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Exosomes released by cardiomyocytes modulate angiogenic response in heart ischemia

Dissertação para a obtenção do grau de Mestre em Investigação Biomédica
sob orientação científica do Doutor Henrique Girão e co-orientação de Teresa Rodrigues
apresentada à Faculdade de Medicina da Universidade de Coimbra

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Dissertação de Mestrado em Investigação Biomédica, na especialidade de Oncobiologia, apresentada à Faculdade de Medicina da Universidade de Coimbra para obtenção do grau de Mestre.

Orientadores: Doutor Henrique Girão e Teresa Rodrigues

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Abbreviations

BrdU	5-bromo-2'-deoxyuridine
BSA	Bovine serum albumin
CHMP4	Multivesicular body protein
CVDs	Cardiovascular disease
Cx	Connexin
DII4	Delta-like ligand 4
DMEM	Dulbecco's Modified Eagle Medium
DTT	Dithiotheitol
EBV	Epstein- Barr virus
ECGF	Endothelial cell growth factor
ECs	Endothelial cells
ESCRT	Endosomal Sorting Complex Required for Transport
EVs	Extracellular vesicles
FBS	Fetal bovine serum
Flt-4	FMS-like tyrosine Kinase
IA	Intussusceptive angiogenesis
IHD	Ischemic heart disease
ILVs	Intraluminal vesicles
JAG-1	Jagged-1
MCEC	Cardiac endothelial cells
MI	Myocardial infarction
MT1-MMP	Membrane type 1- matrix metalloproteinase
MVBs	Multivesicular bodies
NO	Nitric oxide

PBS	Phosphate buffer saline
PDGF-b	Platelet derived growth factor-b
PFA	Paraformaldehyde
RIPA	Radioimmunoprecepitation assay
ROS	Reactive oxygen species
SA	Sprouting angiogenesis
SDS -Page	Sulfate polyacrylamide gel electrophoresis
Slac2b	Exophilin 5
Slp4	Synaptotagmin-like 4
TBS-T	Tris- buffered saline Tween 20
TSG101	Protein tumour susceptibility
UNCSb	Unc-S homolog b
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VSP	Vacuolar protein sorting
WHO	World Health Organization

Resumo

O coração é um orgão complexo formado por diversos tipos de células que dependem de uma rede de comunicação intercelular organizada que assegura diversas funções, incluindo, a manutenção da estrutura, a contração cardíaca e um eficaz bombeamento sanguíneo.

A doença de isquémia cardíaca é fundamentalmente caracterizada pela redução do fornecimento sanguíneo ao miocárdio devido a obstrução de uma artéria coronária, causando deste modo um fornecimento insuficiente de nutrientes a determinadas áreas do músculo cardíaco. Devido à alta exigência metabólica do miocárdio, a carência em O_2 e nutrientes poderá conduzir a profundas implicações na função cardíaca. Estudos recentes têm demostrado que as células endoteliais cardíacas apresentam um papel importante na sobrevivência e contração do miocárdio. Contudo, a maquinaria molecular que permite a comunicação intercelular entre cardiomiócitos e células endoteliais, *via* exossomas, no contexto de isquémia cardíaca, permanece em grande parte inexplorada.

Assim, o principal objetivo deste estudo consistiu em caracterizar a comunicação estabelecida entre cardiomiócitos e células endoteliais, via exossomas, e compreender de que forma a isquémia poderá afetar esta interação no contexto de promoção de angiogénese.

Os resultados obtidos neste estudo, usando diferentes abordagens, demostram que os exossomas secretados por cardiomiócitos, submetidos a isquémia, promovem a angiogénese de células endoteliais cardíacas. De facto, mostramos que exossomas isquémicos incubados em células endoteliais promovem a sua proliferação, migração, formação de tubos, sprouting e sprouts em anéis de aorta. Em suma, estes resultados sugerem fortemente que os exossomas isquémicos têm o potencial para promover a angiogénese no coração.

Para validar os resultados obtidos *in vitro,* decidimos avaliar a formação de vasos usando a membrana corioalantóica da galinha *ex vivo*. Seguramente, os resultados obtidos com este ensaio mostram que os exossomas isquémicos promovem o crescimento de um maior número de vasos, reforçando a ideia de que os exossomas isquémicos comunicam com as células endoteliais a fim de manter a homeostase do coração.

Para além disso, mostramos que os exossomas isquémicos induzem uma diminuição significativa na permeabilidade celular, sugerindo que a integridade das proteinas de junção das células endoteliais se encontram afetadas pelos exossomas. Em concordância com este resultado, observamos que os exossomas isquémicos induzem uma redistribuição da proteina ZO-1 na monocamada de células endoteliais, quando comparados com os exossomas controlo. Tendo em consideração os resultados obtidos no ensaio de permeabilidade celular, fomos avaliar o efeito destes exossomas libertados por cardiomiócitos na resistência elétrica transendotelial.

Surpreedentemente, e contrariamente aos ensaios de permeabilidade, os valores da resistência eléctrica transendotelial diminuem na presença de exossomas isquémicos, quando comparados com os exossomas controlo. Todos estes resultados no seu conjunto sugerem que os exossomas têm a capacidade de afetar a permeabilidade e a resistência elétrica transendotelial.

Em resumo, prevemos um modelo de comunicação entre cardiomiócitos e células endoteliais, via exossomas, com o objetivo de manter a homeostase cardíaca. Os resultados obtidos neste estudo fornecem novas evidências sobre a rede de comunicação estabelecida entre cardiomiócitos e células endoteliais, o que poderá vir a contribuir para o desenvolvimento de novas estratégias destinadas a preservar a função cardíaca na doença de isquémia.

Abstract

The heart is a complex organ formed by a diversity of cell types that depends on a well-regulated intercellular communication network to perform its functions, namely to provide structure, ensure regulated heart contraction, and efficient pumping of blood.

Ischemic heart disease (IHD) is primarily associated with the reduction of myocardial blood supply, mainly as a result of the obstruction of a coronary artery causing a defective nourishment of certain areas of the heart muscle. Given the high metabolic demand of the myocardium, the lack of O_2 and nutrients in the sequence of artery occlusion can have profound effects on heart function. Recent studies have demonstrated that endothelial cells (ECs) play a critical role in supporting cardiomyocytes survival and myocardial contraction. However, the molecular machinery underlying the intercellular communication between cardiomyocytes and ECs, via exosomes, namely in the context of ischemia, remain largely underexplored.

Therefore, the main objective of this study is to characterize the communication established between cardiomyocytes and ECs, through exosomes, and determine how ischemia can affect this interplay, within the context of angiogenesis promotion.

The results presented in this study, using different complementary approaches, demonstrate that exosomes released by cardiomyocytes, subjected to ischemia, stimulate angiogenesis of cardiac ECs. Indeed, we show that ischemic exosomes incubated with EC present an ability to promote proliferation, migration, tube formation, angiogenic sprouts and aortic ring sprouts. These results suggest that ischemic exosomes may play a pro-angiogenic effect in the heart. To confirm these data, we decided to validate the results obtained *in vitro* in a more physiological and relevant biological model, using *in vivo* chick chorioallantoic membrane (CAM) assay. This assay confirms and validate the previous studies *in vitro,* suggesting that ischemic exosomes derived from cardiomyocytes communicate with ECs in order to maintain heart homeostasis.

Moreover, we showed that ischemic exosomes induce a significant decrease in cellular permeability, suggesting that the integrity of ECs junction's proteins is being affected by exosomes. In agreement, we demonstrate that ischemic exosomes induce a redistribution of ZO-1 on the endothelial cell monolayer, when compared with control exosomes.

Considering the results obtained in the cellular permeability assay, we also evaluated the effect of these exosomes released by cardiomyocytes on the transendothelial electrical resistance (TEER). Surprisingly, and contrary to the permeability assays, the TEER values decreased in the presence of ischemic exosomes, in comparison with control exosomes. Together, these results suggest that exosomes differentially affect permeability and TEER.

In conclusion, we envision a model whereby cardiomyocytes and endothelial cells communicate via exosomes to maintain cardiac homeostasis. Altogether, this study provides new evidence regarding the communication network established between cardiomyocytes and endothelial cells, which may contribute for the development of new strategies that aim to preserve cardiac function in ischemic heart disease.

1. Introduction

1.1 The heart

Life, from early embryogenesis to the whole adult life, depends on the uninterrupted function of the heart, capable of contracting and relaxing repeatedly, pumping blood to all tissues and organs. The human heart (Figure 1) is composed by four-chambers: two atria, that receive the pulmonary oxygenated blood (left atria) and venous blood from the rest of the organs (right atria), and two ventricles, that pump blood to the lungs (right ventricle) or throughout the organism (left ventricle)[1].

The contractile capacity of the heart relies on a muscle tissue, commonly known as myocardium, composed by multiple highly specialized myocardial lineages, including those of the ventricular and atrial myocardium, and the specialized conduction system. The rhythm of the heart is mainly determined by the activity of a specific population of cells, localized at the sinoatrial and atrioventricular nodes, which possess intrinsic depolarization activity, thus acting as pacemakers. However, to work in a synchronized and coordinated manner, the electrical impulses, generated at the nodes have to be rapidly and efficiently propagated to the rest of myocardium[2].

Indeed, to function as a syncytium, the heart relies on a network of intercellular low resistance channels, localized in specialized cell-to-cell junctions called intercalated discs. These intercellular channels, termed gap junctions, are formed by six subunits of a protein called connexin (Cx), and allow for the correct and efficient electrical signal anisotropic propagation. This ensures a highly synchronized and powerful contraction of cardiomyocytes, required to pump blood out of the atria and ventricles to the blood vessels of both systemic and pulmonary circulatory systems[3].

Aside of cardiomyocytes, the heart is comprised by other cells, such as endothelial cells (ECs), fibroblasts, smooth muscle cells, immune cells, pacemaker cells and Purkinje cells, that are fundamental to normal heart function providing structural, biochemical, mechanical and electrical properties[4]. The mammalian heart contains 2 to 4 billion cardiomyocytes, which are nourished by a network of blood vessels that transport oxygen and nutrients to support the high metabolic activity and O_2 consumption of cardiomyocytes.

During myocardial infarction (MI) in which the blood supply to some areas of the heart is compromised due to coronary arteries occlusion, more than 25% of the myocytes can be damaged[5]. To prevent this damage, the heart has developed different strategies to deal with the lack of O_2 and nutrients, including biological and structural adaptations, which include among others, the growth of new vessels (angiogenesis).

ECs are critical for cardiomyocyte survival and myocardial contraction. In response to stress conditions, cardiomyocytes can affect EC function, primarily leading to angiogenesis promotion. For this reason, it is pivotal to guarantee the crosstalk between the different cell types and consequently the integrity of the heart.

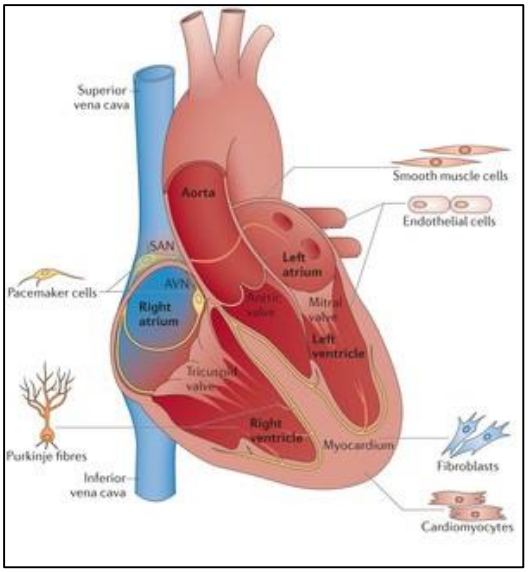

Figure 1. Schematic representation of mammalian heart.
The heart are composed by four chambers and different cardiac cell types: cardiomyocytes, endothelial, smooth muscle, pacemaker and Purkinje cells. Adapted from Xin, et al. (2013).

1.2 Ischemic Heart disease

According to the World Health Organization (WHO), cardiovascular diseases (CVDs) are the first cause of global morbidity and mortality in humans**.** Among CVDs, MI is considered the first cause of death worldwide and frequently associated with the development of heart failure.

Ischemic heart disease (IHD) is primarily caused by the reduction of myocardial blood supply mainly as a result of a coronary artery blockage, leading a partition of heart tissue without O_2 and nutrients. Given the high metabolic demand of the myocardium, the lack of blood supply can have profound effects on heart function. In less complex species such as amphibians and zebrafish, regeneration of the myocardium occurs immediately after myocardial injury, but in the human heart this regenerative capacity is lost early in life, and cardiomyocytes that die after ischemic episodes are not adequately replaced, resulting in the loss of ventricular function. There are several risk factors associated with IHD that include high blood pressure, diabetes, lack of exercise, obesity, high blood cholesterol and poor diet. Heart ischemia is associated with severe metabolic changes, which include increase of oxidative stress, mitochondrial DNA damage, creatine phosphorylation, ATP-depletion, enhanced anaerobic glycolysis, accumulation of inorganic phosphate and development of intracellular acidosis [6,7].

Indeed, during ischemia, intracellular pH decreases and the accumulation of lactic acid, reactive oxygen species (ROS) and other damaging metabolites accounts for the loss of cell integrity and function[8]. Furthermore, alterations in normal intercellular communication between different cardiac cell types have been implicated in changes during IHD. For example, a decrease in nitric oxide (NO) and ROS signalling has been often associated with a reduction of communication between cardiomyocytes and endothelial cells, with the concomitant loss of vascular integrity [9]. Hence, it would be important to understand the mechanisms that regulate the interaction between different cardiac cell types and clarify the impact of the deregulation of these mechanisms in the context of disease.

1.3 Intercellular Communication

1.3.1 Exosome-mediated communication

In multicellular organisms, intercellular communication between cells enables the correct coordination of cell function, which is essential to maintain tissue homeostasis. In addition to cardiomyopathies, failure in transmission of information has been implicated in several other diseases such as cancer, diabetes, Alzheimer, Parkinson, and atherosclerosis.

Intercellular communication can occur directly, between neighbour cells via gap junctions, or indirectly, at longer distances through soluble factors and extracellular vesicles (EVs) released under basal or stress conditions to the extracellular space (Figure 2)[10-12].

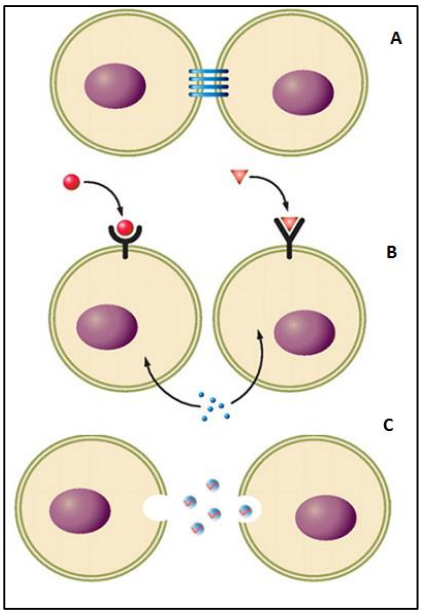

Figure 2. Intercellular communication.
Intercellular communication between neighbour cells via GJ (A), or indirectly, at longer distances through soluble factors (B) and extracellular vesicles (EVs) released under basal or stress conditions (C). Adapted from Meda, et al. (2011)

According to their size, composition and subcellular origin, EVs can be divided in apoptotic bodies, microvesicles and exosomes [13]. Some of these EVs originate from the endocytic pathway that is responsible for internalization of molecules from the plasma membrane that can be recycled, degraded and even released into the extracellular space. This pathway is characterized by several events that begin with the formation of endosomes through the budding of the plasma membrane. As they mature to late endosomes, invagination of the limiting membrane occurs and intraluminal vesicles (ILVs) are formed resulting in the generation of multivesicular bodies (MVBs). At the molecular level, the process of MVBs formation is coordinated by the Endosomal Sorting Complex Required for Transport (ESCRT) machinery and consists of four complexes: ESCRT-0 responsible for cargo clustering, ESCRT-I and ESCRT-II that induce bud formation and ESCRT-III involved in vesicle

secretion. Originally, it was thought that MVBs fuse with lysosomes to degrade their intraluminal cargo. However it has become clear that MVBs can also fuse with the plasma membrane, in an exocytic manner, releasing the ILVs, as exosomes, into the extracellular environment[14,15].

Exosomes are small membrane vesicles, released by many, if not all, living cells, thus ascribing to these group of small vesicles an important role as intercellular messengers, conveying the transference of biological information between donor and acceptor cells [16].

1.3.2 Exosome biogenesis

The molecular details underlying exosome biogenesis remained unclear for many years. However, it is now known that exosomes biogenesis can occur either dependently or independently of the ESCRT machinery [17]. In several cellular models, exosome formation and release is impaired upon ESCRT dysfunction namely by silencing ESCRT-0 members HRS and STAM1, the ESCRT- I protein tumour susceptibility gene 101 (TSG101), the ESCRT- II component vacuolar protein sorting 22 (VSP22), and the ESCRT-III components charged multivesicular body protein 4 (CHMP4), vacuolar proteins sorting 4A and 4B (VPS4A and VPS4B) and ALIX [14,18]. A study showed that ALIX also plays a crucial role in intraluminal budding of vesicles in endosomes and hence exosome biogenesis through a direct interaction with syntenin, the cytoplasmic adaptor of heparan sulphate proteoglycan receptors[19].

More recently, evidence indicates the existence of a mechanism that does not require ESCRT machinery, is sphingomyelinase dependent, and relies on the ceramide pathway enzyme. Indeed, it was demonstrated that ceramide, because of the size of its head group, promotes negative curvature in the membrane bilayer, required for membrane deformation. Importantly, this ESCRT- independent mechanism was initially demonstrated in an oligodendroglial cell line that contains exosomes with higher levels of ceramide and derivatives, suggesting that this mechanism is responsible for the formation of raft-like domains required to sort specific cargo molecules into exosomes[20]. In contrast, in human melanoma cells, the inhibition of sphingomyelinases does not affect MVB biogenesis[21]. In

this model, the formation of MVB relies on a CD63-dependent mechanism, previously described as central for targeting the Epstein – Barr Virus (EBV)-encoded LMP1 protein to ILVs and for exosome release[22].

After their formation, ILV are released into the extracellular milieu, following MVB fusion with the cell surface (Figure 3). However, little is known about the mechanisms that govern MVB fusion with the plasma membrane. Several studies have shown that multiple Rab GTPases and tetraspanins, highly enriched in exosomes, play a crucial role in the formation and secretion of exosomes[15,18].

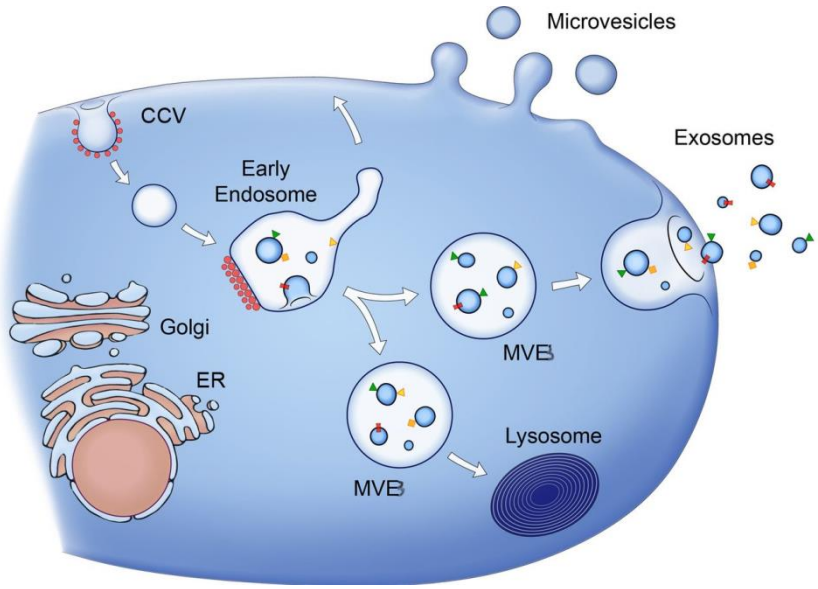

Figure 3. Exosomes biogenesis.
Exosomes biogenesis occurs inside their MVB and its release occurs through the fusion of the MVB with the plasma membrane. Adapted from Raposo, et al. (2013).

1.3.3 Exosome composition

As stated above exosomes convey biological information between cells that likely relies on their composition in terms of proteins, lipids and genetic material [10]. During the last years, several studies have been performed in order to identify the content of exosomes. The emergence of new technology has lead to the substitution of antibody-based techniques

by proteomic analysis techniques, which are more accurate and allow for an unbiased large-scale identification of non-predetermined proteins.

Exosome protein composition varies among cell type and tissue, but as a consequence of their endosomal origin, contains a common set of evolutionary-preserved proteins, such as, TSG101, flotillin, ALIX, Annexins, SNAREs that are embedded during exosomes biogenesis[18,23]. However, different classes of cytosolic proteins commonly detected in exosomes include, Rabs, GTPases, tetraspanins (CD63, CD81, and CD9), heat-shock proteins (HSP70, HSP60, HSPA5, HSP90), antigen-presentation (MHC-I, MHC-II) co-presentation molecules (CD86), cell adhesion proteins (integrins, MFGE8) and cell structure and motility proteins (actins, myosin, tubulin)[10,15,18]. However, the mechanisms that regulate exosomal content selection remain unknown.

In addition, the lipid composition of exosomes also differs, from the plasma membrane of the originating cells. Indeed, when comparing secreted vesicles, including exosomes, with the cellular plasma membrane, an enrichment in lipid raft components, namely, cholesterol, sphingolipids, ceramide or its derivative, ganglioside GM3 can be observe[15,24].

Besides proteins and raft-lipids, exosomes are enriched in DNA and coding and noncoding RNA molecules, namely miRNAs. Moreover, it is known that miRNAs secretion is highly regulated, being modulated by various stress stimuli and disease conditions. For example, hypoxia, a pathological condition observed in the core of large tumours or upon vascular injury, modulates protein and RNA composition of exosomes released by endothelial [25] and tumour [26]cells.

1.3.4 Mechanisms involved in the release of exosomes

Multiple pathways are involved in the fusion of MVB with the plasma membrane resulting in bursts of exosome secretion. These pathways require the cytoskeleton (actin and myosins), associated molecular motors (Kinesins and myosins), molecular switches (small GTPases) and the fusion machinery (SNAREs and tethering factors)[18].

Exosomes are released from cells via two different mechanisms: a constitutive pathway, regulated by specific RAB GTPases[27,28], hetrotrimeric G-protein and protein Kinase D[29], and an inducible pathway triggered by several stress stimuli, such as increased intracellular Ca^{2+}[30,31], DNA damage[32,33], thrombin, extracellular ATP[34], hypoxia, and LPS stimulation[35]. Rab GTPases are members of the RAS GTPase superfamily and are considered regulators of vesicle formation, trafficking, tethering and fusion with target organelles. In a recent study, it was demonstrated that Rab27a and Rab27b serve both common and different roles, most likely through the Slp4 (synaptotagmin-like 4) and Slac2b (exophilin 5) effector proteins, in the intracellular trafficking of MVBs, leading to exosome secretion[28].

Furthermore, Rab11 and Rab35 appear also to be involved in exosome release; however, selective inactivation of each of these Rabs only partially affected this pathway, thus suggesting that the role played by GTPases vary according to the cell type. Alternatively, it is also conceivable that Rab GTPases act indirectly by regulating pathways upstream of exosome secretion[15].

Other important players in exosome secretion are SNAREs, a large protein superfamily consisting of more than 60 members in yeast and mammalian cells. Regarding exosomes, several studies have reported that SNARE complexes are involved in the fusion of different subpopulations of MVBs with the plasma membrane. Moreover, changes in the levels of one SNARE protein might affect the secretion of only a particular subpopulation of exosomes, thus ascribing a role to SNARES in selective sorting of exosomes[15,36].

1.3.5 Intercellular communication of exosomes with target cells

It is now clear that exosomes can have specialized functions and play a key role in intercellular signalling, essential for multicellular organisms to maintain their vital functions. Nevertheless, during several decades, most of the extracellular vesicles were considered as mere cell debris, or signs of cell death. Increasing evidence has shown that exosomes have four possible mechanisms whereby they communicate with the target cell and influence the behaviour of the recipient cells by exchanging material and information (Figure 4)[10,24].

Classical means of cell communication are represented by exosomal membrane proteins with capacity to interact with the plasma membrane of recipient cells via specific receptors, thus activating of downstream signalling. Another possible mechanism is the cleavage of exosomal membrane proteins by proteases with the resulting fragment acting as a soluble ligand which binds to surface receptors on the acceptor cells. The most interesting aspect of exosomes is its involvement in the transfer of exosomal information, through the fusion of exosomes membranes with the plasma membrane of acceptor cells and the release of their content, thereby protecting the genetic information within the exosomes against extracellular degrading enzymes. Lastly, exosomes can be internalized by endocytic mechanisms, including, phagocytosis, macropinocytosis or receptor-mediated endocytosis[10,18,37].

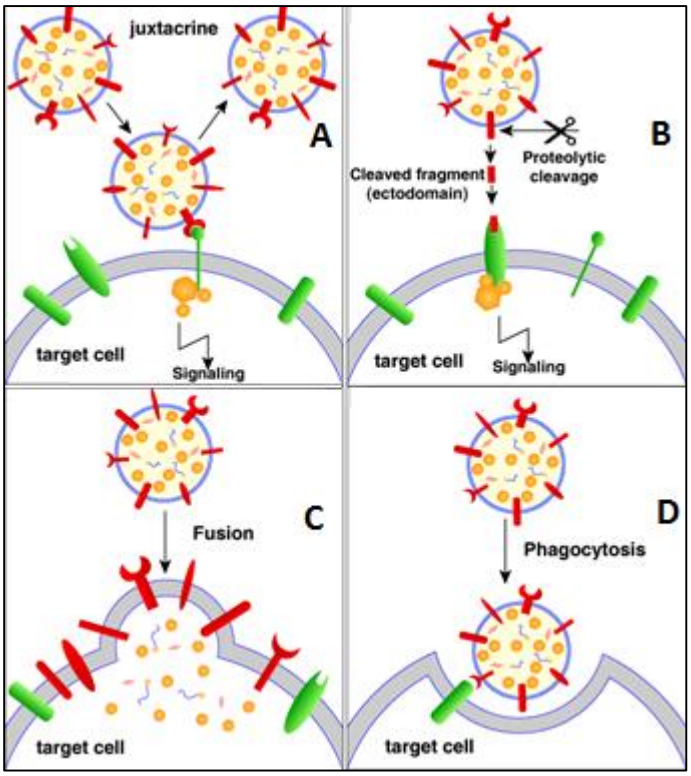

Figure 4. Mechanisms by which exosomes communicate with target cells.
(A) direct ligand-receptor interaction, resulting in activation of signalling cascades, (B) extracellular proteases cleave exosomal membrane proteins, delivering soluble ligands that bind to target receptors of acceptor cells, (B) direct membrane fusion with target cell membranes and release of their content into the acceptor cell or, (D) internalization of exosomes by endocytic mechanisms (macropinocytosis, phagocytosis or receptor mediated endocytosis). Adapted from Mathivanan, et al. (2012).

1.3.6 Cardiac cell communication: the role of exosomes in physiology and disease

The heart is an organ formed by different cell types that have to efficiently communicate in order to maintain heart homeostasis and function. Cardiomyocytes, the main contractile unit of the heart, are not considered a typical secretory cell. Nevertheless, several evidences suggest that, in the heart, cardiomyocytes secrete exosomes both in healthy and ischemic conditions mediating the communication between healthy and damaged cells. Considering the formation and secretion of exosomes from a variety of cardiac cells, it is reasonable to expect that these small vesicles are involved in many cardiovascular physiological and pathological disorders[17,38,39].

Following cardiac injury, there are dramatic shifts in the various cardiac cell populations that can affect cardiac communication and cardiac function. For example, cardiomyocyte

progenitor cell-derived exosomes have been shown to modulate cell differentiation, proliferation, migration and survival after induced ischemia[40].

Furthermore, the content of secreted exosomes may vary depending on the cardiac stimuli, triggering different signalling pathways in recipient cells. Recent studies described exosomes as a conveyer of anti and pro angiogenic factors in blood vessel walls under pathological situation such as, hypertension, metabolic syndrome, diabetes mellitus and MI[41].

Therefore, depending on their origin, exosomes may also possess therapeutic capacity in angiogenesis. This effect likely relies on the capacity of exosomes to reprogramme endothelial cells, inducing changes in the number, phenotype, and function of endothelial progenitor cells that might ultimately result in an increase of capillary-like tubes in vitro and the generation of new vessels *in vivo*[42].

1.4 Angiogenesis

In small and less complex animal species, such as the nematode *Caenorhabditis elegans* and the fruitfly *Drosophila melanogaster*, oxygen is capable of diffusing throughout their small body reaching all cells. Nevertheless, in more complex and larger organisms, a vascular network capable of distributing oxygen and nutrients in the blood to distant cells is required, thus providing an adequate environment for cell metabolism[43].

During embryonic development, blood vessels provide the growing organs with the oxygen they require for development. Besides this major function, the vessels supply instructive trophic signals to promote organ morphogenesis. Blood vessel networks can occur by an assembly process, designated vasculogenesis or through the coordinated expansion of a pre-existing network, namely angiogenesis (Figure 5)[43,44].

Vasculogenesis[45-47] relates to *the novo* formation of blood vessels, and relies on the local differentiation of mesoderm-derived angioblasts into ECs that assemble into a primary capillary plexus.

In contrast, angiogenesis[48] is the formation of new capillaries from the pre-existing vasculature and has a variety of morphogenic events during which pre-existing ECs coordinately sprout, branch, split, differently growth vessels, and rearrange themselves, into a highly and functional vascular network.

Angiogenesis is a mechanism controlled by a number of growth factors and different signalling pathways resulting from a balance between pro and anti angiogenic factors[49]. Afterwards, nascent ECs sprouts are covered by pericytes and smooth muscle cells. During this process, the ECs migrate, proliferate, establish junctions and apical-basal polarity, and deposit a stabilizing basement membrane which provides strength and allows regulation of vessel perfusion. The dysregulation of this process can lead to abnormalities during development. For example, mutation or inactivation of genes involved in blood vessel development may result in embryonic lethality[50].

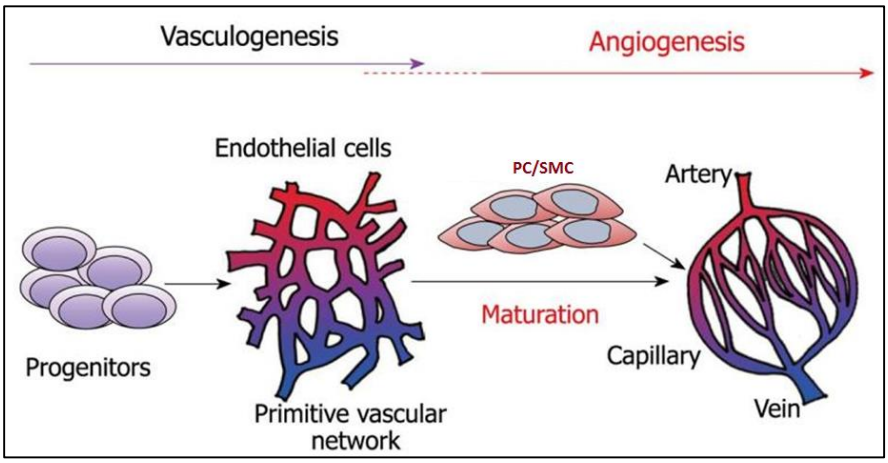

Figure 5. Schematic representation of the development of vascular systems.
Vasculogenesis and angiogenesis. During vasculogenesis the endothelial progenitor cells give rise to a primitive vascular labyrinth of arteries and veins. During subsequent angiogenesis, the network expands, pericytes (PCs) and smooth muscle cells (SMCs) cover nascent endothelial channels, and a stereotypically organized vascular network emerges. Adapted from Kazuaki Yoshioka, et al. (2012).

There are two specific mechanisms involved in the formation of new vessels during angiogenesis, namely, intussusceptive angiogenesis (IA) or sprouting angiogenesis (SA). SA

can occur by formation of new capillaries from the existing vessels, whereas IA, also named splitting angiogenesis (Figure 6), occurs by dividing pre-existing capillaries.

IA is the mechanism characterized by the formation of so called intraluminal tissue pillars that arise from the invagination of the capillary walls into the vascular lumen, through a complex multistep process. The hallmark of this type of angiogenesis is the formation of pillars that result in the establishment of a tissue that intersect the vascular lumen. Two opposite capillary walls establish a zone of contact and the endothelial cell junctions are reorganized allowing the vessel bilayer to be perforated so that growth factors and cells (pericytes and myofibroblasts) penetrate into the lumen, invading interstitial tissue. These cells produce collagen fibers into the core of the lumen vessel providing an extracellular matrix important for the growth process[51,52].

Finally, the core is fleshed out with no alterations to the basic structure. Unlike sprouting, IA is especially important since it requires a relatively low rate of endothelial cell proliferation and has low vascular permeability. This type of angiogenesis has been described in a wide range of tissue outgrowth stages, including, the mammary gland and glomeruli development. Although less frequent, IA also occurs in skeletal muscle, liver, brain and heart muscle[51].

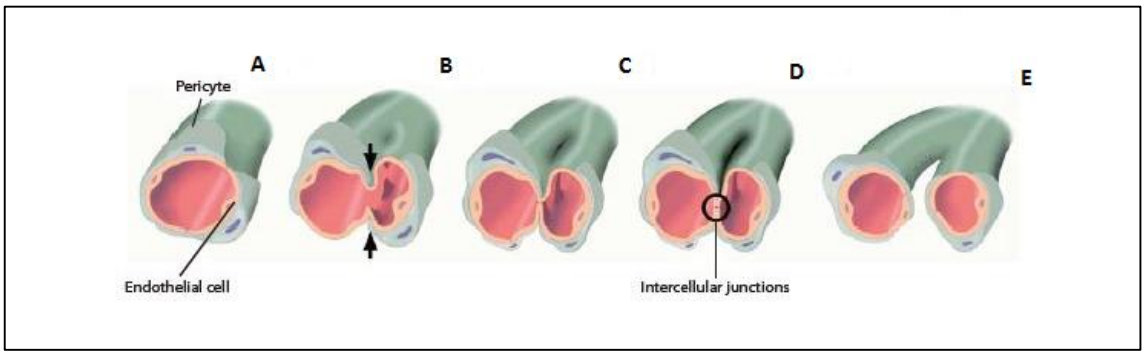

Figure 6. Schematic representation of intussusceptive angiogenesis.
Intussusceptive angiogenesis protrusion of opposing capillary walls into the lumen (A and B), intraluminal pillar formation (C), and rearrangement of intercellular junctions of the opposing endothelial cells (D). Further growth of the pillar leading to splitting of the blood vessel into two new vessels (E). Adapted from De Spiegelaere et al. (2012).

SA also called tubular morphogenesis (Figure 7) occurs when nutritional and oxygen demands within a tissue are higher than which is supplied by the existing capillary (or venule). The process of sprouting angiogenesis requires the local breakdown and

reorganization of the extracellular matrix, mediated by matrix metalloproteinases, like membrane type 1-matrix metalloproteinase 1 (MT1-MMP), heparinases, chymases, tryptases and cathepsins. The pericytes detach and interendothelial contacts are weakened. Furthermore, ECs migrate into the connective tissue and an endothelial sprout is formed. Subsequently, proximal to the migrating front, lumen starts to form and contiguous tubular sprouts anastomose originating functional capillary loops. Simultaneously, the recruitment of pericytes and synthesis of the new basement membrane ensures the establishment of stable and mature blood vessels. Although initially the blood vessel sprouting progresses without cell division, the sustainability of the growth requires cell proliferation[48,53]. The initiation of sprouting requires the specialization of ECs into a tip and stalk cells with distinct cell phenotypes based on their expression profiles and the functional specifications of ECs within a newly formed sprout.

Tip cells are migratory and polarized with capacity to extend long filopodia that respond to the presence of attractant and repellent signals and consequently migrate requiring a minimal proliferation activity[54]. Contrarily, stalk cells, proliferate during sprouting extension and form the nascent vascular lumen. This mechanism that differentiates tip and stalk EC phenotypes is controlled by differential gene expression between pro-angiogenic signals, vascular endothelial growth factor (VEGF) and Jagged-1 (JAG-1), and suppressors of endothelial cell proliferation[55].

Another pivotal key in the regulation of the specification of tip and stalk cells is the Notch signalling pathway. During angiogenesis, activated Notch signalling inhibits tip cell formation and promotes the stalk cell phenotype, but the presence of specific markers such as delta-like ligand 4 (Dll4), platelet derived growth factor-b (PDGF-b), unc-5 homolog b (UNC5b), vascular endothelial growth factor receptor 2 (VEGFR2) and VEGFR-3/ FMS-like tyrosine kinase 4 (Flt-4), and low levels of Notch signalling activity, allows the activation of tip cells. The balance between migration of tip cells and the proliferation of stalk cells allows adequately shaped nascent sprouts[56,57]. However, during the transition from active sprouting to quiescence endothelial cells tips cells adopt a “phalanx” phenotype, that is non-proliferating and immobile cells that promote promotes vessel integrity and stabilizes the vasculature through increased cell[58].

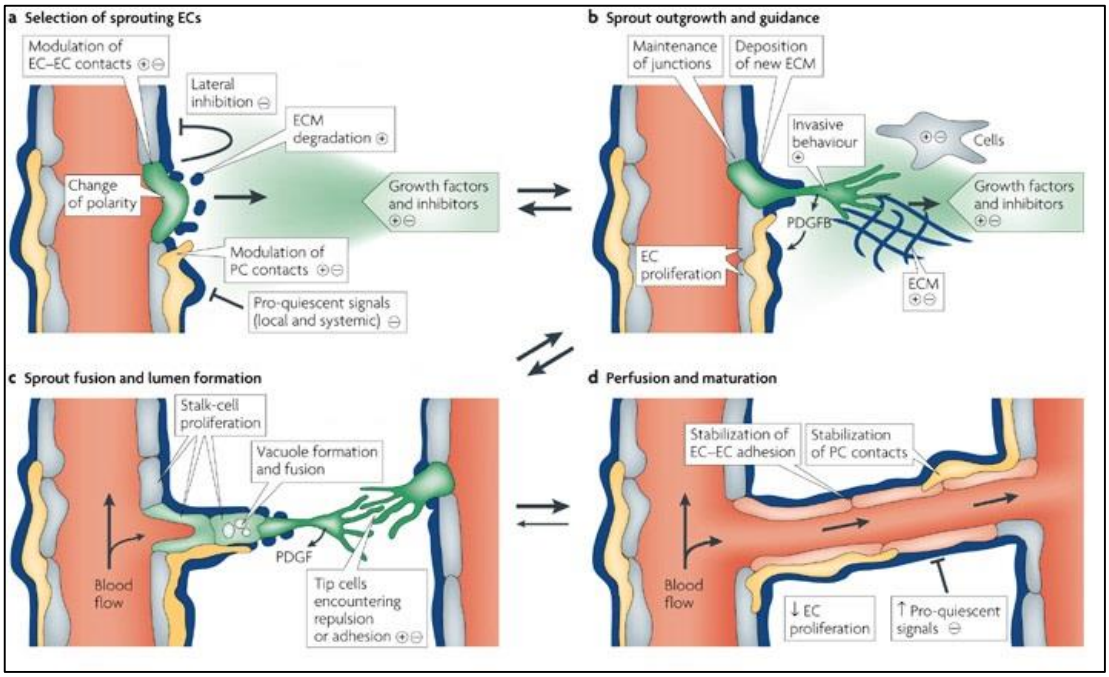

Figure 7. Schematic illustration of the cellular steps involved in sprouting angiogenesis.
Selection of EC sprouting is controlled by the balance between pro-angiogenic factors (eg. VEGF) and factors that promote queiscence (a); sprout outgrowth and guidance requires invasive and motile activity, modulation of cell-cell contacts and local matrix degradation to form tubes (b); sprout fusion and lumen formation due to adhesive or repulsive interactions occur when tip cells encounter each other (c) perfusion and maturation leads to the establishment of a continuos lumen that allows oxygen delivery and reduction of pro-angiogenic, hypoxia-induced signals as well as stabilization of cell junctions, matrix deposition and tight PC attachment (d). Adapted from Adams and Alitato (2007).

Angiogenesis is a central process during fetal growth, from placenta formation to the circulatory system ramification. During adulthood, most blood vessels remain quiescent and angiogenesis plays a pivotal role in specific processes, such as cycling ovary and in the placenta during pregnancy[59]. However, this mechanism is not only a physiological phenomenon, and has been indirectly involved in a different number of pathological conditions. For example, the aggressive outgrowth of blood vessels is involved in cancer, rheumatoid arthritis, atherosclerosis and diabetic retinopathy, whereas insufficient blood vessel formation follows MI and chronic wound healing failure.

Furthermore, during IHD the angiogenic switch is insufficient and responsible for EC dysfunction and vessel malformation or neovascularization regression, leading to severe consequences and even tissue death[43,50,60]. In the mammalian heart, ECs play a critical role in

cardiomyocyte survival and myocardium contraction[61]. However the role of cardiomyocytes in the regulation of endothelial cell function is still unclear. Recent studies indicate that angiogenesis can be modulated by a group of extracellular vesicles constitutively secreted by mammalian cells, namely exosomes. It was already demonstrated that cardiomyocyte-derived exosomes contain a large amount of Hsp20 that remarkably promotes angiogenesis in ECs and also that exosomes released by cardiomyocytes from type 2 diabetic rats, impaired angiogenesis through the exosomal transfer of miR-320 into ECs[62].

However, the effect of exosomes, released by cardiomyocytes in the sequence of ischemic heart, on endothelial cells behaviour has never been established. Therefore, this work focuses on the responses of endothelial cells elicited by exosomes secreted by cardiomyocytes either under basal or ischemic conditions. For that, we will use a comprehensive approach that intends to investigate several aspects usually associated with angiogenesis. A better knowledge of these processes may pave the way towards the development of new therapeutic strategies against ischemia induced heart lesion.

2. Objectives

The heart is an organ with a complex network of interactions between the different types of cardiac cells. Therefore, a highly regulated interplay between these cells is vital to facilitates proper myocardial contractility, permit sufficient perfusion and ensure balanced myocardial extracellular stiffness. Intercellular communication between heart cells can occur directly, via gap junctions, or indirectly through exosomes. Recently, extracellular vesicles, namely exosomes secreted by various cell types have gained much attention regarding their multiple roles in mediating the cell-to-cell communication, cell survival and angiogenesis.

An impairment of intercellular communication between different cardiac cells have been associated with several cardiomyopathies, including IHD. IHD is characterized by a reduction of myocardial blood supply, which supports the high metabolic demand of the myocardium, leading to ECs dysfunction.

Although the communication between cardiomyocytes and ECs via soluble mediators has been extensively investigated, the involvement of exosomes in this interplay remains largely unclear. Therefore, the main objective of this study is to evaluate the communication between cardiomyocytes-derived exosomes either in control or ischemic conditions on ECs function and their ability to modulate the myocardial angiogenesis.

More specifically, we intend to unveil the effect of exosomes, released by cardiomyocytes under control or ischemic conditions in ECs angiogenic response, using different complementary approaches, such as, proliferation, migration, tube-like formation, sprouting, rat aortic ring assay, cellular permeability and TEER assay. To further confirm these *in vitro* studies in a more physiological and relevant biological model, we use *in vivo* CAM assay. This may be of particular relevance in the context of a cardiac lesion, namely myocardial infarction, where altered communication between cardiac cells may lead to adverse remodeling of the heart, including angiogenesis. The knowledge of the exosomes role as an extracellular communicator in cardiac diseases can be important for the revaluation of the mechanisms involved in ischemic heart disease leading to a development of cardiac therapeutics.

3. Material and methods

3.1 Cell Culture and treatments

The cardiac endothelial cell line (MCEC-1) was developed by Faculty of Medicine, National Heart & Lung Institute in London, United Kingdom and nobly provided by Professor Justin Mason. The MCEC-1 was maintained in Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, Carlsbad, CA), supplemented with 10% fetal bovine Serum (FBS), 1% Penicillin/Streptomycin (100 U/mL:100 µg/mL) and 1% GlutaMAX (Life Technologies, Carlsbad, CA), with 10 U/ml heparin (Invitrogen), 75 ug/ml endothelial cell growth factor (ECGF) Sigma. The cardiac endothelial cell line was cultured in 1% gelatin (Sigma-Aldrich, St. Louis, MO), and expanded at 33ºC under 5% CO_2, of then plated at 37ºC with 5% CO_2 24h-48h prior to experimentation.

The cardiomyoblast cell line H9c2 (Sigma-Aldrich, St. Louis, MO) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, Carlsbad, CA), supplemented with 10% FBS, 1% Penicillin/Streptomycin (100 U/mL:100 µg/mL) and 1% GlutaMAX (Life Technologies, Carlsbad, CA), at 37ºC under 5% CO_2.

Metabolic ischemia was induced by a buffer exchange to an ischemia-mimetic solution (in mM: 118 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 1.2 $CaCl_2$, 25 $NaHCO_3$, 5 calcium lactate, 20 2-deoxy-D-glucose, 20 Na-HEPES, pH 6.6) and by placing the dishes in hypoxic pouches (GasPakTM EZ, BD Biosciences), equilibrated with 95% N_2/5% CO_2.

Cells were stimulated with, unless otherwise stated, 2.5 µg/mL of exosomes in exosome-depleted medium, for the indicated time periods.

3.2 Exosomes purification

Serum was depleted of exosomes by ultracentrifugation at 154.000 g, for 16 hours[63], in a 1:1 dilution to minimize serum proteins loss due to its viscosity. Exosomes derived from cultured cells were isolated from conditioned medium after culture in either exosome-depleted medium or ischemia-mimetic solution, for 2 hours. Afterwards, the medium was collected and exosomes were isolated by ultracentrifugation[64]. In brief, the supernatants were subjected to differential centrifugation at 4°C, starting with a 300 g centrifugation, for 10 min followed by a 16.500 g centrifugation for 20 min. To remove cellular debris and larger particles, the supernatants were filtered with a 0.22 μm filter unit, and then ultracentrifuged at 120.000 *g*, for 70 min. The resultant pellet was washed with phosphate buffered saline (PBS) (1.5 mM KH2PO4, 155 mM NaCl and 2.7 mM $Na_2HPO_4 \cdot 7H_2O$ pH 7.4) and, after a second ultracentrifugation, exosomes were ressuspended in PBS (sterile when used for biological assays).

3.3 Western blot (WB) analysis

After appropriate treatments, cells were washed twice in ice-cold PBS, scraped off the dishes and lysed in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, 1% NP-40 and 0.1% SDS, pH 7.5), containing protease and phosphatase inhibitors. Cell lysates were incubated for 30 min on ice, and the solubilized fraction was recovered in the supernatant after centrifugation at 12.000 g for 10 min, at 4^{o}C. Protein concentration was determined by the Pierce™ BCA Protein Assay Kit (Thermo Scientific, Waltham, MA, USA), after which the supernatants were denatured with 4x Laemmli buffer, and heated at 95^{o}C for 5 min.

Exosomes were re-suspended in ice-cold PBS and protein concentration was determined using Pierce™ BCA Protein Assay Kit, after which were denatured with 4x Laemmli buffer and heated at 95^{o}C for 5 min.

Cell and exosomal lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat milk in Tris-buffered saline-Tween 20 (TBS-T) (20

mM Tris, 150 mM NaCl, 0.2% Tween 20, pH 7.6), probed with appropriate primary antibodies and horseradish peroxidase (HRP)-conjugated secondary antibodies. All antibodies used in this work are listed in Table 1. The proteins of interest were visualized by chemiluminescence using a VersaDoc system (BioRad).

3.4 Transmission electron microscopy (TEM)

Exosomes pellet were fixed with 2% paraformaldehyde (PFA) and deposited on Formvar-carbon coated grids (TAAB Laboratories Equipment, Berks, United Kingdom). Samples were washed with PBS and fixed with 1% glutaraldehyde for 5 minutes. Following a total of 8 washes using distilled water, grids were contrasted with a uranyl-oxalate solution, pH 7 for 5 minutes, and tranferred to methyl-cellulose-uranyl acetate for 10 minutes on ice, according to Théry, et al.[64] Observations were carried out under Tecnai G2 Spirit BioTWIN electron microscope (FEI, Oregon, USA) at 80kV.

3.5 Particle size and concentration distribution measurement with Nanosight tracking analysis (NTA).

Exosomes isolated from H9c2 cells (5 µg), cultured in exosome-depleted medium or ischemic-mimetic solution for 2h, were ressuspended in 1 mL of PBS, after which Nanosight tracking analysis (NTA) was performed using NanoSight LM 10 instrument, (NanoSight Ltd, Amesbury, UK). The analysis settings were optimized and kept constant between samples and each video was analyzed to give the mean size and estimated concentration of particles. Data were processed using NTA 2.2 analytical software.

3.6 Immunofluorescence

Cells grown on glass coverslips were fixed with 4% paraformaldehyde (PFA) in PBS, for 10 min. The samples were then washed three times with PBS, permeabilized with 0.2% v/v Triton X-100 in PBS, for 10 min, and blocked with 2.5% bovine serum albumin (BSA) for 20 min. Incubation with primary antibodies proceeded for 1 hour at room temperature. The samples were then washed three times with PBS before incubation with the secondary antibody for an additional hour at room temperature. Actin was stained using Alexa Fluor® 568 Phalloidin concomitantly to the secondary antibody. All antibodies used in this work are listed in Table 1 (end of chapter). Nuclei were stained with DAPI. The specimens were rinsed in PBS and mounted with MOWIOL 4-88 Reagent (Calbiochem). All solutions were made in 0.25% w/v BSA in PBS. Fluorescence microscopy images were obtained using a Zeiss Axio HXP IRE 2 (Carl Zeiss AG, Jena, Germany).

3.7 Exosomal PKH26 dye uptake

H9c2 cells were cultured either in exosome-depleted medium or ischemic-mimetic solution for 2 hours, after which exosomes were obtained by ultracentrifugation from cell culture supernatants. For dye uptake assays, 5 μg of exosomes per condition were labeled with PKH26 Fluorescent Cell Linker, ressuspended in Diluent C, followed by 5 min incubation with PKH26. Excess dye was washed by exosomal floatation on a sucrose gradient, where the exosomes were placed at the bottom of the ultracentrifuge tube and filled with a discontinuous gradient of sucrose (from 2.5 M to 0.4 M) and ultracentrifuged overnight, at 160.000 g. Fractions from 4-10 were collected and washed with PBS by ultracentrifugation. Recipient cells, grown on glass coverslips, were stained with Alexa Fluor 488 Phalloidin for 10 min at 37^{o}C, washed 3 times with sterile PBS, and incubated with exosomes for 30 min. Cells were fixed with 4% PFA. The specimens were rinsed in PBS and mounted with MOWIOL 4–88 Reagent. Nuclei were stained with DAPI. Fluorescence microscopy images were collected with a Zeiss Axio HXP IRE 2 (Carl Zeiss AG, Jena, Germany).

3.8 Evaluation of Endothelial Cell Monolayer Integrity

MCEC-1 were cultured on filters with 0.4-µm pores (Transwell; Corning Costar, Acton, MA). After, control or ischemic exosomes derived from H9c2 cells and 10 µM fluorescein isothiocyanate- dextran (70 kDa) were added to the apical chamber of inserts. Samples were removed from the basolateral chamber every 30 min during a 4 hours period, after which a sample from the medium in the apical chamber was collected. Fluorescence quantification was performed using an ELISA automatic microplate reader (SLT, Austria) at 492 nm absorbance/518 emissions.

The rate of diffusive flux (P_o) was calculated by the following formula:

$$P_o = [(F_A/\Delta t)V_A]/(F_L A) \qquad \text{(Eq. 1)}$$

where P_o is in centimeters per second; F_A is basolateral fluorescence; F_L is apical fluorescence; Δt is change in time; A is the surface area of the filter (in square centimeters); and V_A is the volume of the basolateral chamber (in cubic centimeters). Transendothelial electrical resistance (TEER) of monocultures was measured, in the experimental settings described above, using a STX-2 electrode coupled to an EVOM resistance meter (World Precision Instruments, Hertfordshire, UK). TEER readings of cell-free inserts were subtracted from the values obtained with cells, and results were expressed as % of control exosomes.

3.9 Wound healing assay

The wound healing assay was employed to measure MCEC-1 migration. ECs migration was assessed by scratching a confluent layer of cells in the center the well of a 12-well plate using the extremity of a 20 to 200 µL sterile pipette tip. Detached cells were removed by a PBS wash, and 1ml test medium (with control or ischemic exosomes) was added, followed by incubation at 37^{o}C in a 5% CO_2. Phase contrast were taken immediately after the scratch and 4 and 8 hours later using a Zeiss Axio HXP IRE 2 microscope (Carl Zeiss AG, Jena, Germany). The area of wound reduction was quantified using ImageJ.

3.10 Matrigel angiogenesis assay

The angiogenic potential of MCEC-1 cells was tested by Matrigel angiogenesis assay. For that 35000 cells were seeded (in a total volume of 50 µl of medium with control or ischemic exosomes) onto 10 µL solidified Matrigel (Corning® Matrigel® Matrix Growth Factor Reduced) in a µ-Slide Angiogenesis (Ibidi, Martinsried, Germany). The images were collected 8 hours after seeding, by phase-contrast images using Zeiss Axio HXP IRE 2 microscope (Carl Zeiss AG, Jena, Germany), and analyzed using plugin of angiogenesis for ImageJ.

3.11 Endothelial sprouting assay

Endothelial sprouting was assessed by a modification of the procedure described by Nakatsu, et al. [65]. Briefly, microcarrier beads coated with gelatin (Cytodex® 3; Sigma Aldrich, St. Louis, MO, USA) were seeded with MCEC-1 cells. When cells reached confluence on the beads, equal numbers of MCEC-1-coated beads were embedded in fibrin gel in Millicell EZ slide 8-well.

For preparation of fibrin gels, bovine fibrinogen type I (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in PBS in a concentration of 2.0 mg/mL. Aprotinin (Sigma Aldrich, St. Louis, MO, USA) was added at a concentration of 0.15 Units/mL, and the solution was then filtered through a 0.22 µm-pore-size filter. After beads resuspension in the fibrinogen solution at a concentration of~500 beads/ mL, the solution was transferred to Millicell Ez slide 8-well and clotting was induced by the addition of 0.625 Units/mL of thrombin (Sigma-Aldrich, St. Louis, MO, USA).

After clotting was complete, gels were equilibrated by adding exosome-depleted medium. Following 30 minutes of incubation, the overlying medium was substituted by medium containing control or ischemic exosomes from H9c2 cells. Individual beads were imaged 24 hours after inclusion in the gel using a Zeiss Axio HXP IRE 2 microscope (Carl Zeiss AG, Jena, Germany). A minimum of 15 beads per condition was imaged in each of the independent experiments and the number of sprouts was counted in a blind fashion manner.

3.12 Aortic Rings assay

Wistar rats were obtained from our local breeding colony (Faculty of Medicine of University of Coimbra, Coimbra, Portugal). All animals received care in accordance with the Portuguese Law on Experimentation with Laboratory Animals (last amendment, 2004), which is based on the principles of laboratory animal care as adopted by the EC Directive 86/609/EEC for animal experiments.

Aortic Ring assay was assessed by a modification of the procedure according to Baker, et al.[66] The Wistar rats (4 months old) were anesthetized and then sacrificed by cervical dislocation. The thoracic cavity was opened and the thoracic aorta was removed and placed in 60 mm cell culture dish containing 2 ml Opti-MEM. After dissecting, exterior fat and extravenous brachings were removed and the aorta was cleaned, and cut in equal rings ~ 5 mm in length (total of 20-25 rings were obtained). Aortic rings were placed into a 96-well plate coated with Matrigel (Corning® Matrigel® Matrix Growth Factor Reduced, and then covered with another layer of Matrigel. Aortic rings were cultured overnight with Opti-MEM (supplemented with 2.5% (vol/vol) FBS and antibiotics (100 U/ML penicillin, 100 µg/ml streptomycin). Afterward, the medium was replaced with Opti-MEM (supplemented with 2.5% (vol/vol) FBS and antibiotics (100 U/ML penicillin, 100 µg/ml streptomycin) supplemented with control or ischemic exosomes. Aorta preparations were cultured for 6 days and growth was assessed after this period. The presence of ECs in new microvessel growths was confirmed by staining the aorta ring with BS1 lectin-FITC (data not shown) Phase contrast images were taken with a Zeiss Axio HXP IRE 2 microscope (Carl Zeiss AG, Jena, Germany) and the number of sprouts was counted in a blind fashion manner.

3.13 Chicken embryo CAM assays

Fertilized chick embryos were incubated for 10 days at 37 °C with 70% humidity. Briefly, exosomes isolated from control or ischemic H9c2 cells were placed on top of E10 growing CAM into 3 mm silicon ring under sterile conditions. After 72 hours, the embryos were euthanized and the CAM was excised from the embryos, photographed *ex ovo*, at 20×

magnification (Olympus, SZX16 with a DP71 camera). To evaluate angiogenesis, the number of new vessels (<15 µm diameter) was counted in a blind fashion manner.

3.14 Statistical analysis

All data are representative of at least three independent experiments. Data were analyzed with GraphPad Prism 6 for Windows, version 6.01 (GraphPad Software, Inc.). Results are expressed as mean ± S.E.M. Student's t-test with Welch correction was applied. Differences were considered significant at $p<0.05$.

Table 1. List of primary and secondary antibodies used for the WB and immunofluorescence.

Antibody	Host/Clonality	Clone/Cat#	Application	Dilution	Company
Calnexin	goat polyclonal	AB0041	WB	1:5000	SICGEN (Cantanhede, Portugal)
Flotilin-1	rabbit polyclonal	F1913	WB	1:500	Santa Cruz Biotechnology (Texas, USA)
HSC70	Rat monoclonal	1B5	WB	1:1000	ENZO (Texas, USA)
KI67	Rabbit monoclonal	16667-500	IF	1:50	Abcam (Cambridge, UK)
ZO-1	Rabbit polyclonal	61-7300	IF	1:50	Life Technologies (Carlsbad, CA)
Alexa 488 anti-rabbit	goat	A-11034	IF	1:200	Molecular Probes, Life Technologies (Carlbad, CA)
Anti- rat-HRP	goat	172-1019	WB	1:5000	BioRad (Hercules, CA, USA)
anti-goat-HRP	rabbit	61-1620	WB	1:5000	BioRad (Hercules, CA, USA)
anti-rabbit-HRP	goat	656120#	WB	1:5000	BioRad (Hercules, CA, USA)

4. Results

4.1 Characterization of exosomes collected from cardiomyoblast cell line

It has been widely shown that exosomes, small vesicles of endocytic origin secreted by various cell types, play multiple roles in various biological processes, including, cell survival, immune responses and angiogenesis. Of particular relevance are the cardiomyocytes, that are not considered a typical secretory cell, however, have been intensively studied in the context of exosomes and their involvement in cardiac diseases.

Given their importance to supply cardiac cells with O_2 and nutrients, ECs play a critical role in the maintenance of heart homeostasis, namely in the context of myocardial contraction. However, it is believed that this effect is reciprocal, and other cardiac cells have a role in the maintenance of a functional and healthy cardiac vascular system, especially the cardiomyocytes. Therefore, it is conceivable that in response to a stressful stimulus, like ischemia, cardiomyocytes release signals that affect arteries function and structure leading to a modification in the coronary blood flow. It is plausible that in the absence of O_2 and nutrients, cardiomyocytes secrete messages in order to recruit new vessels to the affected area, with the objective of replenishing the heart with O_2 and nutrients essential to all survival. Despite its importance, this interplay between cardiomyocytes and ECs, via exosomes, namely in the context of ischemia, is largely unknown.

In this study we hypothesized that exosomes release by cardiomyoblast upon ischemia can trigger the response of cardiac endothelial cells. To test this hypothesis we started by isolating exosomes, by differential ultracentrifugation, from cell culture supernatants of the heart cardiomyoblast cell line H9c2 after incubation for 2 hours in exosome-free medium upon control (Exo-Ct) or ischemic conditions (Exo-Isch).

To confirm that the isolation procedure employed give rise to a vesicle population highly enriched in exosomes, we used nanosight tracking analysis (NTA) and electron microscopy (TEM) to assess the size of the vesicles. Our data show that the isolated extracts are enriched in vesicles with approximate size between 80 and 200 nm (Figure 8A), within the exosome-size range, and present the typical exosome morphology (Figure 8B). Moreover, NTA data shows that there is no significant difference in the number of particles

release by cardiomyoblast cells under control or ischemic conditions (2,2±0,2x10^8 and 2,1±0,3x10^8 particles/ml respectively).

Additionally, we used WB to evaluate the presence of exosomal markers like flotillin-1 and Hsc70, proteins commonly enriched in exosomes (Figure 8C). Calnexin was used to demonstrate that the isolated exosomes were free of cytoplasmic protein contamination. Altogether these results demonstrate that our isolation method allows us to obtain an exosome enriched population of vesicles.

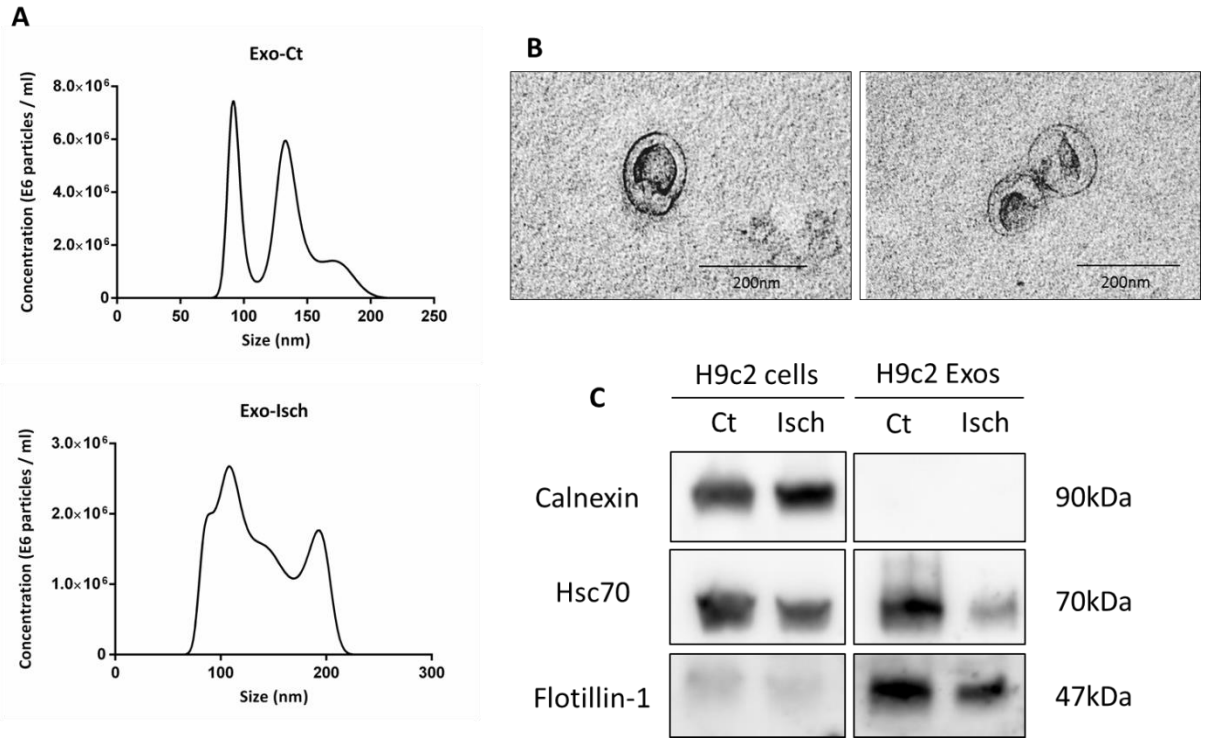

Figure 8. Characterization of exosomes isolated from control and ischemic cardiomyoblats.
(A) Exosomes isolated from control and ischemic cardiomyoblast cells (5x10^6 cells/condition) were ressuspended and analyzed by Nanosight tracking analysis (NTA). Representative graph of exosomal concentration (E6 particles/ml) and size distribution as measured by NTA. (B) TEM was performed in exosomes isolated from control and ischemic cardiomyoblasts; Scale bars 200 nm. (C) Exosomes were isolated from control and ischemic cardiomyoblasts by differential ultracentrifugation, after which WB analysis was performed in exosomal (Exos) and cell lysates to assess the presence of exosomal markers Hsc70 and Flotillin-1. 30 µg of exosomal and cell extracts was used. Calnexin was used as control for cell debris contamination.

4.2 Exosome uptake by ECs

Accumulating evidence has shown that exosomes interact with target cells and are taken up in many ways, including, receptor-mediated endocytosis, phagocytosis, micropinocytosis, or direct fusion with the plasma membrane. To determine if the exosomes secreted by control and ischemic cardiomyoblast can be internalized by ECs, the purified exosomes, labeled with green fluorescent lipidic dye PKH26, were incubated with ECs for 30 minutes. Fluorescence microscopy analysis revealed that ECs have the capacity to take up both control and ischemic-derived exosomes, as observed by the intracellular presence of PKH26 stained vesicles (Figure 9).

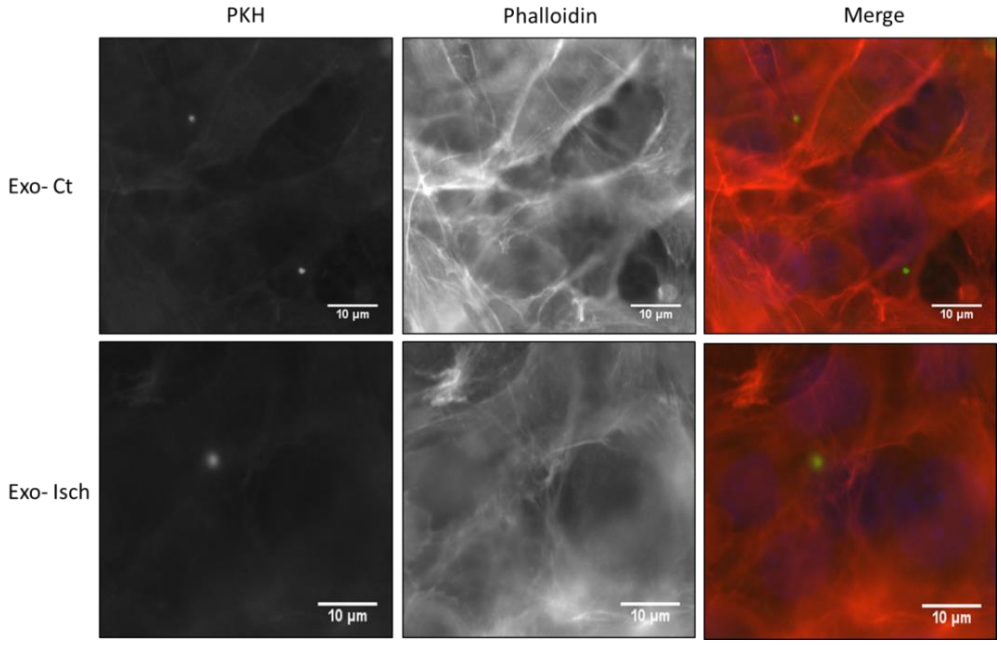

Figure 9. Exosome uptake by ECs (recipient cells).
Cardiac ECs were incubated in exosome-depleted medium, with exosomes stained with PKH26, obtained from either control or ischemic cardiomyoblasts. After incubation F-actin was stained with Phalloidin and nuclei were stained with DAPI (blue) Representative images of fluorescence microscopy analysis. All images were taken at 630x magnification. Scale bar 10 µm.

4.3 Exosomes from ischemic cardiomyoblats decrease endothelial cell permeability and promote transendothelial electrical resistance (TEER)

It is well established that vascular ECs play a major role in maintaining cardiovascular homeostasis by providing physical barrier between the vessel wall and lumen to maintain blood vessel (vascular) tone. The integrity of ECs layer is retained by the cross talk of tight junctions (TJs, zona occludens, claudins) and adherent junctions (VE-cadherin). However, in pathological conditions, such as myocardial ischemia, these junctions can be disrupted, affecting the selective barrier function of the endothelium and eventually raising vascular permeability. Due to the important role of the vascular endothelium as a first line of defense, we hypothesized that cardiomyoblast cells-derived exosomes affect the barrier of ECs.

To assess the effect of exosomes on permeability, ECs cultivated as confluent monolayers in transwells were stimulated with control or ischemic exosomes, being the permeability determined by measuring the passage of FITC-labelled dextran (molecular mass: 70 kDa) across ECs monolayers. Our results, demonstrate that stimulation with ischemia-derived exosomes lead to a decrease, of approximately 0.16-fold, in the permeability of vascular endothelial when compared with control-derived exosomes treated cells (Figure 10A).

We subsequently asked whether specific TJ protein remodeling could be triggered by the exosomes, namely ZO-1 distribution. Our analysis of ZO-1 staining by immunofluorescence shows that ZO-1 in monolayers stimulated with control derived exosomes is arranged into an irregular series of fine and course undulations (Figure 10B upper image). On the other hand when ECs were stimulated with ischemia derived exosomes, ZO-1 profile is generally in the form of smooth arc-like structures (Figure 10B down image), suggesting that ischemia derived exosomes induce a strengthening of endothelial junctions.

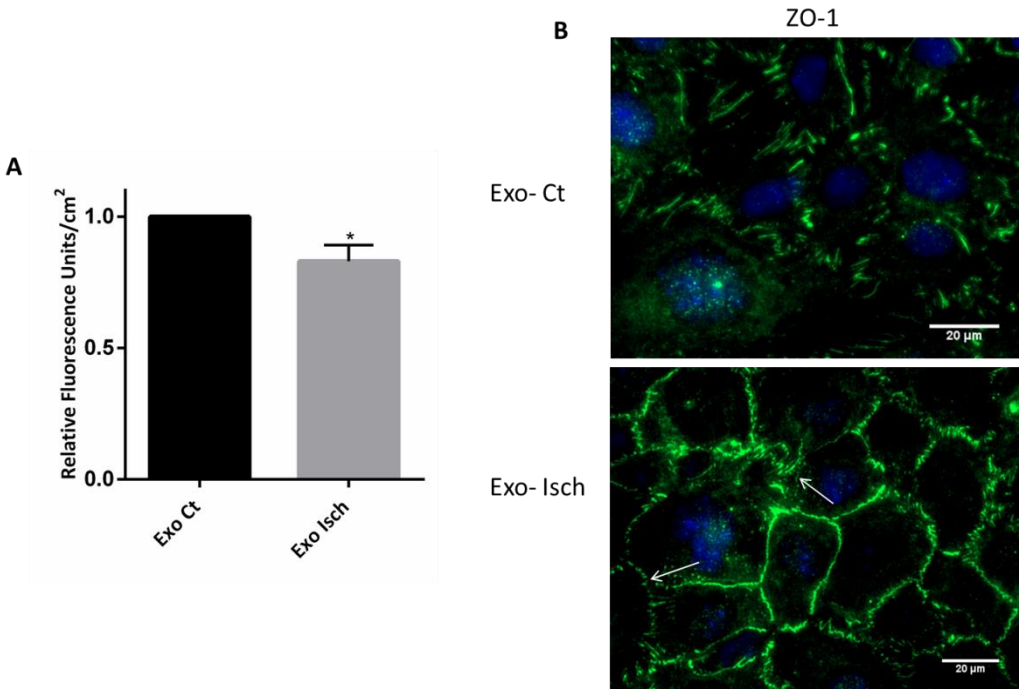

Figure 10. Exosomes derived from ischemic cardiomyoblast decrease ECs permeability and promote ZO-1 reorganization.
(A) ECs were plated in the upper part of a transwell and were cultivated until the formation of a tight monolayer. Then, FITC-labelled dextran was added together with control exosomes (exo-ct) or ischemic exosomes (exo-isch). Fluorescence was quantified in lower chamber and results were normalized to the Exo-Ct. Results represent mean ± S.E.M. of duplicate samples from three independent experiments. *p<0.05 vs. Exo-Ct; (B) Representative immunofluorescence images showing ZO-1 distribution (4 hours after incubation with Exo-Ct and Exo-Isch) ZO-1 in monolayers stimulated with Exo-Ct are organized into irregularly undulating patterns. Exo-Isch causes ZO-1 profiles to be reorganized into smooth and arc-like structures. Only occasional mild undulations are present (arrow). Nuclei stained with DAPI (blue). All images were taken at 400x magnification. Scale bar 20 µm.

Taking this into account, we decided to evaluate the effect of the exosomes released by cardiomyoblasts on transendothelial electrical resistance (TEER). TEER is a measure of the electrical current that is carried by ions in the medium and therefore, will reflect the permeability of ECs monolayer to ions.

Our results show that TEER values of cardiac ECs were significantly decreased after exposure to ischemic exosomes up until 4 hours when compared with control exosomes, indicating that the endothelial barrier was compromised (Figure 11). Our results suggest that the decrease in TEER that usually correlates with an increase in permeability was due to defects in the paracellular difusion barrier.

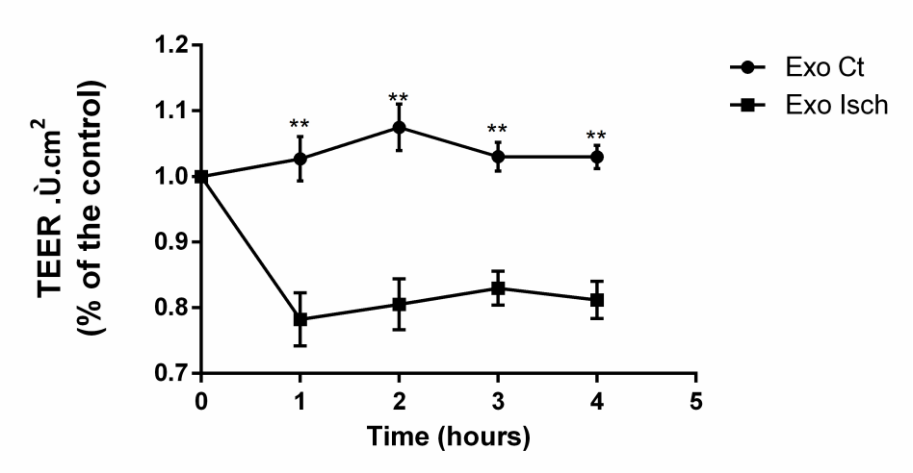

Figure 11. Effect of exosomes derived from cardiomyoblast on transendothelial electrical resistance (TEER).
Control exosomes (Exo-Ct) and ischemic exosomes (Exo-Isch) was added to a ECs tight monolayer at time 0. Resistance was recorded over 4 hours and normalized to the time 0 values. A gradual increase in endothelial permeability (reduced TEER) was observed with presence of ischemic exosomes (Exo-Isch). Results represent mean ± SEM of duplicate samples from three independent experiments. **p<0.01

4.4 Exosomes derived from ischemic cardiomyoblast cells promote proliferation of ECs

It is well established that the lack of oxygen and nutrients during IHD can lead to cardiomyocyte dysfunction that in turn can impact on ECs. To test whether exosomes release by ischemic cardiomyoblast regulate EC proliferation, we evaluated by immunofluorescence, the presence of Ki67, a nuclear protein expressed in all phases of the cell cycle except the resting phase (G_0), and widely used as a marker of proliferation positive cells for Ki67 were counted by Image-Pro Plus 6.0.

The results presented in figure 12A-B demonstrate that ECs treatment with ischemia derived exosomes significantly increased the percentage of Ki67-positive cells (13%) compared with control derived exosomes treated cells. These data suggest that ischemic-derived exosomes promote endothelial cell proliferation.

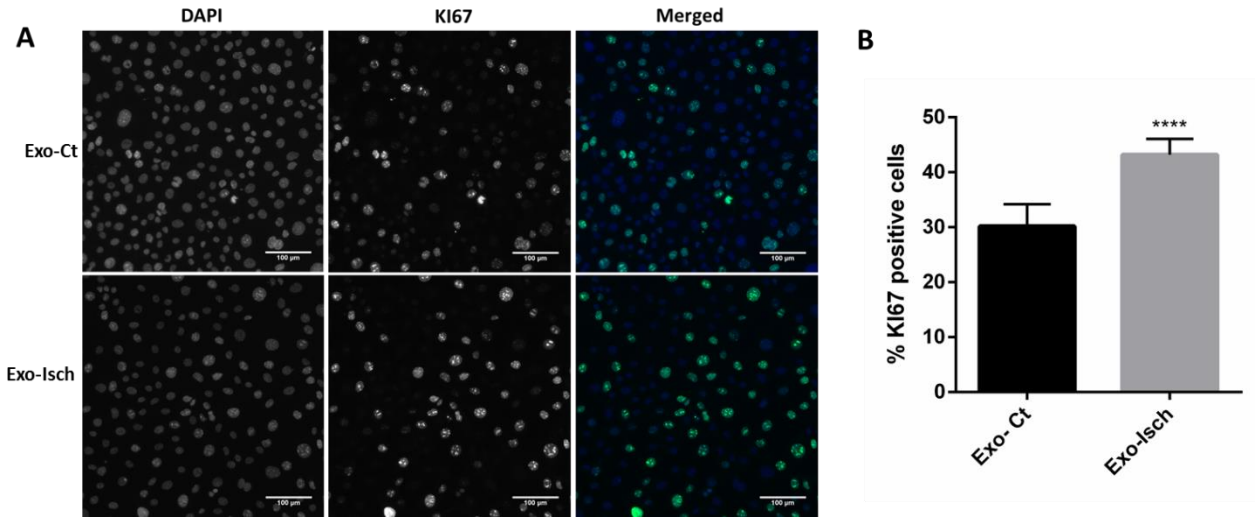

Figure 12. Ischemic exosomes derived by cardiomyoblast promote endothelial cells proliferation. EC were cultured in exosomes-depleted medium with exosome isolated from either control (Exo-Ct) or ischemic (Exo- Isch) cardiomyoblast for 8 hours. Cell proliferation was evaluated using the Ki67 positive cells (A-B). All images were taken at X20 magnification, scale bar 100 µm. Data is presented as mean ± S.E.M. ****p<0.0001; n=3 per group.

4.5 Exosomes from ischemic cardiomyoblasts promotes cardiac ECs migration and tube formation

Recently, exosomes have been recognized as a potential vehicle of intercellular communication with capacity to modulate angiogenic responses. Several studies have ascribed pro- and anti- angiogenesis function to exosomes that depend not only on the producing cell type, but also on the environment at the time of genesis and release, leading to different responses in the target cell.

To evaluate whether exosomes released by cardiomyocytes impact on ECs, we started by investigating the effect of exosomes derived by cardiomyoblast under control or ischemic conditions, on cardiac ECs migration. With that purpose we used a scratch assay, in which, cell migration into the wound space was quantified.

The results presented in Figure 13A, demonstrate that ECs incubated with ischemia derived exosomes migrate more than those incubated with control derived exosomes, suggesting that ischemia-derived exosomes play a pro-angiogenic role in heart ECs (Figure 13B).

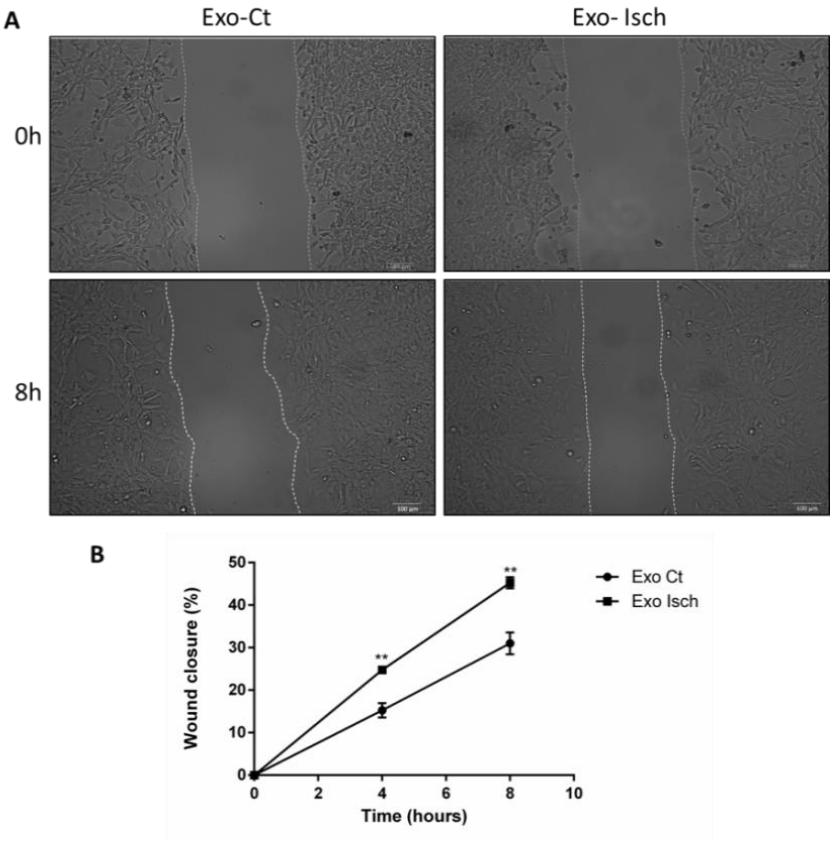

Figure 13. Exosomes derived from ischemic cardiomyoblast promote migration of cardiac ECs. (A) Migration capacity of ECs was analyzed by the wound healing assay. ECs confluent monolayer was scratched and medium was replace by exosome-depleted medium supplemented with either control (Exo-Ct) or ischemia-derived (Exo-Isch) exosomes. Cell migration was monitored for an 8 hours period after the scratch. (A) Representative phase-contrast images at time 0 (immediately after the scratch) and 8 hours after the scratch are shown. The lines indicate the wound edge. All images were taken at 100x magnification. Scale bar 100 µm. (B) Quantification was performed in images taken at the beginning of the assay and at the indicated times after the scratch. Cell-free area was measure, using ImageJ, and results represent mean ± S.E.M. percentage of recovered area of triplicate samples from three independent experiments. **p<0.01

Furthermore, we investigated the effect of exosomes on endothelial tube formation as a measure of *in vitro* angiogenesis. To assess this question, ECs were seeded on Matrigel and incubated with exosome-depleted medium supplemented with control or ischemia derived exosomes, after which we evaluated the formation of capillary-like structures (Figure 14A).

A more detailed analysis of the data, reveal that the total number of meshes, nodes and master segments lenght were significantly increased (a 0.2, 0.11 and 0.13-fold increase, respectively) in the presence of ischemia derived exosomes, when compared with control

exosomes (Figure 14B). Put together, all of these parameters consistently suggest that ischemia-derived exosomes promote tube formation *in vitro.*

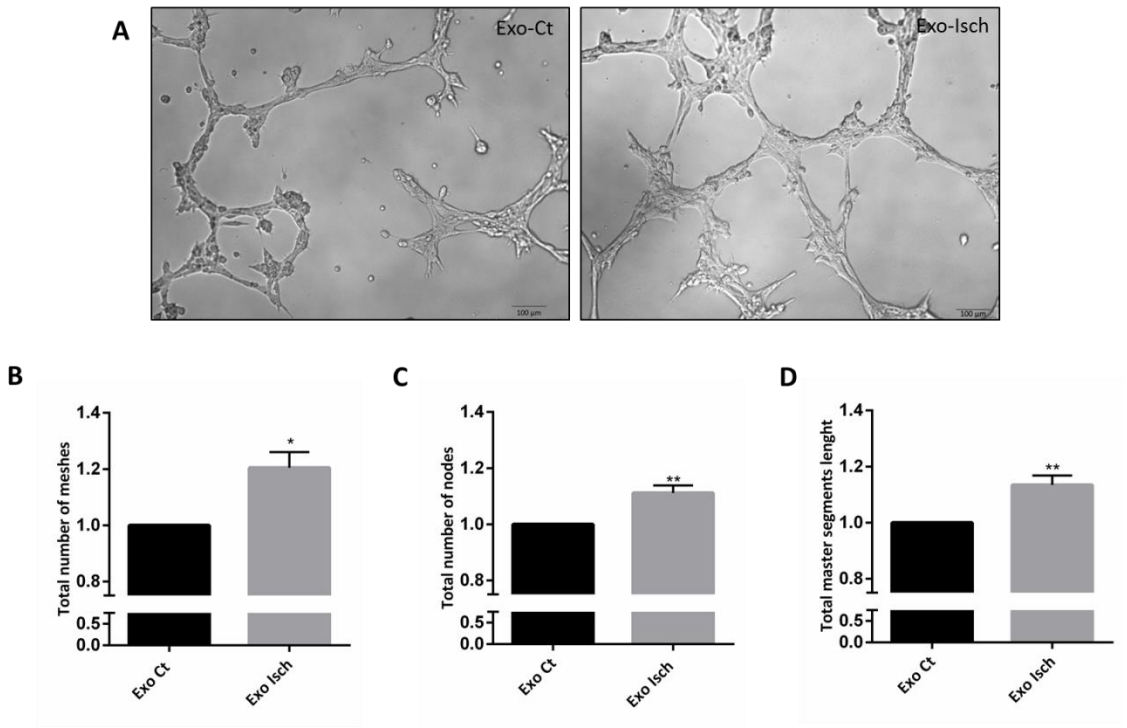

Figure 14. Exosomes derived from ischemic cardiomyoblast promote ECs differentiation into capillary-like structures *in vitro*.
Capillary-like tube formation assay on Matrigel was assessed 5 hours after ECs seeding with exosome-depleted medium supplemented with either control (Exo-Ct) or ischemia-derived (Exo-Isch) exosomes. (A) Representative phase-contrast images are shown. All images were taken at 100x magnification. Scale bar 100 µm. Quantitative assessment of (B) total number of meshes, (C) nodes and (D) master segments lenght was performed by analytical software Image Pro Plus 5.1 and results were normalized to the Exo-Ct. Results represent mean ± S.E.M. of triplicate samples from three independent experiments.*p<0.05, **p<0.01.

4.6 Exosomes from ischemic cardiomyoblasts promote ECs sprouting

In a subsequent stage of this work, we proceeded to evaluate the effect of exosomes secreted by ischemic or control cardiomyoblast cell line in the formation of endothelial sprouts. To accomplish this task EC seeded on cytodex beads were cultured within 3D fibrin gel and stimulated with either control or ischemic exosomes (Figure 15A). As observed in Figure 15B, ischemic exosomes induce higher number and longer sprouts, 1.4 fold, when

compared with control exosomes, after incubation for 24 hours. These data suggest that ischemic exosomes have a pro-angiogenic effect by promoting the formation of endothelial sprouts.

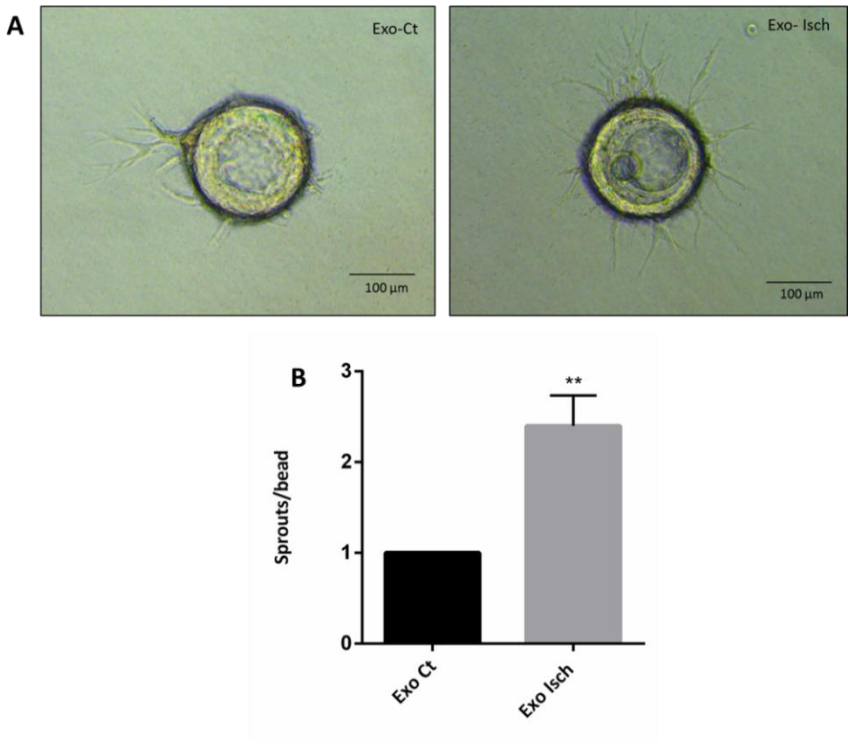

Figure 15. Ischemic exosomes induce sprouting angiogenesis *in vitro.*
ECs on beads in three-dimensional fibrin gels incubated with exosomes derived by either control (Exo -Ct) or ischemic (Exo- Isch) cardiomyoblast were observed after 24 hours. (A) Representative phase-contrast images are shown. All images were taken at 100x magnification. Scale bar 100 µm. (B) Quantitative assessment of number of sprouts per bead was performed in at least 15 beads per condition and results were normalized to the Exo-Ct. Results represent mean ± S.E.M. of samples from five independent experiments. **p<0.01

To further confirm these results we decided to proceed the studies using a three-dimensional *ex vivo* assay, the aortic ring assay. For this purpose, we used transverse sections of rat aortic tissue embedded in Matrigel. The aortic rings were incubated with exosomes derived from cardiomyoblast cells under control or ischemic conditions. After 5 days, the aortic rings were fixed and endothelial cells were identified using BS1 lectin-FITC (data not shown), a specific marker of endothelial cells, and the endothelial sproutings were counted in bright field images (Figure 16A).

Not surprisingly, our results show that ischemic derived exosomes significantly increase, in the sprouts number in 1.4 fold compared with control exosomes (Figure 16B). Together, these results confirm that ischemic exosomes significantly promote sprouting *in vitro* and *ex vivo*.

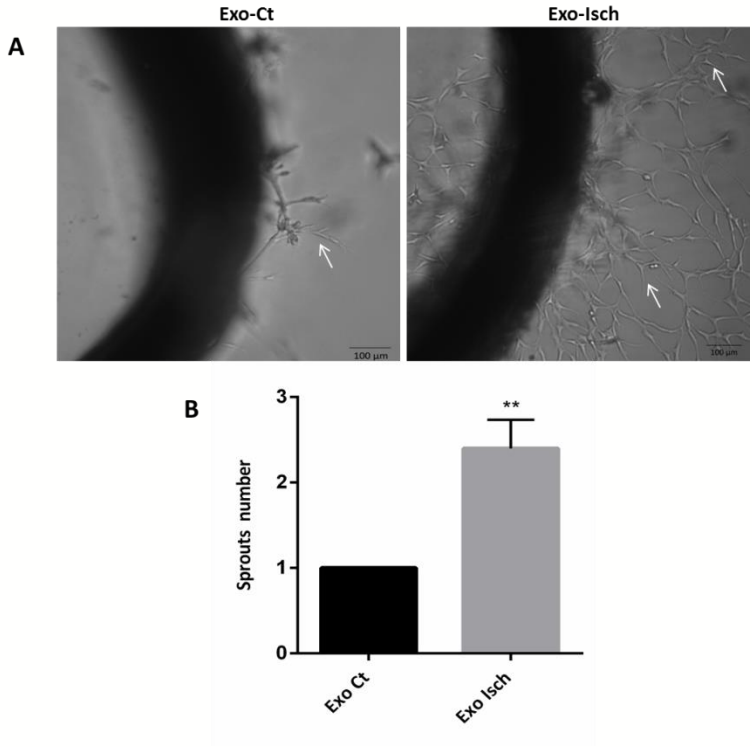

Figure 16. Ischemic exosomes induce sprouting angiogenesis *ex vivo.*
Cultures of rat aortic rings were stimulated with exosomes derived from either control (Exo -Ct) or ischemic (Exo- Isch) cardiomyoblast, 5 days after the treatment aortic rings were fixed and analyzed by microscopy. (A) Representative phase-contrast images are shown. All images were taken at 100x magnification. Scale bar 100 µm. (B) Quantitative assessment of number of sprouts per aortic ring and results were normalized to the Exo-Ct. Results represent mean ± S.E.M. of triplicate samples from four independent experiments. **p<0.01

4.7 Exosomes from ischemic cardiomyoblasts promote angiogenesis *ex vivo*

The results presented up to this point constitute an important contribution to establish the effect of exosomes produced by cardiomyocytes, either in control or ischemia conditions on angiogenic activity of ECs in *in vitro* and *ex vivo* models. Therefore, in the final stage of this work, we evaluated whether these exosomes exhibit angiogenic effect *in vivo.* For that purpose, we used the chick chorioallantoic membrane (CAM) assays. Fertilized

chick eggs were treated with exosomes derived from control or ischemic cardiomyoblats (Figure 17A), and the angiogenic response was evaluated 72 hours after inoculation.

CAM results revealed that ischemia derived exosomes lead to an increase of about 30% in the vascular density (Figure 17B). Importantly, these results confirm the pro-angiogenic potential of exosomes produced by ischemic cardiomyoblasts.

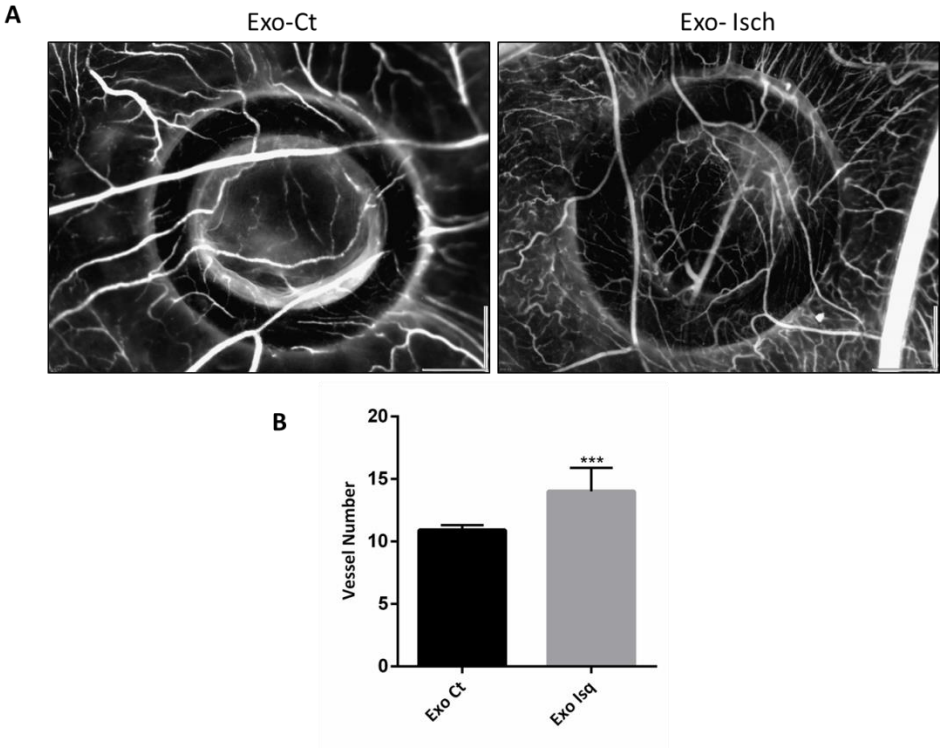

Figure 17. Ischemia-derived exosomes induce angiogenesis *ex vivo.*
Fertilized chicken eggs were exposed to exosomes derived from control and ischemic cardiomyoblasts. After incubation for 72 hours, the CAMs were photographed and visible branches counted. (A) Representative images of the vascular branches in CAM. All images were taken at 20x magnification (B) Quantification of the newly formed blood vessels. Results represent mean ± S.E.M. of five to eight eggs per condition from three independent experiments. **p<0.01

5. Discussion

The heart is an organ formed by a diversity of cell types, including cardiomyocytes, endothelial cells, fibroblasts, smooth muscle cells, immune cells, pacemaker cells and Purkinje cells. To pump the blood to the various organs and tissues of the organism, the heart relies on a well-organized intercellular communication network, essential to provide structure and ensure regulated and synchronized heart contraction[1,67].

It has been demonstrated that efficient communication between cardiac cells, both directly, via gap junctions, and indirectly, via exosomes, is crucial to maintain heart function[13,68]. In agreement, several cardiomyopathies, including IHD, have been associated with on impairment of the mechanisms underlying intercellular communication between different cardiac cells[61,69,70]. Given their high energetic demand, to maintain heart contraction, cardiac muscle cells (cardiomyocytes) depend on continuous blood supply, to an nourishment.

It is widely accepted that IHD develops as a result of reduced coronary blood flow usually due to coronary atherosclerotic plaques formation. In under this circumstance the heart activates strategies to compensate the lack of O_2 and nutrients caused by myocardial ischemia[8,71]. The formation of new vessels that replenish the affected area consists of one of these strategies, giving rise to collateral circulation.

Although it is well established that endothelial cells play a critical role in cardiomyocyte survival and myocardial contraction[72], the interplay between cardiomyocytes and endothelial cells, namely via exosomes, remains largely underexplored.

According to recent studies, exosomes released by different types of cardiac cells appear to play a role in the angiogenic process, representing a new way to disseminate either pro- or anti- angiogenic signals[41]. The message conveyed in exosomes depends not only on the producing cell type, but also on the environmental conditions where these vesicles are produced and released, leading to different responses in the target cell[73]. However, the effect of exosomes secreted by cardiomyocytes subjected to ischemia, on endothelial cells has never been addressed.

The work presented herein aims to fill this gap in knowledge by evaluating whether exosomes secreted by the cardiomyoblast H9c2 cell line affects endothelial cell function.

Our first goal was to characterize exosomes secreted by the cardiomyoblast H9c2 cell line under control or ischemic conditions. First, using nanoparticle tracking analysis (NTA) we showed that vesicles we isolate are within exosome-size range (up to 200nm). By transmission electron microscopy (TEM) we demonstrated that, these vesicles present the typical morphology of exosomes. Moreover, the nature of these vesicles was confirmed by the presence of exosomal markers, such as flotillin-1 and Hsc70. Altogether, these results demonstrate that our isolation procedure gives rise to a population of vesicles enriched in exosomes.

It is conceivable that signals emitted by injured cardiomyocytes, in the sequence of an ischemic event, can modulate the angiogenesis process. Based on this hypothesis, we proposed that exosomes secreted by cardiomyocytes regulate endothelial cells function. To address this question, we evaluated the effect of exosomes derived by cardiomyocytes, under control or ischemic conditions, in EC function and behavior.

It is well established that ECs that cover the internal surface of blood and lymphatic vessels, play a major role in maintaining cardiovascular homeostasis. However, during IHD, the integrity of EC tight junctions and adherent junctions are disrupted, which affect the selective barrier function of the endothelium and eventually raise vascular permeability[74].

In order to understand the role of the endothelium as a first line of defense, we assessed the impact of exosomes, derived by cardiomyocytes under control or ischemic conditions, on EC barrier properties. For that, we evaluated barrier properties through two different but complementary approaches: cellular permeability and transendothelial electrical resistance.

To address paracellular permeability we used a fluorescently labelled dextran (70 KDa FITC-dextran) on a monolayer of ECs incubated in the presence of either control or ischemic exosomes. The results obtained in this study demonstrate that incubation of ECs with ischemic exosomes induces significant decrease in EC permeability when compared with control exosomes, thus suggesting that ischemic exosomes promote the adhesion though of ECs junctional proteins. To further confirm this hypothesis, we evaluated the effect of these exosomes on the subcellular staining of tight junction adaptor protein ZO-1. Our analysis of ZO-1 distribution by immunofluorescence shows that ZO-1 in monolayers stimulated with control derived exosomes is arranged into an irregular series of fine and course undulations. On the other hand when ECs were stimulated with ischemia derived

exosomes, ZO-1 profile is generally in the form of smooth arc-like structures. This result is supported by the previous study that suggests that the local disruption in tight junction architecture is a consequence of ZO-1 undulations[75]. Another study demonstrated that ZO-1 forms a cooperative unit that functions as a master regulator of endothelial tight junctions through the stimulation of junctional actomyosin, following activation of JACOP and P114RhoGEF. According to this work, ZO-1 regulates endothelial barrier formation, cell-cell tension, and angiogenic potential by the recruitment of additional actomyosin regulators, such as vinculin and PAK2[76].

Taking this into account, we decided to evaluate the effect of exosomes release by cardiomyocytes on transendothelial electrical resistance (TEER). TEER was determined using the two-compartment transwell, where electrical resistance is measured directly across a monolayer using an electrical resistance meter, by inserting probes into the luminal and abluminal compartments. The resistance of the filter membrane is first measured in the absence of cells, and then is subtracted from the total resistance in the presence of a confluent cell monolayer to yield the resistance of the cell monolayer per unit surface area (specific resistance; Ohm cm^2).

Surprisingly and contrary to the permeability assays, the TEER values decreased in the presence of ischemic exosomes when compared with control exosomes. This apparent contradictory result can be explained by the fact that exosomes differentially affect tight junction properties regarding the permeation of macromolecules and ions. It is plausible that ischemic exosomes induce tight junction remodeling that excludes macromolecules but allows permeation of ions.

A previous study have demonstrated that ion channels are present at tight junctions, where they mediate ion transport across lipid bilayers. This new class of paracellular-tight junction channels (PTJC) facilitates the transport of ions through adjacent cells[77]. Taking into consideration these results, we can suggest that ischemic exosomes promote the passage of ions but exclude larger molecules thus accounting for the altered stability of the endothelial barrier.

ECs proliferation is an essential process for developing capillaries during angiogenesis process. We assessed the ECs proliferation using Ki67, a nuclear protein expressed in all mitosis phases excepting G0. To evaluate whether exosomes release by ischemic cardiomyoblast regulate EC proliferation, ECs were incubated with control or ischemic

exosomes for 8h and then an immunofluorescence assay was performed using a KI67 antibody. The results demonstrate that ECs treatment with ischemia derived exosomes significantly increased the Ki67-positive cells compared with control derived exosomes treated cells.

Furthermore, we demonstrated that exosomes secreted by cardiomyoblast subjected to ischemia, promote endothelial cell migration and tube formation, when compared with control exosomes. Taken together, these data suggest that ischemia, induces the release of exosomes that have an ability to promote angiogenic responses on cardiac ECs.

Our results are in agreement with previous studies showing that exosomes are a potential mode of intercellular communication with capacity to modulate angiogenic responses in target cells[40,62,78]. Interestingly, a recent study demonstrated that cardiomyocytes-derived exosomes contain a large amount of Hsp20 that remarkably promotes ECs migration and tube-like formation. Mechanistically, the exosomal Hsp20 interacts with VEGFR2 and activates its downstream Akt/ERK signaling cascade. Given that coronary angiogenesis is instrumental in functional compensation and restoration of the heart, increased angiogenic potential in cardiac specific Hsp20 overexpressing may provide a new mechanism underlying Hsp20-mediated protective effects against potential cardiac injury[79]. Nevertheless future studies are needed to determine the exosomal components that vary from control to ischemic exosomes secreted by cardiomyoblast and understand how they influence the biological activity of EC.

It is established that angiogenesis is defined as a new blood vessel sprouting from pre-existing vessels. In order to better understand the crosstalk established between cardiomyocytes and ECs, we also investigated the role of exosomes released by either control or ischemic cardiomyoblast in endothelial sprouting usually used as a way to evaluate angiogenic potential. In a first approach using EC coated beads that were embedded in a fibrin gel, we demonstrated that exosomes secreted by ischemic cardiomyoblast induce more sprouts when compared to control exosomes. However, the simplicity of our *in vitro* model, involving a single type of cells, does not reproduce the *in vivo* biological environment of ECs, where they are interacting with other types of cells and

subjected to external stimuli. Indeed, a recent report showed that exosomes also provide signaling cues for EC, stimulating sprouting angiogenesis, through miR-214, which is highly expressed in endothelial cells[80].

Therefore, we proceeded to evaluate the effect of exosomes on EC sprouting using the *ex vivo* rat aortic ring sprouting assay in which developing microvessel undergo many key features of angiogenesis over a timescale similar to that observed *in vivo*. The vascular outgrowths are very similar to normal blood vessels and are composed of the same cell types in animal model.

The aortic rings embedded in Matrigel were incubated with exosomes derived from control or ischemic cardiomyoblast, after which we evaluated the number of sprouts. Our results show that ischemic exosomes caused a significant increase in cell sprouting when compared with control exosomes. These observations confirm the results we obtained with EC coated beads, reinforcing the idea that ischemic exosomes promote sprouting angiogenesis. Several studies demonstrated that the genetic content of exosomes include pro- angiogenic factors that increase the number of sproutings in ECs that are essential to provide nutrients and oxygen[80-82]. It is therefore conceivable to suggest that exosomes released by ischemic cardiomyoblast transport pro-angiogenic factors, which in turn trigger signaling pathways in ECs, that leads to the formation of EC sprouts.

Nevertheless additional studies are required to determine the exosomal content and the mechanism whereby ischemic cardiomyoblasts released exosomes induce an increased in sprouting angiogenesis.

Lastly, we intended to validate the results obtained *in vitro* in a more physiological and relevant biological model. To evaluate the effect of exosomes in angiogenesis *ex vivo*, we performed *ex vivo* chick chorioallantoic membrane (CAM) assays that have been widely used to study angiogenesis. In accordance with the results discussed above, the CAM assay revealed that ischemic exosomes caused higher recruitment of blood vessels compared to control exosomes, thus demonstrating an angiogenic effect. This assay confirms and validates our previous *in vitro* studies, strongly suggesting that cardiomyocytes communicate with ECs, via exosomes, most likely in an attempt to maintain heart homeostasis.

In conclusion, the results presented in this study using a comprehensive and integrated strategy, establishes, for the first time, that exosomes released by cardiomyoblast, subjected to ischemia, stimulate angiogenesis of cardiac ECs.

This study highlights the importance of cardiomyocyte-EC intercellular communication, through exosomes, in the heart and its implications in ischemia associated loss of heart function. Based on these data, we envision a model whereby cardiomyocytes and ECs communicate via exosomes to maintain cardiac homeostasis.

Therefore, it is tempting to speculate that strategies that amplify this process can be beneficial to improve angiogenesis after ischemic episodes and, consequently heart function. Although future studies are required to better elucidate the implications of these communication systems in cardiac physiopathology, we believe that identifying the mechanisms involved, in this exosomes mediated pro-angiogenic response could represent a promising new therapeutic strategy against IHD.

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