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Abstract

Intercellular communication is one of the most important events in cell population behavior. In the last decade, tunneling nanotubes (TNTs) have been recognized as a new form of long distance intercellular connection. TNT function is to allow molecular and subcellular structure exchange between neighboring cells via the transfer of molecules and organelles such as calcium ions, prions, viral and bacterial pathogens, small lysosomes and mitochondria. New findings support the concept that mesenchymal stem cells (MSCs) can affect cell microenvironment by the

release of soluble factors or the transfer of cellular components to neighboring cells, in a way which significantly contributes to cell regulation and tissue repair, although the underlying mechanisms remain poorly understood. MSCs have many advantages for their implementation in regenerative medicine. The TNTs in these cell types are heterogeneous in both structure and function, probably due to their highly dynamic behavior. In this work we report an extensive and detailed description of types, structure, components, dynamics and functionality of the TNTs bridging neighboring human umbilical cord MSCs obtained from Wharton”s jelly. Characterization studies were carried out through phase contrast, fluorescence, electron microscopy and time lapse images with the aim of describing cells suitable for an eventual regenerative medicine.

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Characterization of Tunneling Nanotubes in Wharton's jelly Mesenchymal Stem Cells. An Intercellular Exchange of Components between Neighboring Cells

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Abstract Intercellular communication is one of the most important events in cell population behavior. In the last decade, tunneling nanotubes (TNTs) have been recognized as a new form of long distance intercellular connection. TNT function is to allow molecular and subcellular structure exchange between neighboring cells via the transfer of molecules and organelles such as calcium ions, prions, viral and bacterial pathogens, small lysosomes and mitochondria. New findings support the concept that mesenchymal stem cells (MSCs) can affect cell microenvironment by the release of soluble factors or the transfer of cellular components to neighboring cells, in a way which significantly contributes to cell regulation and tissue repair, although the underlying mechanisms remain poorly understood. MSCs have many advantages for their implementation in regenerative medicine. The TNTs in these cell types are heterogeneous in both structure and function, probably due to their highly dynamic behavior. In this work we report an extensive and detailed description of types, structure, components, dynamics and functionality of the TNTs bridging neighboring human umbilical cord MSCs obtained from Wharton"s jelly. Characterization studies were carried out through phase contrast, fluorescence, electron microscopy and time lapse images with the aim of describing cells suitable for an eventual regenerative medicine.

Keywords Tunneling nanotubes · Mesenchymal stem cells · Mitochondrial transfer · Intercellular communication · Intercellular bridges

Introduction

Intercellular communication is one of the most important events in cell behavior. Classically, cell-cell communication included gap junctions, synapses and membrane surface receptor-ligand binding. Most of these communications are of fundamental importance in essential processes such as embryonic development, tumor growth, immune response, organ function and homeostasis.

In the last decade, tunneling nanotubes (TNTs) have emerged as a newly discovered form of long distance intercellular communication. TNTs were initially described by Rustom et al. [1] in PC12 cell cultures as intercellular bridges formed by thin cell processes. TNTs do not contact the substratum, extending up to 100 μm in length and having diameters ranging from 50 to 200 nm. They hover in the medium, extending as thin cytoplasmic bridges surrounded by a continuous membrane between both connected cells. Owing to the lack of specific TNT markers, the identification of these

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structures was mainly based on morphological criteria and on the presence of cytoskeletal components. Since the description by Rustom et al. [1], the presence of TNTs has been shown in numerous cell types including myeloid-lineage dendritic cells and monocytes [2], co-cultures of human endothelial progenitor cells with rat cardiomyocytes [3], human B and natural killer cells [4] and mesenchymal stem cells (MSCs) [5], among others. Besides cell-type specific differences in TNTs, evidence has also shown different kinds of TNTs in a single cell type [6]. TNT function seems to be to allow molecule and small subcellular structure exchange between neighboring cells by the transfer of ions, prions, viral and bacterial pathogens, lysosomes and mitochondria [3, 7].

The potential of stem cell-based therapy for different diseases has been explored in numerous animal studies and clinical trials. Recent studies suggest a mechanism of subcellular material transfer by TNTs between stem cells and damaged tissue [8, 9]. MSCs have considerable advantages for their use in regenerative medicine [10], although the mechanisms underlying MSC rescue of damaged cells remain poorly understood. New descriptions show that TNTs in these cell types are very heterogeneous in both structure and function, probably as a consequence of their highly dynamic behavior. For these reasons, great interest has been raised on the knowledge of MSC-TNT constitution and dynamics.

Among the different types of mesenchymal cells capable of generating TNTs, our interest focused on Wharton's jelly mesenchymal stem cells (WJ-MSCs) as effective donor cells for transplantation therapy in many debilitating disorders, as they are easy to obtain and have reduced immunogenicity [11].

This report presents an extensive and detailed description of types, structure, components, dynamics and function of TNTs bridging neighboring human WJ-MSCs, using phase contrast, fluorescence and electron microscopy, as well as time lapse images.

Materials and Methods

WJ-MSC Culture

Umbilical cords were obtained and cultured as previously described [12]. The procedure was approved by the Ethics Committee of Facultad de Ciencias Médicas, Universidad de La Plata, Argentina. Briefly, Wharton"s jelly was carefully exposed and the vascular vessels were discarded. The jelly was cut in 2–4 mm small pieces and placed in plastic cell culture dishes with a low volume of medium during the first 48 h. Approximately after a week, WJ-MSCs proliferated from these pieces and reached confluence. WJ-MSCs were then suspended in a minimum essential medium α-MEM (Life Technologies, 11,900–016) supplemented with 10% fetal bovine serum (Natocor Argentina), penicillin (100/ units/ml), streptomycin (100 μg/ml) (Life Technologies, 15,140–148), gentamicin (0.3 μg/ml) and fungizone (50 μg/ml) (Life Technologies, 15,290–018). Cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 and culture media were changed three times a week. WJ-MSCs were first cultured at a plating density of 10^6 cells/ml in T25 culture flasks (Corning) and finally cultured in glass slides until the desired confluence (50–70%). Passages 2 to 4 were used in all experiments.

Flow Cytometry

WJ-MSCs were characterized by flow cytometry analyses performed in a BD Accuri cytometer following a standard protocol for cell staining. Briefly, adherent cells were washed with PBS and a single-cell suspension was obtained through incubation with Accutasse (Life Technologies). After incubation, cells were washed with PBS plus 0.5% albumin and incubated for 30 min at room temperature with specific antibodies (CD90, CD73, CD105, CD19, CD74a, CD45, CD14, CD11b, HLA-DR). Cells were analyzed after washing with PBS plus 0.5% albumin, and also after stimulation with IL-2. At least 5000 events were counted. Multilineage differentiation of WJ-MSCs was done following standard protocols [12].

Immunocytochemistry

WJ-MSC cultures in glass slides at 50–70% confluence were washed twice with phosphate buffered saline (PBS) with culture medium removal by aspiration. The cells were then fixed with 4% *w*/*v* paraformaldehyde (Sigma, P6148) in PBS for 30 min, rinsed twice with PBS for 5 min and blocked with 1% *v*/v goat serum (Natocor Argentina) in PBS at 37 °C for 1 h. Cells were incubated overnight at 2–8 °C with antibodies against surface markers CD90 (Millipore, CBL415, 1:100), CD105 (Invitrogen, MHCD10500, 1:100), CD73 (Invitrogen, 410,200, 1:100), CD34 (Invitrogen, 073403, 1:100), CD45 (Invitrogen, MHCD4501, 1:100) and α-tubuline (Sigma, T6199, 1:500). Subsequently, cells were incubated with 2 μg/ml secondary antibodies Alexa Fluor®488 goat anti-rabbit (Invitrogen, A1108, 1:1000) or Alexa Fluor®594 goat anti-mouse (Invitrogen, A1105, 1:1000) for 2 h according to the primary antibodies used. For actin detection phalloidin-TRITC labeled (Fluka, 77,418) was employed. Finally, images were obtained with a 20X objective (Olympus LUCPLAN FLN; NA: 0.45, Japan) or a 60X objective (Olympus UPLANS Apo; oil; NA: 1.35) using an inverted IX81 microscope (Olympus). The corresponding micrographs were edited with Adobe Photoshop 7.0 (Adobe Systems) and Image Pro Plus 6.0 (Media Cybernetics Inc.).

Mitochondrial Labeling

In order to evaluate mitochondrial presence and transport, WJ-MSCs (5 × 10^5cells/well) were grown up to 50%–70% confluence onto cover glass dishes and mitochondria were labeled with a mitochondrial target sequence linked to a fluorescent dye. Transfection was performed with a reagent mixture including 0.8 μg pSUPER-retro plasmid (Oligoengine,VEC-PRT-0001) containing the mitochondrial target sequence constituted by a cytochrome oxidase subunit and 0.8 μg yellow fluorescence protein mt-YFP (Clontech, 632,443) in Opti-MEM medium (Life Technologies, 31,985–070), adding 2 μl lipofectamine 2000 transfection reagent (Invitrogen, 11,668–019) according to the manufacturer's instructions. Cells were incubated with this mix in DMEM (Life Technologies, 12,800–082) supplemented with 10% *v*/v fetal bovine serum in PBS without antibiotics for 5 h at 37 °C and 5% CO_2. Culture medium was then replaced by one supplemented with 10% *v*/v fetal bovine serum in PBS and antibiotics (100 U/ml penicillin/streptomycin, 0.3 μg/ml gentamicin and 50 μg/ml fungizone) under the same conditions for 48 h.

Movie and Kymograph Analysis

TNT Formation Dynamics and Stability Culture dishes containing WJ-MSCs were transferred to an epifluorescence microscope (Olympus IX81) connected to a CCD camera (Olympus DP71/12.5 megapixels). Cultures were maintained in an IX81 microscope adapted culture chamber (Olympus) at 37 °C using a heating stage and under 5% CO_2. Images were taken every 3 min over a period of 12 h using a 60X Olympus UPLANS Apo (oil NA: 1.35) objective, and then stacked at 125 mseconds/frame rate (8 Hz)

Mitochondrial Kinetics In order to evaluate mitochondrial transport, imaging and kymograph analyses were conducted as previously described by Noble et al. [13]. Briefly, 48 h after transfection with mt-YFP, cells were recorded in the same microscope and culture chamber described above. Cultures were kept under a 60X UPLANS Apo. Movies and photomicrographs were obtained every 20 s for a period of 25 min and stacked at 125 mseconds/frame rate. Mitochondria were identified as particles moving in the green channel. Kymographs were plotted with Image J using the multiple kymograph plug-in, and average net velocity, distance and direction were calculated for analyses and processed using MATLAB routines (The Mathworks, USA).

Another set of transfected cultures was fixed with 4% (*w*/*v*) paraformaldehyde in PBS for 20 min at room temperature, washed with PBS and submitted to immunocytochemical assays.

Electron Microscopy

The following protocol was used to obtain a representative image of TNT ultrastructure: cells were fixed by removing half the volume of the culture medium and adding equal volumes of fixative solution [4% (*w*/*v*) paraformaldehyde and 0.25% (*v*/v) glutaraldehyde] in potassium phosphate buffer (KPB) 0.1 M pH 7.5, repeating this step twice until the pure fixative solution was maintained for one hour at 4 °C. After washing in KPB, cells were postfixed in 1% (*w*/*v*) osmium tetroxide in the same KPB for 30 min, dehydrated in an ethanol gradient to 70° and contrasted with 5% (*w*/*v*) uranyl acetate in ethanol 70°. Cultures were carefully raised with a scraper to obtain overlapping sheets which were then embedded in Spurr Low Viscosity Kit (Ted Pella, USA) or Epoxy Embedding Medium Kit (Sigma, USA). Ultrathin sections parallel to the sheets were obtained and stained with lead citrate (standard method). Images were acquired on a Zeiss 109 transmission electron microscope (TEM, Carl Zeiss, Germany) and photographed with a GATAN CCD camera (USA).

Results

Phenotypic Evaluation of Cultured WJ-MSCs

As a first step, the present report characterized the cultures of WJ-MSCs from the umbilical cords employed, which were identified through flow cytometry and immunocytochemistry using specific surface markers. As expected, the cell cultures expressed CD105, CD73 and CD90, and did not express any of the hematopoietic surface markers (CD45, CD19, CD14, CD11b or HLA-DR). The combination of positive and negative expression of specific molecules identified the studied cells as WJ-MSCs. Furthermore, WJ-MSCs differentiated to chondrocytes, osteoclasts and adipocytes as expected. Finally, WJ-MSCs presented potent immunomodulatory ability (Supplementary data 1).

Diversity of TNT Morphology in WJ-MSCs

Optical microscopy observations of WJ-MSC cultures showed TNTs either to extend between two neighboring cells (Fig. 1a-c) or to occasionally form a network connecting various neighboring cells (Fig. 1b). In both cases, TNTs were seen as long rectilinear extensions whose length ranged between 100 and 700 μm with a constant diameter in all the length. The number of TNTs per microscopic field was quantified in 10X magnification images from cultures with a cell confluence between 60 and 70% (approximately 300 cells/field). The number of TNTs per field was 27 ± 3 corresponding to photographs taken from 4 independent cultures.

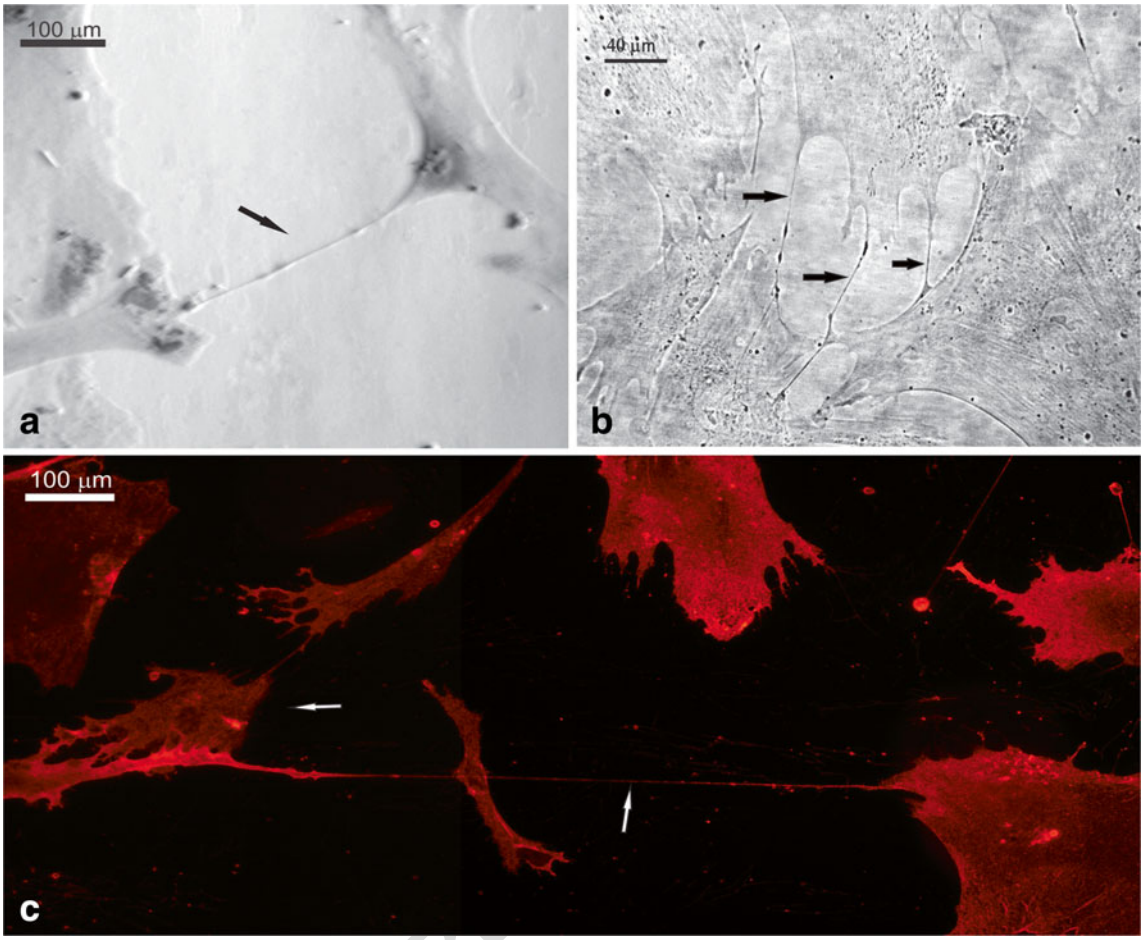

Fig. 1 TNT detection. **a** Phase contrast microscopy image showing a TNT between two neighboring cells. **b** Phase contrast microscopy image showing a TNT network connecting several cells. **c** Immunocytochemical detection of WJ-MSCs expressing stem cell marker CD105. This microphotograph shows a long TNT between two neighboring cells

Two different types of TNTs were identified through immunocytochemical studies, according to cytoskeletal components. The first type presented only actin filaments (Type I), while the second type presented both actin and tubulin filaments (Type II) (Fig. 2a-c and d-e, respectively).

WJ-MSC cultures were also videotaped for 12 h for the analysis of TNT dynamic formation and stability. TNTs were observed to appear mainly through a mechanism known as cell dislodgement, which consists in the appearance of a TNT when attached cells depart from one another. Time-lapse imaging showed that the period between TNT formation and disappearance was about 120 min, which provides evidence of TNT life time (Supplementary data 2).

Mitochondrial Presence and Behavior

WJ-MSC cultures treated with the mitochondrial target sequence probe (mt-YFP) showed more than one mitochondrion per TNT (Fig. 3). In turn, in vivo time lapse imaging employing the same marker showed mitochondria lined up and moving along the TNT (Supplementary data 3). The study of mitochondrial dynamics in the TNTs of WJ-MSC cultures showed that these organelles move both up and down, in both directions, in the TNTs extending between neighboring cells (Fig. 4) at a kymograph calculated velocity of 0.8 ± 0.2 μm/ min ($p < 0.05$; $n = 12$).

Ultrastructural Analysis

Due to TNT nanoscale size and TNT heterogeneity and fragility, as shown by their no adherence to the substrate and their photolability, electron microscopy was used for ultrastructural analyses, with high resolution approaches which were essentially required for detailed TNT description. Once again, two types of TNTs were observed, varying in thickness and composition, in accordance with the optical microscopy classification (Fig. 5). Type I TNTs corresponded to thinner tubes which did not exceed 100 nm diameter. They exhibited no organelles inside, contained only soluble cytoplasmic molecules and a scarce number of actin filaments under the plasmatic membrane (Fig. 5a, b). Type II TNTs were thicker, with a diameter

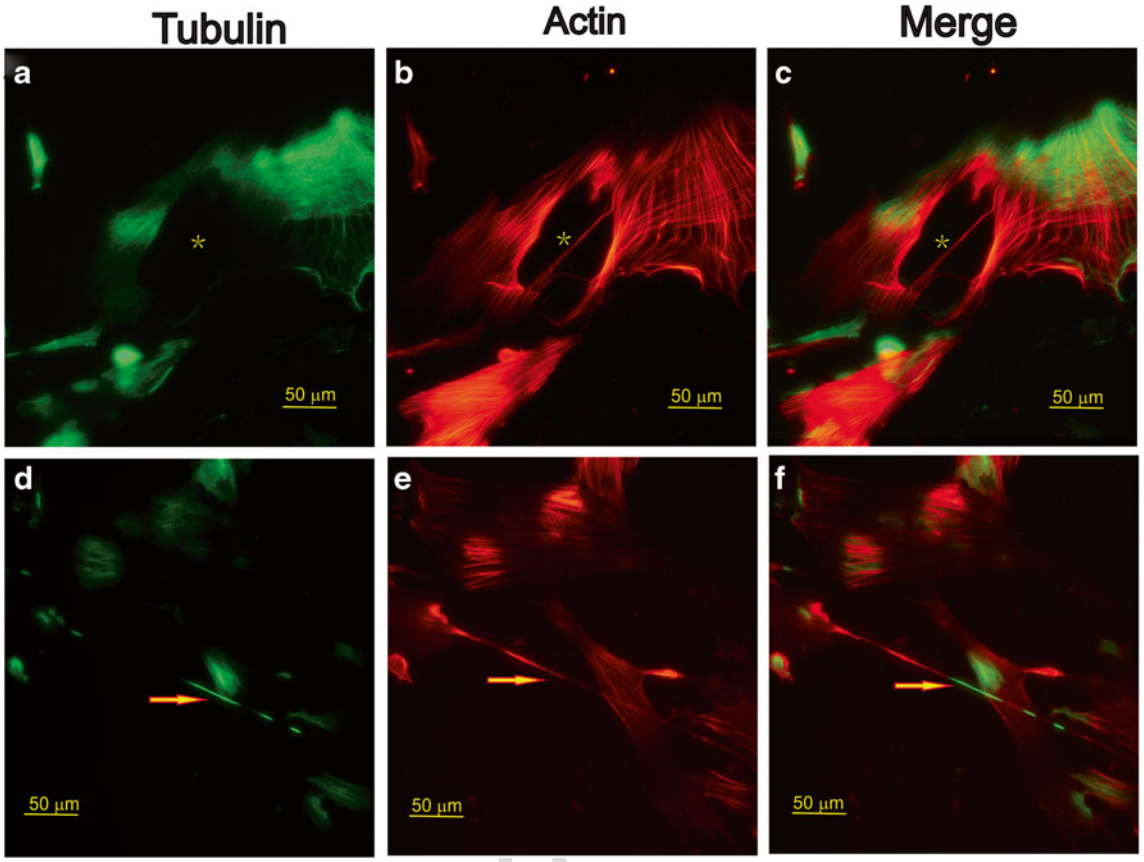

Fig. 2 Heterogeneous TNT composition. WJ-MSCs are connected by both types of TNTs. **a-c** Immunocytochemical detection of type I TNT containing F-actin (stained by phalloidin; red). **d-f** Immunocytochemical detection of type II TNT containing F-actin and microtubules (stained with phalloidin; red and mAb against -tubulin; green)

of 600–700 nm, and contained polyribosomes, cisterns of rough endoplasmic reticulum, vesicles and mitochondria (Fig. 5c, d, e). The cell surface of both types of TNTs exhibited caveoles, suggesting endocytic activity (Fig. 5b, d). Some TNTs also showed bundles of F-actin located near the plasmatic membrane and microtubules oriented parallel to the major axis of TNT. Studies conducted on a total of 50 TNTs through electron microscopy rendered approximately 70% type I and 30% type II TNTs, in agreement with the immunocytochemical characterization.

In addition, two different types of TNT-target connection were observed, one of them consisting in a linear contact between both cellular membranes (Fig. 6a) and the other one showing a concave-convex surface contact (Fig. 6b).

Discussion

Cell-to-cell communication is a critical requirement for multicellular organism development, tissue regeneration and normal

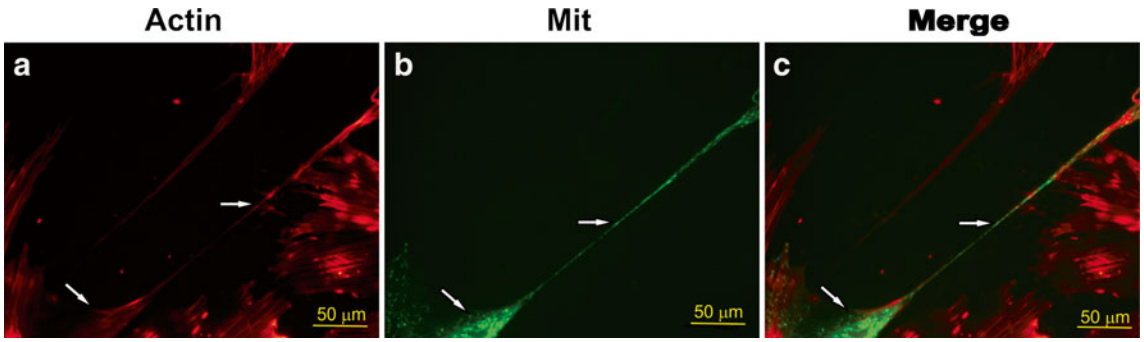

Fig. 3 Mitochondria in TNT. Immunocytochemical detection of mitochondria in a TNT employing a mitochondrial target sequence and mt-YFP. These microphotographs show the presence of several mitochondria both in the cell body and along the TNT

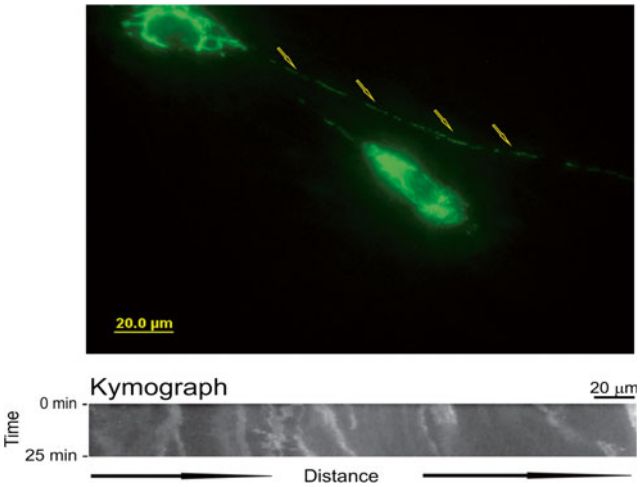

Fig. 4 Mitochondria transport. **a** Fluorescence image of several mitochondria (arrows) moving along a TNT. **b** The kymograph for the time-lapse sequence shows several streaked trajectories corresponding to motile mitochondria within the nanotube. Scale bar 20 µm

physiology. TNTs constitute a newly discovered biological communication mechanism between neighboring cells, allowing the direct transfer of organelles, proteins, genetic material, ions and small molecules [14].

Different types of MSCs have great potential for therapeutic applications, although clinical use requires thorough understanding of their specific biological characteristics. In particular, the first report providing robust evidence that Wharton's jelly stromal cells can be classified as MSCs was published in 2004 [17].

In recent years, considerable attention has been given to the exciting properties of umbilical cord MSCs derived from Wharton's jelly, because of their more primitive nature as compared to bone marrow MSCs. These characteristics along with the simplicity by which WJ-MSCs are isolated by non-invasive means, the provision of a large number of cells without risk for the donor and the fact that this cell population can be rapidly expanded have generated much enthusiasm regarding their potential applications in cell-based therapies [11]. In this context, the present work focuses on the characterization of WJ-MSC TNTs and their capacity to transfer their own components to neighboring cells. In accordance with previous reports [18] referring to other types of MSCs, our studies demonstrate that WJ-MSCs present both types of TNTs described, type I and II, and that even a single cell can develop both types at the same time. Also, employing time lapse movies, we determined that TNTs result from the dislodgement of adjacent cells, a mechanism similar to that described by Veranic et al. [19] in urothelial cell cultures.

As shown by previous reports on different models, TNT membrane continuity enables direct and fluent cargo transport between neighboring cells along tubes constituted

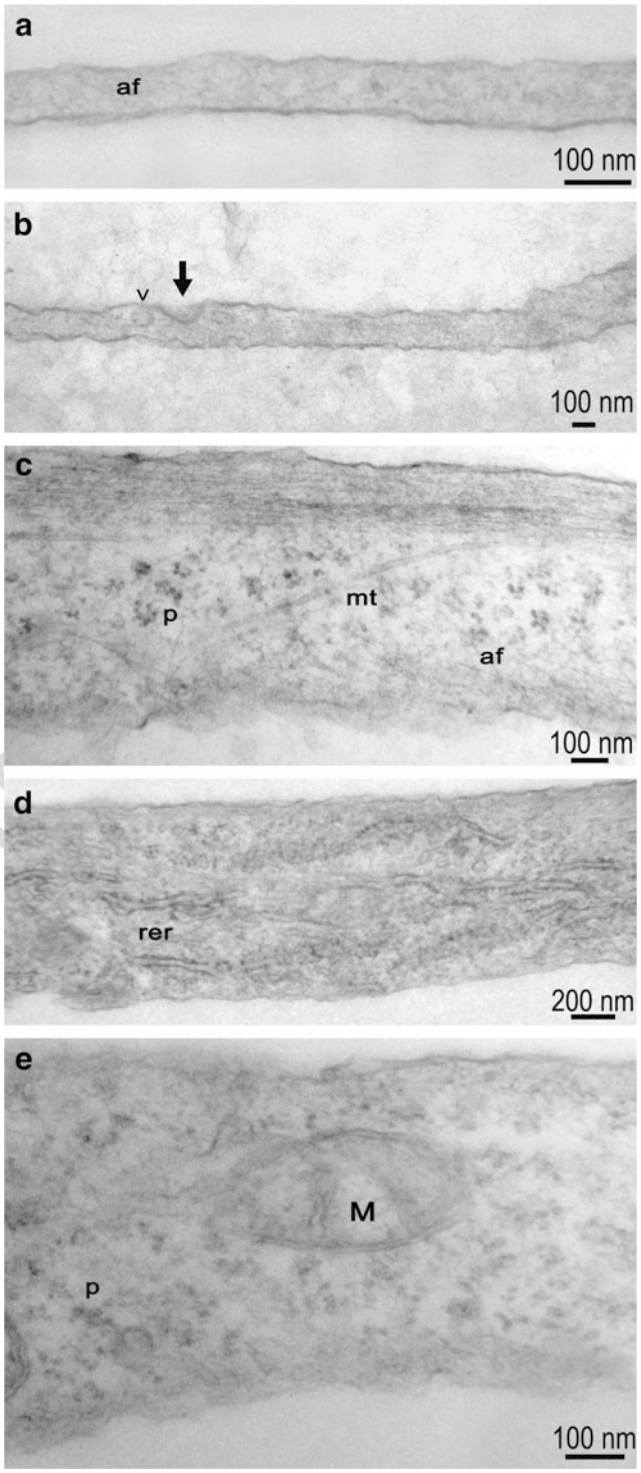

Fig. 5 Ultrastructure of TNTs. Electronmicrographs of: (**a**) a type I TNT of less than 100 nm of thickness without organelles inside. In this TNT only actin microfilaments (af) are observed. Scale bar 100 nm. **b** a type I TNT with a diameter between 100 and 200 nm. Arrow shows a caveole and an endocytic vesicle (v). Scale bar 100 nm. **c** a type II TNT with a diameter near 700 nm containing two type of cytoskeletal components microtubules (mt) and actin filaments (af). Also present poliribosomes (p). Scale bar 100 nm. **d** a type II TNT with a diameter near 700 nm containing rough endoplasmic reticulum (rer) Actin microfilaments (af) are observed too. Scale bar 200 nm. **e** a type II TNT with a diameter near 500 nm showing a mitochondria (M) and poliribosomes (p). Scale bar 100 nm

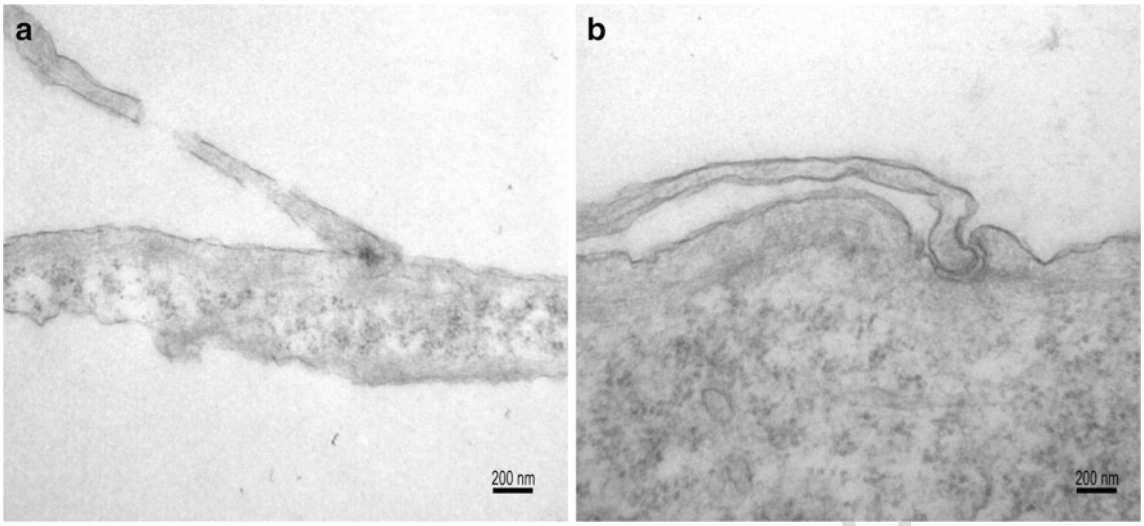

Fig. 6 Ultrastructure of TNT contacts. Electronmicrographs of two types of contact between neighboring cells through TNTs. **a** linear contact between the both cellular membranes of TNTs. Scale bar 100 nm. **b** concave-convex surface contact between a TNT and a cell body. Scale bar 100 nm

by cytoskeletal elements for the transfer of molecules and cell constituents [20]. In the present report, both optic and electronic microscopy results revealed the presence of mitochondria and other organelles such as endoplasmic reticulum and lipid droplets inside WJ-MSC TNTs. This finding indicates that TNTs may transport not only mitochondria but also other types of cytoplasmic components between connected cells, suggesting that TNTs may be involved in important biological processes.

Our data show that mitochondria move along TNTs at a rate of 1 μm/min. As the velocity of mitochondrial transport along microtubules is known to be approximately 6 μm/min, it may be assumed that WJ-MSC mitochondrial transport is a track-dependent movement along F-actin organized in bundles down the axis of TNTs. This assumption is in agreement with images obtained through electronic and optical microscopy in this report, which show the prevalence of TNTs type I in WJ-MSCs.

In addition, our time lapse movies show mitochondria moving in both directions, i.e. to and away from the cell body. This bidirectional movement may be due to the fact that all cells in the population observed have the same degree of stress. However, numerous reports show that co-cultures combining healthy and damaged cell populations exhibit mitochondria moving from the healthy to the damaged cells only. In this respect, Han et al. [15] reported that MSCs can rescue cardiomyocytes from apoptosis employing a co-culture of cardiomyocytes supplemented with bone marrow MSCs and submitted to ischemia. The authors reported that this type of MSCs shows antiapoptotic ability and rescues cardiomyocytes from death. The phenomenon may be attributed to a recovery of mitochondrial function promoted by TNT mitochondrial transfer. This mitochondrial transfer has also been reported in macrophage phagocytic processes in in vitro and in vivo models of acute respiratory distress syndrome (ARDS) and sepsis [16]. Moreover, Plotnikov et al. [21] reported that MSCs obtained from human fetal long bones can act in the cell therapy of heart disorders. These cells donate functional mitochondria to cardiomyocytes and restore their energetic state when their mitochondria are damaged or dysfunctional.

As one of the most important contributions of the present work, electron microscopic images show the presence of different cytoplasmic components inside TNTs, such as polyribosomes and cisterns of rough endoplasmic reticulum. This could reflect active protein synthesis inside TNTs or the transport of protein synthesis machinery. In turn, the presence of caveoles indicates a role of WJ-MSC TNTs in endocytosis and exocytosis of tissue components, reflecting dynamic interaction with the extracellular matrix.

In this respect, Koyanagi et al. [3] showed endothelial progenitor cells to differentiate into cardiomyocytes in the same time window in which they receive mitochondria from cardiomyocytes in a TNT-dependent manner, which may hint at the transfer of transcription factors.

Further investigation of the protective effects of stem cells through TNT-mediated mitochondrial transfer may provide novel insights into the therapies for different pathologies. At this point, Watson et al. [22] have provided an update of recent TNT preclinical and clinical applications.

In conclusion, WJ-MSCs may be thought to constitute a promising therapeutic tool as donors of mitochondria and other cytoplasmic components to injured cells. The results reported in this work, together with the HUC-MSC great capacity to generate long-enduring TNTs and the possibilities for MSC culture, pave the way for their use for autologous implant in the treatment of different diseases.

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Compliance with Ethical Standards

Disclosures The authors indicate no potential conflicts of interest.

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