Ischaemia impacts TNT-mediated communication between cardiac cells

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Efficient contraction of the heart relies on a highly regulated communication network between cardiac cells. Direct intercellular communication is mediated by gap junctions but can also occur through tubular structures named tunnelling nanotubes (TNTs), which connect the cytoplasm of neighbouring cells and facilitate the transport of various cargoes. Although the formation of TNTs between cardiomyocytes has been reported, the effect of ischaemia on this process remains unclear. In this work, we assessed the impact of ischaemia and oxidative stress on TNT-mediated communication between cardiac cells. We found that cardiac cell lines and neonatal primary cultures of cardiomyocytes subjected to in vitro ischaemia form more TNTs than control cells. Moreover, antioxidants prevented ischaemia-induced TNT formation, suggesting that oxidative stress regulates this process. Furthermore, we identified troponin T as a new specific marker of cardiomyocyte-derived TNTs, which allows for the identification of heterocellular TNT connections between cardiomyocytes and other resident cells in the heart, such as fibroblasts. We also determined the presence of TNT-like structures in rat and human hearts. Rat hearts subjected to global ischaemia in the ex vivo Langendorff system showed increased formation of TNTs. Altogether, this study demonstrates that ischaemia affects the formation of TNTs in the heart and sheds new light on the regulation of TNT-mediated communication between cardiomyocytes.

1. Introduction

Efficient communication between the different cells that form the tissues and organs is essential to ensure the maintenance of an organism's homeostasis. Soluble factors and extracellular vesicles mediate long-range communication, whereas gap junctions and tunnelling nanotubes (TNTs) ensure the exchange of information between physically connected cells (Simons and Raposo, 2009; Nielsen et al., 2012; Rustom et al., 2004). TNTs, which are thin and long F-actin-enriched membrane structures with a diameter of 20–500 nm and a length of up to 100 μm, permit the transfer of diverse materials including miRNAs, proteins, organelles, prions and pathogens between connected cells (Rustom et al., 2004; Sowinski et al., 2008; Gousset et al., 2009; Onfelt et al., 2006; Ribeiro-Rodrigues et al., 2017a; Abounit and Zurzolo, 2012). Unlike other F-actin membrane projections (e.g. filopodia), TNTs do not attach to the substrate, are highly dynamic and fragile, being sensitive to external factors such as light exposure, mechanical stress and chemical fixation. This, as well as the lack of specific TNT markers, has hampered the observation of these structures in their natural environment within tissues (Rustom et al., 2004; Abounit and Zurzolo, 2012). Despite these limitations, several studies have demonstrated the biological relevance of TNTs during important cellular states and processes such as...
embryogenesis (Caneparo et al., 2011; Pyrgaki et al., 2010), differentiation (Teddy and Kulesa, 2004), wound healing (Wood et al., 2002), stress resistance (Cselényik et al., 2010), immune response (Chinnery et al., 2008; Kumar et al., 2017) and cancer progression (Lou et al., 2012). The functionality of TNTs has been demonstrated in a variety of cell types in vitro including fibroblasts (Kim et al., 2019), epithelial (Wang et al., 2010), immune (Sowinski et al., 2008), neural (Wang et al., 2011) and cardiac cells (Koyanagi et al., 2005), as well as in numerous cancer cells (Lou et al., 2012; Osswald et al., 2015). Spurred by recent advances in microscopy techniques (Lou et al., 2017), TNTs or TNT-like structures have also been described in vivo, for example in the skin (Su and Igyártó, 2019), the cornea (Chinnery et al., 2008) and the heart (He et al., 2011). In particular, TNTs appears to be highly present in many tumours, for example astrocytomas (Osswald et al., 2015), mesotheliomas (Ady et al., 2014) and pancreatic cancer (Desir et al., 2018). Indeed, although TNTs can form under normal physiological conditions, several studies suggest cellular stress including pro-inflammatory stimuli (Chinnery et al., 2008), viral infection (Eugenin et al., 2009), hypoxia (Desir et al., 2016), oxidative stress (Zhu et al., 2005) and ischaemia (Han et al., 2016) can trigger this process. It is known that ischaemia, which is stress resistance (Cseleny et al., 2008), viral infection (Eugenin et al., 2009), hypoxia (Desir et al., 2016), oxidative stress (Zhu et al., 2005) and ischaemia (Han et al., 2016) can trigger this process. It is known that ischaemia, which is defined by an inefficient blood supply with a consequent shortage of oxygen and nutrients to cells and tissues, has a severe impact in different aspects of cell function and homeostasis. Mounting evidence has demonstrated the dramatic impact of ischaemia on intercellular communication mediated by either gap junctions or extracellular vesicles (Martins-Marques et al., 2015a; Ribeiro-Rodrígues et al., 2017b; Almeida Paiva et al., 2018; Sluijter et al., 2018; Davidson et al., 2018). However, to the best of our knowledge, the effect of heart ischaemia on intercellular communication through TNTs remains to be studied. In the present study, we aimed to evaluate the impact of ischaemia on TNT-mediated communication between cardiac cells.

2. Material and methods

2.1. Cell cultures

The rat cardiomyoblast H9c2 cell line (Sigma-Aldrich, St. Louis, MO via European Collection of Cell Cultures (ECACC)) was cultured in Dulbecco’s Modified Eagle Medium (DMEM) (Life Technologies, Carlsbad, CA), supplemented with 10% fetal bovine serum (FBS) (Life Technologies) and Penicillin/Streptomycin (100 U/mL; 100 μg/mL) (Life Technologies). Cells were maintained at 37 °C in a humidified chamber with 5% CO2.

The mouse atrial cardiomyocyte cell line HL-1 (clone 6) was obtained from Dr Emmanuel Dupont (Imperial College London, London, United Kingdom), established from the HL-1 parental cell line previously immortalized by Dr W. C. Claycomb (Louisiana State University Health Centre, New Orleans, LA, USA) (Claycomb et al., 1998; Dias et al., 2014). HL-1 cells were cultured in gelatin/fibronectin (0.02% gelatin/1 mg/mL fibronectin) coated culture vessels and maintained in Claycomb medium (Sigma-Aldrich) supplemented with 0.1 mM Norepinephrine (Sigma-Aldrich), 2 mM L-Glutamine (Sigma-Aldrich), 10% FBS and Penicillin/Streptomycin (100 U/mL; 100 μg/mL), at 37 °C under 5% CO2 (Martins-Marques et al., 2015a).

2.2. Cell treatments

When indicated, H9c2 cells were incubated with 250 μM of Hydrogen peroxide (PanReac AppliChem, Chicago, IL, USA) for 30 min, 200 μM of L(+) Ascorbic acid (Riedel-de Haen®, Seelze, Germany) or 500 μM of Glutathione (Sigma-Aldrich) for 18 h. Unless stated otherwise, all chemicals were purchased from Sigma-Aldrich.

2.3. Animal models

Wistar rats were obtained from our local breeding colony (Faculty of Medicine of the University of Coimbra, Coimbra, Portugal). All animals were handled according to European Union guidelines for the use of experimental animals (2010/63/EU). Experiments were approved by the Ethics Committee of the Faculty of Medicine, University of Coimbra (ORBEA-IBIL, permit 13/2015).

2.4. Neonatal primary cultures of rat cardiomyocytes

Primary cell cultures were obtained from 3-day-old (P3) Wistar rats euthanized by decapitation. Hearts were removed and cardiac cells were isolated from the ventricles by enzymatic digestion with Trypsin/Collagenase, as previously described (Fu et al., 2005). Briefly, after excising the ventricles, hearts were separated from the atria, rinsed with Hank’s Balanced Salt Solution, and incubated with 0.1% trypsin-EDTA over-night, at 4 °C. Type II collagenase (Gibco, 75 μU/mL) digestion was further performed for 30 min, at 37 °C, followed by mechanical dissociation of the tissue. Enzyme inactivation was performed by the addition of DMEM containing 10% FBS, after which digested tissues were transferred through a screen (70 μm). Cells were recovered by centrifugation and plated into 1% (w/v) gelatin-coated dishes for 3 hous. After that, non-adherent cells (enriched in cardiomyocytes) were plated at a density of 3.3x10^5 cells/cm^2 on gelatin/fibronectin-coated coverslips, resulting in a 70% confluent cell culture. Cells were maintained in DMEM, supplemented with 10% FBS, Penicillin/Streptomycin (100 U/mL; 100 μg/mL), at 37 °C, under 5% CO2.

2.5. Human heart samples

Human heart samples were obtained from surgical septal myectomy specimens of patients submitted to aortic valve replacement for severe aortic stenosis and concomitant subaortic septal myectomy (Center of Cardiothoracic Surgery, University Hospital and Faculty of Medicine of Coimbra, Coimbra, Portugal). The work has been carried out in accordance with The Code Ethics of the World Medical Association (Declaration of Helsinki). Human heart slices were embedded in optimum cutting temperature (OCT) matrix (Tissue-Tek®, Histolab, Sweden) for cryosectioning.

2.6. In vitro ischaemia model

Ischaemia was induced in vitro by a buffer exchange to an ischaemia-mimetic solution (118 mM NaCl, 4.7 mM KCl, 1.2 mM KH2PO4, 1.2 mM MgSO4, 1.2 mM CaCl2, 25 mM NaHCO3, 5 mM calcium lactate, 20 mM 2-deoxy-D-glucose, 20 mM HEPES, pH 6.6) and by placing the dishes in hypoxic pouches (GasPakTM EZ, BD Biosciences), equilibrated with 95% N2/5% CO2.

2.7. Langendorff heart perfusion model

Wistar rat hearts were perfused in a Langendorff apparatus, with modified Krebs-Henseleit buffer, as described before (Martins-Marques et al., 2015b). Perfusion was stabilized for 10 min followed by further perfusion during 60 min for control conditions or exposed to no-flow ischaemia for 60 min. After control or ischaemia, hearts were embedded in OCT for cryosectioning before storage at −80 °C.

2.8. Fluorescence staining

H9c2, HL-1 cells and neonatal primary cultures of rat cardiomyocytes cultured on gelatin/fibronectin-coated glass coverslips were fixed with two different fixative solutions (fixative 1: 2% PFA, 0.05% glutaraldehyde, 0.2 M HEPES, followed by fixative 2: 4% PFA, 0.2 M HEPES, both diluted in PBS), as described before (Abounit et al., 2015). Fixed cells were washed with PBS and labelled with Alexa Fluor 488-conjugated wheat germ agglutinin (WGA; Sigma-Aldrich; 1:200 dilution) and Phalloidin-Tetramethylrhodamine B isothiocyanate (Sigma-Aldrich; 1:300 dilution) for 20 min in the dark, at room temperature. For

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immunofluorescence staining, H9c2 cells or primary cardiomyocytes cultured on fibronectin-coated glass coverslips were fixed with 4% PFA, 0.05% glutaraldehyde in PBS (fixative 3). Fixed cells were washed with PBS, permeabilized with 0.1% Triton X-100 in PBS and blocked with 2% BSA for 20 min prior to incubation with primary mouse anti-vinculin antibody (Abcam; ref: ab11194; dilution 1:400) or primary mouse monoclonal anti-Cardiac troponin T antibody (Abcam; ref: ab33589; dilution 1:500) in 2% BSA for 1 h at room temperature. The samples were then washed three times with PBS before incubation with the secondary antibody for 40 min at room temperature in the dark. The specimens were rinsed in PBS and mounted with MOWIOL 4–88 reagent (Calbiochem, San Diego, CA, USA).

For rat and human heart samples (20 μm cryosections), fixation was performed as described before (fixative 1 and 2 for WGA and phalloidin staining, or fixative 3 for vinculin or troponin T staining). The samples were then washed three times with PBS, permeabilized with 0.1% Triton X-100 and blocked with 2.5% BSA for 30 min prior to incubation with WGA, phalloidin and/or primary mouse monoclonal anti-Cardiac troponin T antibody and primary mouse monoclonal anti-Lamp1 antibody (Calbiochem; ref. 428017; dilution 1:25) for 2 h at room temperature in the dark. The samples were then washed three times with PBS before incubation with the secondary antibody for 1 h at room temperature, protected from light. The specimens were rinsed in PBS and mounted with MOWIOL 4–88 Reagent. The images were collected by fluorescence microscopy using a widefield (Zeiss Axio HXP IRE 2; Carl Zeiss AG, Jena, Germany) or a confocal microscope (Zeiss LSM 710).

2.9. TNT quantification

Cells were grown until they reached about 70% confluence which was found as the optimal confluence to allow TNT formation. For TNT counting, only projections that are 1) positive for both WGA and phalloidin, 2) connect remote cells and 3) present in middle and/or upper stacks of the picture in Z dimension (not attached to the substrate) are considered as TNTs. The total number of TNTs and cells were counted manually in each image. For statistical analysis, 30 fields for each experimental condition were collected, from at least three independent experiments for each cell type. In heart slices images, the total number of TNTs were counted manually in each image.

2.10. LysoTracker and MitoTracker-positive vesicle intercellular transfer

H9c2 cells and neonatal primary cultures of rat cardiomyocytes grown on gelatin/fibronectin-coated μ-Dish 35 mm, high (Ibidi, Cat. No: 81156, Gräfelfing, Germany) were incubated with 50 nM LysoTracker® Red DND-99 (Life Technologies) or 100 nM MitoTracker® Green FM (Life Technologies) following manufacturer’s instructions. Pictures and videos were obtained using a Zeiss Axio HXP IRE 2 microscope (Carl Zeiss AG).

2.11. Statistical analysis

Data are representative of three independent experiments. Data were analyzed with GraphPad Prism 6 for Windows, version 6.01. Unless stated otherwise, results are expressed as individual data points with mean ± SEM. As appropriate, Mann-Whitney or Kruskal-Wallis tests were applied. Differences were considered significant at p < 0.05.

3. Theory/calculation

This study demonstrates that ischaemia impacts on the formation of TNTs between cardiac cells, which brings new insights concerning the mechanisms involved in the regulation of cardiac intercellular communication. The knowledge gathered in this work allowed the identification of a new marker of TNT of cardiomyocyte origin and the mechanisms that trigger their formation elicited by ischaemia, paving the way towards the development of innovative therapeutic strategies tackling defects of TNT-mediated communication implicated in cardiac diseases.

4. Results and discussion

4.1. Ischaemia increases the number of TNTs formed between cardiac cells

It is known that ischaemia affects intercellular communication in different tissues and organs including the heart (Ribeiro-Rodrigues et al., 2017b; Almeida Paiva et al., 2018; Martins-Marques et al., 2015c; Johansen et al., 2011). Although intercellular communication through gap junctions and extracellular vesicles has been a matter of intense research, the impact of ischaemia on TNT-mediated communication between cardiac cells remains elusive. In this work, we aimed to characterize the formation of TNTs between cardiac cells and assess how ischaemia impacts the dynamic of these structures. For this purpose, we used a comprehensive approach that encompassed cell culture systems including cardiac cell lines and primary co-culture systems to investigate the formation of TNTs between cardiomyocytes, fibroblasts and cardiomyocytes and fibroblasts, under basal and ischaemic conditions, as well as rat and human heart samples.

We started by evaluating the number of TNTs formed by H9c2 cardiomyoblast cells and the atrial cardiomyocyte cell line HL-1. Despite their limitations, both cell lines have been widely described as suitable in vitro cardiomyocyte models that respond to ischaemia as expected (Martins-Marques et al., 2015a; Almeida Paiva et al., 2018). To evaluate whether ischaemia affects the number of TNTs in vitro, we incubated H9c2 and HL-1 cells in an ischaemia-mimetic solution under hypoxia for 1 h. Cells were fixed and labelled with wheat germ agglutinin (WGA) to stain membranes, and phalloidin to stain F-actin (Abouin et al., 2015). Results presented in Fig. 1A and B shows that the number of TNTs formed per cell doubled when H9c2 cells were subjected to ischaemia (Fig. 1A and B). Likewise, HL-1 cells subjected to ischaemia formed four times as many TNTs per cells compared to control cells (Fig. 1C and D). To demonstrate that these F-actin enriched structures were not substrate-attached membrane protrusions, we performed Z-stack confocal analysis to show that they were not in contact with the substrate (Figs. S1A and B and Movie S1,S2). We also demonstrated that the majority of these tubules were negative for vinculin, a protein usually associated with cell-to-substrate attachment (Fig. S2) (Delage et al., 2016).

Supplementary video related to this article can be found at https://doi.org/10.1016/j.crcbio.2020.04.001

Next, we investigated whether the TNTs are functional and facilitate the passage of organelles between connected cardiac cells. To address this question, we assessed the presence and/or trafficking of lysosomes and mitochondria between TNT-connected cells in culture. Figs. S3A and S3B and Movies S3–S8 show the presence of lysosomes and mitochondria moving along the TNTs connecting H9c2 cells and primary cardiomyocytes maintained under control or ischemic conditions, ascribing a functional role to these structures in mediating the transfer of organelles. Nevertheless, the TNT-mediated movement of vesicles appeared to be slower in cells cultured under ischaemic conditions when compared with control cells, suggesting that ischaemia-induced ATP depletion compromises TNT-mediated organelle transport. In agreement, ATP and the motor proteins kinesin, dynein and myosin are required for cargo transfer along TNTs (Sisakhtnezhad and Khosravi, 2015). However, the exact mechanisms and signals underlying this process are not completely understood. It was recently reported that intercellular transport of mitochondria through TNTs can rescue injured H9c2 cells in an in vitro ischaemia-reperfusion model (Han et al., 2016). Also, previous studies by Koyanagi and co-workers (Koyanagi et al., 2005) suggested that mesenchymal stem cells can donate functional mitochondria to cardiomyocytes to restore their energetic state when mitochondria are damaged or dysfunctional. Although we observed a slower lysosomal and mitochondrial movement through TNTs in cells subjected to ischaemia, this
Trafficking pathway can be viewed as an essential mitochondrial repair mechanism in cardiac cells damaged under ischaemia (Koyanagi et al., 2005). Interestingly, we show that TNTs formed between beating cardiomyocytes in culture are maintained, suggesting these TNTs are relatively resistant structures that are preserved in cells subjected to dynamic morphological changes of plasma membrane.

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Although our data demonstrate that ischaemia induces TNT formation in cardiomyocytes, the mechanisms and signals regulating this response, and the biological and pathophysiological relevance of this process, remain to be determined. Grounded on these results, we cannot ascertain whether this is a beneficial or detrimental process. It can be speculated that the formation of TNTs constitutes a protective response to preserve the homeostasis and tissue viability, as part of a strategy that helps the cells to cope with adverse conditions. Indeed, TNTs may mediate the passage of “protective” molecules and/or organelles during ischaemia, including the proteostasis machinery, from better-fitted cells to injured cells in an attempt to rescue the damaged cells (Ham and Raju, 2017). In accordance it was shown that distressed cardiomyocytes may also employ TNTs to activate mesenchymal stem cells for repair purposes (Figeac et al., 2014). On the other hand, communication through TNTs may mediate spreading of noxious molecules, including protein aggregates and damaged organelles, which can either have a protective dilution effect or may contribute to injury spreading. Since metabolites released by stressed cells may elicit the formation of TNTs in neighbouring cells (Abounit and Zurzolo, 2012), it is also plausible that TNT-mediated communication exacerbates the propagation of noxious substances.

4.2. Antioxidants prevent ischaemia-induced formation of TNTs between cardiac cells

After uncovering that ischaemia increases the number of TNTs formed between cardiac cells we proceeded to elucidate the molecular mechanisms underlying this process. Previous studies have demonstrated that oxidative stress can trigger TNT formation (Zhu et al., 2005), an observation that we confirmed by treating H9c2 cardiomyoblasts with hydrogen peroxide for 30 min. We hypothesized that the ischaemia-associated oxidative environment could be a causative factor for the increased formation of TNTs. We therefore pre-incubated H9c2 cells with antioxidant agents (ascorbic acid or glutathione) for 18 h, after which cells were subjected to 30 min of ischaemia. As shown in Fig. 2A and B, treatment with antioxidants prior to ischaemia led to a 50% decrease in the number of TNTs compared with control cells subjected only to ischaemia. In support of these observations, pre-treatment with hydrogen peroxide further stimulated TNT-formation under ischaemic conditions. Altogether, these results suggest that oxidative stress potentiates the formation of TNTs during ischaemia, which can be prevented by the use of antioxidants. Besides ischaemia, it is conceivable that the imbalance between the oxidant and antioxidant agents, caused by an increased formation of oxidative species and/or a decrease of antioxidant defences, influences TNT-mediated intercellular communication. It has been reported that oxidative stress is involved in the pathophysiology of
several cardiac disorders and conditions, including ischaemia/reperfusion injury (Kurian et al., 2016; González-Montero et al., 2018). Although TNTs are present under physiological conditions, studies have shown that stressful environments, such as inflammation, oxidative stress, low serum, bacterial toxins, viral infection and hypoxia, can enhance the formation of TNTs (Ribeiro-Rodrigues et al., 2017a; Sisakhtnezhad and Khosravi, 2015). In accordance, it was demonstrated that hydrogen peroxide, an oxidative agent, increases the number of TNTs between astrocytes (Wang et al., 2011; Zhu et al., 2005). However, to the best of our knowledge, this is the first study establishing the effect of oxidative stress and antioxidants on TNT formation between cardiac cells.

4.3. Troponin T is present in TNTs formed between cardiomyocytes

Besides cardiomyocyte-cardiomyocyte communication, heart function also relies on the efficient crosstalk between cardiomyocytes and other cardiac-resident cells, including fibroblasts. To compare the impact of ischaemia on the number of TNTs connecting cardiomyocytes (CM-CM), cardiomyocytes and fibroblasts (CM-FB) and between fibroblasts (FB-FB), we used a mixed culture of primary neonatal rat ventricular myocytes (NRVMs) and fibroblasts. To distinguish cardiomyocytes from fibroblasts, cultured cells were labelled with the cardiomyocyte-specific marker troponin T. Ischaemia increased the number of TNTs formed...
between all the cell types present in the culture, compared to cells maintained in control conditions (Fig. 3A and B, Fig. S1C). Importantly, besides staining the cytoskeleton of the cardiomyocytes, troponin T was present in TNTs formed between cardiomyocytes, whereas TNTs between fibroblasts were devoid of this protein, suggesting that troponin T could be a suitable marker to identify TNTs formed between cardiomyocytes (Fig. 3A, Movie S9). In line with these results, we hypothesize that in heterocellular conditions this staining pattern can indicate the cellular origin of the TNTs in which cardiomyocyte-derived TNTs are troponin T-positive. The identification of novel and more specific molecular markers can help to elucidate the biogenesis of TNTs in cardiac health and disease, constituting an important step forward in the field of TNT biology (Abounit and Zurzolo, 2012; Delage et al., 2016).

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4.4. TNTs are present in heart in vivo and increase in numbers upon ischaemia

Once we established the existence of TNTs between cardiac cells in culture and the impact of ischaemia in their formation, we sought to evaluate the presence of TNTs in the heart. Staining heart slices for WGA and troponin T we provide evidence of the presence of TNT-like structures in rat (Fig. 4A) and human (Fig. 4B) hearts. Importantly, some of these structures were positive for troponin T, suggesting these TNTs originated from cardiomyocytes. Fig. S1D and Movie S10 show that these TNT-like structures connected remote cells and were present in middle stacks of images in the Z dimension. These results not only corroborate previous studies by He and colleagues (He et al., 2011), claiming the presence of nanotubular structures in an adult mouse heart based on membrane staining, but also suggest for the first time that troponin T can constitute a cardiomyocyte-derived TNT marker. Of note, the human samples used in this study were from patients with hypertrophic hearts and, without access to healthy human hearts, we cannot establish any correlation between the number of TNTs in the heart and the development of disease.

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We next investigated the impact of ischaemia on the number of TNTs in vivo. We compared the number of TNTs in rat hearts maintained in control conditions or subjected to global ischaemia for 1 h using the ex vivo Langendorff system. Congruent with our observations in cultured cells, hearts subjected to ischaemia formed more TNT-like structures than control hearts (Fig. 4C and D), suggesting that ischaemia affects TNT-mediated communication in the heart.

We then assessed whether the TNT-like structures observed in the heart can allow the passage of organelles, ascertaining a functional role for these structures in vivo. For this, we stained heart slices with anti-

![Fig. 3. Ischaemia increases the number of TNTs in primary cultures of neonatal rat cardiomyocytes and troponin T is present in TNTs formed between cardiomyocytes.](image-url)

(A) Representative images of membrane (WGA), F-actin cytoskeleton (Phalloidin) and troponin T staining of primary culture of rat neonatal cardiomyocytes that were subjected to ischaemia (Ischaemia) or control condition (Control). Membrane nanotubes are highlighted by arrows: simple arrows for troponin T-negative TNTs, formed between fibroblasts, and head arrows for troponin T-positive TNTs, formed between cardiomyocytes or by cardiomyocytes and fibroblasts. Scale bars: 20 μm. (B) Graph shows the number of TNTs per cell in control and ischaemic primary cultures of cardiomyocytes (total of 30 images per condition; n = 3). ***p < 0.001 vs Control CM-CM, ##p < 0.01 vs Control CM-FB, $$$$p < 0.0001 vs Control FB-FB by Mann-Whitney test.
lysosomal-associated membrane protein 1 (Lamp1) antibodies. Fig. S3C and Movie S11 show the presence of Lamp1, together with the markers WGA and troponin T in some of these connected structures, suggesting these tubular structures are implicated in the transfer of organelles in the heart, extending the potential pathophysiological relevance of our findings. The existence of TNTs in vivo raises the question as to which roles these structures may play in a pathophysiological context (Ribeiro-Rodrigues et al., 2017a; Gerdes et al., 2013). It can be speculated that the existence of TNTs in vivo constitutes an additional mode of “cross-cellular” communication between parallel cardiomyocytes in addition to the communication through intercalated discs connecting opposing ends of cardiomyocytes. Moreover, TNTs may mediate the communication between all the types of cells present in the heart, including fibroblasts, immune cells and vascular cells.

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It is well known that the heart strongly relies on a fine-tune regulated gap junction-mediated intercellular communication network to ensure the electric and metabolic coupling between cardiomyocytes required for cardiac contraction (Martins-Marques et al., 2015d). In the heart, the most abundant gap junction protein found is Cx43, which in basal conditions is mostly localized at the intercalated discs, at the longitudinal termini of cardiomyocytes to mediate the rapid anisotropic spreading of the electrical impulse (Martins-Marques et al., 2015b). However, a profound remodelling of Cx43, including the accumulation at the lateral membrane of cardiomyocytes, is associated with heart diseases (Martins-Marques et al., 2020). Besides several studies have been highlighted the role of Cx43 for the maintenance of a proper gap junction-mediated intercellular communication, the use of animal models has boosted the scientific knowledge about its importance in the heart (Verheule and Kaese, 2013). In accordance, this knowledge permitted to known that Cx43 knockout (Cx43<sup>−/−</sup>) mice have abnormal heart development and severe cardiac malformations, dying shortly after birth from respiratory failure due to a right ventricular outflow tract obstruction (Réaume et al., 1995).

Although initially described as a protein exclusively involved in the formation of gap junctions, recent findings have also ascribed to Cx43 other biological roles, namely its participation in other types of intercellular communication, including extracellular vesicles (EVs) and TNTs (Ribeiro-Rodrigues et al., 2017a; Wang et al., 2010; Soares et al., 2015). Regarding long-distance communication through EVs, it has been demonstrated that Cx43 facilitates the communication between EVs and target cells (Soares et al., 2015).
Concerning TNT-mediated intercellular communication, it has been demonstrated that the presence of Cx43 at the end of TNTs mediates the electrical coupling between distant cells, through a transient Ca$^{2+}$ elevation in the recipient cell. Remarkably, the TNT-mediated electrical coupling was abolished in cells that do not express gap junctions, which highlight the need of Cx43 in this process (Wang et al., 2010). Besides its role on TNT-mediated electrical coupling, it was also described that the presence of Cx43 in TNTs ameliorated asthma inflammation by the regulation of the transference of mitochondria through TNTs formed between transplanted mesenchymal stem cells derived from induced pluripotent stem cells (iPSC-MSCs) and airway epithelial cells (Yao et al., 2018). Likewise, a recent work also demonstrated a key role of Cx43 in TNT formation, showing that the knockdown of Cx43 in human trabecular meshwork cells significantly decreased the number of TNTs formed (Li, 2019). Although the molecular mechanisms underlying the role of Cx43 in TNT formation were not well known, there is evidence suggesting that its regulation can be due Cx43 association with cell cytoskeleton network (Giepmans, 2006; Kameritsch et al., 2015). Ground on the importance of Cx43 in heart function allied with the increasing evidence for the existence and function of Cx43 in TNT communication between different cell types, it is conceivable that Cx43 plays also a potential role in TNT-mediated intercellular communication between cardiac cells.

5. Conclusions

During the last decade, research has shown that TNTs have different structural and functional properties, which varies between cells. In contrast to gap junctions and extracellular vesicles, the impact of TNT-mediated cell-cell communication in the context of the heart and ischaemia had never been addressed. Therefore, the present study contributes an important addition to a more comprehensive and integrated perspective of the impact of ischaemia on heart intercellular communication. Importantly, a deeper knowledge of the mechanisms underlying TNT formation in healthy and pathological conditions is necessary not only to unveil new bona fide TNT markers but also to identify potential molecular entities that can be used to design novel therapeutic strategies targeting TNTs.

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Declaration of Competing Interest

No competing interests declared.

CRediT authorship contribution statement

Daniela Batista-Almeida: Conceptualization, Investigation, Writing - original draft, Trond Aasen: Conceptualization, Writing - review & editing. Chiara Zurzolo: Methodology, Writing - review & editing. Henrique Girao: Conceptualization, Funding acquisition, Project administration, Supervision, Writing - original draft, Writing - review & editing.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jcrccbio.2020.04.001.

References
