardiac tissue has not been achieved yet, due to the variability of technological devices used to apply mechanical stretch and the heterogeneity of culture systems.

Despite the recent optimization of media and biomaterials used to recreate the complex cardiac three-dimensional architecture *in a dish*, the models currently available are very heterogeneous, difficult to compare and do not provide a standard for the effective identification and validation of the mechanisms and molecules involved in the EC–CM crosstalk.

Conclusions

While revising and discussing the mechanisms by which ECs and CMs communicate during heart development and disease, it emerged that a substantial amount of information is available on the signals generated by ECs and able to influence CM structure and function. Yet, the way CMs can modulate EC biology in the heart still remains poorly investigated. While it is known that pro-hypertrophic stimuli increase myocardial vascularization through the secretion of angiogenic factors by CMs, the exact mechanisms coordinating the workload with the sprouting of new vessels in the heart still remain elusive. The generation of CM-specific knockout animals has provided conflicting results, indicating that these cells are able to send both stimulatory and inhibitory signals to ECs and that these signals are probably integrated within a complex scenario. Multiple cell types, hormones, metabolites and growth factors contribute to the regulation of survival, function and regeneration of cardiac vessels in both normal and pathological conditions. This complexity could explain, at least in part, the reasons why most of the clinical trials aimed at inducing therapeutic angiogenesis in the heart have failed so far, since in all cases these trials have been based on the delivery of a single factor (van der Laan *et al.* 2009; Mitsos *et al.* 2012).

In addition, scanty information is available on the relevance of the EC–CM crosstalk during a series of pathological conditions that often affect the heart, including the onset and perpetuation of arrhythmias, genetic cardiomyopathies, valve disease and cardiac transplantation.

Most of our current knowledge concerns the role of proteins secreted by either ECs or CMs and capable of acting on either or both cell types in a paracrine or autocrine fashion. However, it is becoming clear that ECs and CMs can communicate through alternative mechanisms, including the establishment of gap junctions and direct cell-to-cell contact, as well as the secretion of vesicles. In addition, ECs act as mechanosensors, coupling myocardial contraction to haemodynamic changes. For instance, TRPC channels, which are diffusely present in both atrial and ventricular CMs (Seo *et al.* 2014), are also expressed by endocardial ECs and increase the amplitude of calcium transients in normal CMs. Notably, this positive inotropic effect is lost in response to acute stretch. Whether these types of inter-cellular connections play a major role during normal heart development and/or disease progression still remains largely unexplored.

Furthermore, for many molecules able to impact on either EC or CM function, the exact cellular source and the stimuli leading to their production are not yet defined. While this review specifically focuses on the direct crosstalk between ECs and CMs, many of these paracrine signals also exert pleiotropic actions on other cell types residing in the heart, such as fibroblasts, endocardial and pericardial cells, resident macrophages, and circulating immune cells. At the same time, other cells in the heart, as well as other organs, produce the same molecules, and their levels are importantly modified by several physiological and pathological conditions. For instance, ageing is known to be associated with both EC and CM dysfunction, resulting in heart hypertrophy and stiffness. During ageing, expression of both APLN and its APJ receptor are decreased and APLN infusion in 15-month-old mice reduces cardiac hypertrophy (Rai *et al.* 2017). On the other hand, ET-1 increases in aged hearts, although its pathogenic role in determining cardiac stiffness seems to be related to its effect on cardiac fibroblasts rather than CMs (Wang *et al.* 2015). Additional complexity stems from the fact the heart is at the centre of a complex organism, in which multiple tissues actively produce large amounts of soluble molecules that flows through the cardiac pump and thus can further modulate cardiac EC and CM function, possibly influencing their crosstalk. For example, the thyroid hormones L-thyroxine (T_4) and 3,5,3′-triiodo-L-thyronine (T_3) are well known inducers of CM hypertrophy and, at the same time, exert a pro-angiogenic function through direct binding to integrin $\alpha v \beta 3$ on the EC surface, also modulating VEGF and FGF signalling (Luidens *et al.* 2010). Furthermore, LPL activity on the EC luminal surface is modulated by angiopoietin-like proteins that can be produced by the heart but also by the liver (Kersten, 2014). Finally, both Ang-II and ET-1 are produced through a complex multi-organ cascade, relying on angiotensinogen secretion by the liver, its conversion to Ang-I by renin produced by the kidney and finally conversion of Ang-I into Ang-II by angiotensin converting enzyme, mainly produced by lung ECs (Boehm & Nabel, 2002). Further investigation is required to understand to what extent these signals are interconnected and whether they result in either synergic or antagonistic effects.

The establishment of more complex *in vitro* models, integrating multiple cell types in a three-dimensional structure, and their miniaturization to render them suitable to screening approaches, rather than investigating the effect of a single molecule on a single cell type, will likely help shed light on several of these outstanding questions.

Finally, how this information could be exploited to interfere with the onset and pathogenesis of cardiac disease is a matter of active research, which might lead to innovative therapeutic approaches in the near future.

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Additional information

Competing interests

The authors have no competing interests to declare.

Author contributions

All authors have contributed to the conception, drafting and revising of the work. All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship and all those who qualify for authorship are listed.

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