Integration Properties of Wharton's Jelly-derived Novel Mesenchymal Stem Cells into Ventricular Slices of Murine Hearts

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Key Words

Wharton's jelly • Novel mesenchymal stem cells • Ventricular slices • Cardiac ischemia • Cellular integration • *In vitro* transplantation

Abstract

Wharton's jelly (WJ) is a rich source of multiple-lineage differentiating cells, recently proposed for cell replacement therapy. However, their ability to integrate into the cardiac tissue has not been elucidated, yet. We employed *in vitro* cardiac transplantation models to investigate the capacity of a novel population of human WJ-derived mesenchymal stem cells (nMSCs) to integrate into both living and ischemic cardiac tissue. nMSCs were characterized for the expression of stem/progenitor cell genes and proteins, as well as for multi-lineage differentiation potential. To assess their integration properties, nMSCs were cocultured with either living or ischemic embryonic murine ventricular slices. Immunohistochemical analyses were performed on cryosections of cocultured preparations to allow human cells tracking within the cocultures. Results showed that nMSCs shared MSC and endothelial colony-forming cell characteristics at gene, protein, and functional levels. nMSCs were

markedly chemoattracted towards the ventricular slices, integrating robustly into the depth of both living and ischemic cardiac tissue. In conclusion, the functional ability of WJ-derived cells to populate the cardiac tissue could be validated *in vitro*. The transplantation models described could be further used to depict the mechanisms of WJ-derived cells integration into the cardiac tissue, contributing to optimization of reliable cell therapies for cardiac repair.

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Introduction

Stem cell research studies have represented a valuable contribution to both fundamental biology of stem cells and life-saving clinical therapy applications [1]. The rising importance of stem cells for regenerative medicine justifies the development of novel experimental transplantation models and controlled strategies to differentiate stem cells into progenitors capable to replace the injured tissues at structural and functional levels.

The increasing socio-economical impacts of cardiovascular diseases have determined the scientific

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community to search for more efficient therapeutic approaches, such as cell replacement therapies. While current treatment of myocardial infarction and pediatric heart failure focuses on early revascularization and medical treatment for the inhibition of further cardiomyocyte loss [2], cell therapy aims at restoration of cardiac function by regenerating the cardiovascular tissue. It has been already shown that various adult stem/progenitor cells have the potential to provide functional endothelium [3-5]; however, despite the improvements seen in cardiac function [6, 7], their differentiation to cardiomyocytes has been controversial [4, 5, 8, 9]. Our group has demonstrated that cardiomyocytes could be generated from adult stem cells at a low frequency, exclusively through cell fusion, but not transdifferentiation [9]; furthermore, bone marrow-derived cells induced bone tissue formation in the infarcted myocardium, which questions the biological basis and clinical safety of using these cells to treat nonhematopoietic disorders [10].

Whereas adult bone marrow-derived mesenchymal stem cells (MSCs) have shown limited therapeutic benefit for organ regeneration, it has been postulated that human umbilical cord mesenchymal tissue, namely, Wharton's jelly (WJ), contains stem cells that might be endowed with superior plasticity properties and a greater expansion capability [11, 12]. WJ seems to become a preferential source of stem cells because of the large donor pool, rapid availability, non-invasive collection with no risk or discomfort for the donor, presumed low risk of rejection upon allogeneic cell transplantation, and no ethical constraints or inducement of teratoma formation as in the case of embryonic stem cells (ESCs) [13]. Several groups have established various protocols for the isolation and characterization of stromal cells from WJ [8, 11, 13-17]. It has been shown that the WJ-derived primitive stromal cells possess multipotent properties between ESCs and adult stem cells, bearing a relatively higher proliferation rate, as well as self-renewal and multiple-lineage differentiating capacities [18], including full differentiation towards the endothelial lineage [19, 20]. Although they share common surface markers with bone marrow-derived MSCs, such as the immunomodulatory molecules [21], they also express certain ESC markers, albeit in low levels [13, 17]. However, despite of a wide range of potential clinical applications [12, 17, 22-28], WJ-derived stem/progenitor cell populations have not been fully characterized, and their ability to engraft into the cardiac tissue, participate to cardiovascular regeneration and improve cardiac function has been investigated at a very low extent [8, 17, 29].

Given their particular plasticity and developmental flexibility, umbilical cord stromal cells deserve to be thoroughly examined in novel transplantation models, feasible for clinical translation [18]. Further work is needed in such models to determine whether WJ-derived cells truly integrate and display self-renewal tissue/organ-specific potency [11], to elucidate the real value of umbilical cord-derived stem/progenitor cells for clinical cell replacement therapy. Furthermore, current knowledge of the mechanisms of adult stem cell integration into the cardiac tissue and physiological reconstitution after transplantation is still incomplete. The investigation of these phenomena at the level of cell cultures have generated valuable data, but of limited relevance to predict the outcomes of *in vivo* transplantation into multicellular tissue structures [30]. On one hand, the multicellular *in vitro* models (e.g. monolayers of dissociated cardiomyocytes) mostly do not preserve the *in vivo* structures and the aggressive dissociation methods, required to prepare isolated cell suspensions, often affect the function of membrane ion channels and receptors [31]. On the other hand, clinical studies and even those involving animal models are too complex to reveal the mechanisms underlying the results [32, 33]. Therefore, multicellular preparations with maintained cell-to-cell interactions, such as tissue slices, have allowed a better understanding of the physiological mechanisms and facilitated studies of cellular function within an intact tissue [34]. The *in vitro* transplantation models consisting of living ventricular tissue slices derived from either embryonic [34], neonatal [35], or adult [36] murine hearts have shown morphologically conserved structures and stable electrophysiological characteristics, comparable to the *in vivo* counterparts. These models facilitated not only electrophysiological studies, but also studies of functional cross-talk between different cell types within the heart, at both cellular and molecular levels. Furthermore, non-vital, non-contractile ventricular slice preparations damaged irreversibly by oxygen and glucose deprivation (OGD) have been used as an *in vitro* transplantation model that simulated severe ischemic injury [37]. Using these models, we previously demonstrated that the direct contact of umbilical cord blood endothelial colony-forming cells with living cardiac tissue is a requirement for vascular tube-like structures formation [38]. Despite the already proven value of these *in vitro* models for ESC and endothelial colony-forming cell transplantation studies [37-39], they have not been used yet for studies of mechanical and functional integration, chemotaxis, proliferation, differentiation and survival of umbilical cord matrix-derived stem/progenitor cells, at the level of the

cardiac niche. Therefore, here we proposed to get further insights into the biology of WJ cells, by investigating the capacity of a novel population of human WJ-derived MSCs (nMSCs) to integrate *in vitro* at the level of both vital and non-vital embryonic murine ventricular tissue.

Materials and Methods

Isolation of nMSCs from Wharton's jelly

Human umbilical cord samples were collected following term delivery at the Department of Obstetrics and Gynecology, "Polizu" Hospital, Bucharest, Romania. The samples were obtained upon written informed consent from the mothers and complied with European Union (EU) and national legislation regarding human samples collection, manipulation, and personal data protection. All samples were tested for the absence of HIV, HBV, and HCV and processed up to 5 hrs from collection.

WJ was isolated from the umbilical cords after dissection and removal of the umbilical cord arteries, vein, and amniotic epithelium (Fig. 1A). The isolated tissue was mechanically minced to generate approx. 2 mm² explants that were immediately seeded onto tissue culture dishes pre-coated with 2 µg/cm² fibronectin and kept in Iscove's Modified Dulbecco's Medium (IMDM), supplemented with 20% fetal bovine serum (FBS), 10 ng/ml basic fibroblast growth factor (bFGF), 10 ng/ml epidermal growth factor (EGF), 20 ng/ml insulin-like growth factor 1 (IGF-1), 40 ng/ml vascular endothelial growth factor (VEGF), 100 U/ml penicillin, 100 µg/ml streptomycin, and 50 µg/ml neomycin (all purchased from Sigma-Aldrich, St. Louis, MO, USA). Tissue explants were maintained at 37°C with 5% CO₂ and 21% O₂ in a humidified atmosphere. To maintain optimal culture conditions, medium was changed twice a week. Cells obtained from passages 2 and 3 (on average, after 3 to 4 weeks of culture) were used for the proposed experiments (Fig. 1B describing the experimental design).

Characterization of nMSCs

A) Morphological characterization. Morphological characterization of cells was done by light microscopy using an inverted microscope (Nikon Eclipse TE300, Nikon, Tokyo, Japan) and a digital camera system for imaging (Nikon Digital Net Camera DN100, Nikon), as well as by transmission electron microscopy (Philips EM 400 Philips, FEI Company, Eindhoven, The Netherlands), using a previously described method [40].

B) *In vitro* differentiation potential. *In vitro* differentiation potential of nMSCs into adipogenic, osteogenic, and chondrogenic lineages was assessed using specifically formulated cell culture media (NH AdipoDiff, NH ChondroDiff, and NH OsteoDiff; Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany). The manufacturer's guidelines were followed with minor modifications. Briefly, for adipocyte and osteocyte differentiation, nMSCs were plated at a density of 5x10³ cells per cm² in 6-well cell culture dishes and kept in cell culture for 21 and 15 days, respectively, with medium changed every 3

Fig. 1. A. Wharton's jelly isolation. A, umbilical cord arteries; V, umbilical cord vein; UC, umbilical cord; WJ, Wharton's jelly; B. Schematic representation of the experimental design consisting of coculture of nMSCs with murine embryonic living or ischemic ventricular slices. nMSCs, novel mesenchymal stem cells; OGD, oxygen and glucose deprivation; UC, umbilical cord; WJ, Wharton's jelly.

days. Adipogenic differentiation was verified by staining with oil red O (0.5% in isopropanol, Sigma-Aldrich) after fixation with methanol. Osteogenic differentiation was visualized by von Kossa staining. For chondrogenic differentiation, 5x10⁵ cells were centrifuged (150xg, 15 min.) at room temperature. The resulting cell pellet was cultured for 24 days in a 15 ml polystyrene centrifugation tube in differentiation media, with media changed every three days. The generated nodular cell cluster was embedded in Tissue Tek O.C.T. compound (Sakura Finetek, Staufen, Germany) and frozen at -80°C. Ten-µm-thick cryosections were stained with alcian blue (Sigma-Aldrich) for chondrogenic extra-cellular matrix containing hyaluronic acids.

C) Expression of cell surface molecules. Expression of cell surface molecules on nMSCs was assessed by MoFlo FACS (Dako, Glostrup, Denmark) using 1x10⁵ cells stained with fluorochrome-conjugated (Phycoerythrin, PE; Fluorescein isothiocyanate, FITC) antibodies against CD13, CD14, CD31, CD34, CD45, CD73, CD90, CD105, CD117, CD146, HLA-DR

Gene	GeneBank® accession number	Sequences of oligonucleotide primers	Predicted size (bp)
CD31	NM_000442	S: 5'-AGCACCACCTCTCACGTCA-3' A: 5'-CTTGGATGGCCTCTTTCTTG-3'	250
CD133	NM_006017	S: 5'-CAGTCTGACCAGCGTGAAAA-3' A: 5'-GGCCATCCAAATCTGTCCTA-3'	200
VE-cadherin	NM_001795	S: 5'-CCTTGGGATAGCAAACCTCA-3' A: 5'-CTTTGCCTCCAGGCAGATAG-3'	283
CD34	NM_001773	S: 5'-TCAGTTCTAGTCTCTCTGGGGC-3' A: 5'-ATAAGGGTTAGGAGCTGATCTGG-3'	328
VEGFR1	NM_002019	S: 5'-CAGCCCATAAATGGTCTTTGCC-3' A: 5'-TAATTTGACTGG GCGTGGTGTG-3'	557
VEGFR2	NM_002253	S: 5'-GTGACCAACATGGAGTCGTG-3' A: 5'-CCAGAGATTCCATGCCACTT-3'	660
VWF	NM_000552	S: 5'-CGACTTCCTTACCCCTCTG-3' A: 5'-GCAGGAGCACACGTCGTAG-3'	247
GATA2	NM_032638	S: 5'-CCCTAAGCAGCGCAGCAAGAC-3' A: 5'-TGACTTCTCCTGCATGCACT-3'	439
GATA3	NM_001002295	S: 5'-ACCCCACTGTGGCGGCGAGAT-3' A: 5'-CACAGCACTAGAGACC-3'	740
GATA4	NM_002052	S: 5'-TTAACAGGTTTGTGGGTTAGGGA-3' A: 5'-CCTGGAAGAAGACGACTGCTAA-3'	333
CXCR4	NM_003467	S: 5'-GATGACAGATATATCTGTGACCGC-3' A: 5'-TTAGCTGGAGTGAAAACCTGAAGA-3'	519
Tie-2	NM_000459	S: 5'-CATACTGGGGAAAGCAATGAAAC-3' A: 5'-ACCACTGTTTTTACCTTCCAAA-3'	281
GAPDH	NM_002046	S: 5'-ACCACAGTCCATGCCATCAC-3' A: 5'-TCCACCACCCTGTTGCTGTA-3'	450

Table 1. Sequences of the oligonucleotide primers used for reverse transcription polymerase chain reaction; A, antisense; bp, base pairs; S, sense.

antibodies (Dako), and CD133 (MACS, Miltenyi Biotec). Accutase-detached cells were washed in 1x phosphate-buffered saline (PBS) containing 2% FBS and incubated for 30 min. at 4°C with the appropriate diluted FITC-/PE-conjugated antibodies. For negative controls, the cells were stained with the corresponding isotype-matched Igs (IgG1, IgG2a/b, Dako). Flow cytometry data were analyzed using the Summit 4.0 software (Dako). CD73 and CD146 cell surface markers were documented with a three-color flow cytometer (FACS Calibur, BD Biosciences, San Jose, CA, USA). Briefly, 2×10^5 cells were washed and blocked with FcR (Miltenyi Biotec) for 10 min. at 4°C. After further washing, cells were stained with PE-conjugated antibodies against CD73 and CD146 (BD Biosciences) and matching isotype controls at recommended dilutions, for 10 min. at 4°C. Cell viability was determined by staining with 7-Amino-Actinomycin D (7-AAD, BD Biosciences) and at least 1×10^5 viable cells were included into data analysis (FlowJo software, Tree Star Inc., Ashland, OR, USA).

D) Cell proliferation. Cell proliferation of isolated nMSCs populations ($n = 5$) was evaluated using the colorimetric MTS assay, according to the manufacturer's instructions (CellTiter96 Non-Radioactive Cell Proliferation Assay, Promega, Madison, USA). Cells were seeded in 96-well plates at a density of 5×10^3 cells/well in IMDM supplemented with 20% FBS, 10 ng/ml bFGF, 10 ng/ml EGF, 20 ng/ml IGF-1, 40 ng/ml VEGF, 100 U/ml penicillin, 100 µg/ml streptomycin, and 50 µg/ml neomycin (all purchased from Sigma-Aldrich). The assay was performed every 24 hrs, in triplicates, with a human umbilical vein endothelial cell (HUVEC) line (Promocell, Heidelberg, Germany) as a control. Absorbance

was measured at a wavelength of 570 nm using a spectrophotometric plate reader (Mithras LB 940, Berthold Technology, Bad Wildbad, Germany).

E) Molecular characterization. Molecular characterization of nMSCs was done using total RNA extracted with the GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich). First-strand cDNA synthesis was performed on 1 µg of total RNA; cDNA samples were thereafter amplified in a thermocycler (Bio-Rad Laboratories, Hercules, CA, USA) for 35 cycles (94°C for 45 sec, 60°C for 45 sec, and 72°C for 45 sec) with specific oligonucleotide primers (Metabion, Martinsried, Germany) to assess CD31, CD34, CD133, CD144, VEGFR1, VEGFR2, von Willebrand factor (VWF), GATA2, GATA3, GATA4, C-X-C chemokine receptor type 4 (CXCR4), Tie-2, and GAPDH, as endogenous control, mRNA expression. A HUVEC line (Promocell) was used as a control. The oligonucleotide primer sequences are shown in Table 1.

F) Protein expression. Protein expression assessment was done by Western blotting analysis, using nMSCs and HUVECs as controls, lysed on ice in a lysis buffer containing 10 mM Tris-base, 5mM ethylenediamine tetra-acetic acid, 10% Triton X-100, 25 mM phenyl methyl sulfonyl fluoride, and 1µM benzamidine. Protein concentration was determined by Bradford method, and proteins were separated by 6% SDS-PAGE. High molecular weight markers (75 to 250 kDa) were used as a size standard, and run in parallel with equivalent amounts (40 µg) of total protein at 120V for 1.15 hr. Gels were subsequently transferred to a nitrocellulose membrane, at 25V for 1.5 hr. The membranes were washed in 0.05% Tween-20 in 1xPBS, blocked in 5% BSA in 1xPBS-Tween-20 for 1 hr, and

incubated with the first antibodies: monoclonal rabbit anti-human VEGFR1, polyclonal rabbit anti-human CXCR4, and polyclonal rabbit anti-human Receptor for Advanced Glycation Endproducts (RAGE), all from Abcam Inc., Cambridge, MA, USA. After 17 hrs, membranes were washed again, and incubated for 1 hr with the secondary antibody, goat anti-rabbit IgG coupled with HRP (Abcam Inc.). The bound antibody was detected by enhanced chemoluminescence technique (SuperSignal® Pico Chemiluminescent Substrate, Thermo Fisher Scientific Inc., Rockford, IL, USA). The density of immunoreactive bands was measured using a microcomputer imaging system (Scion Image for Windows, version 4.0.3.2).

G) Chemotaxis assays. Chemotaxis assays were performed by using 3×10^4 nMSCs plated in IMDM, supplemented with 10% FBS, in the upper chamber of a transwell system with 6.5 mm diameter inserts and 5 μ m pore size (Costar-Corning Incorporated, Corning, NY, USA). The assays were employed in the presence of 50 μ g/ml of either angiopoietin 1 (Ang1), stromal cell-derived factor (SDF)-1 α , SDF-1 β , or VEGF for 6 hrs at 37°C, under normoxic conditions. After incubation, the inserts were gently swabbed with cotton-tipped swabs on the upper side. The down side of each insert was fixed with cold methanol at -20°C for 10 min., stained with Crystal violet for 25 min. at 37°C, washed three times with 1xPBS and then dried at room temperature. The inserts were thereafter examined by light microscopy under the inverted microscope (Eclipse TE300, Nikon, Tokyo, Japan), and the pictures were taken with a digital camera system for imaging (Digital Net Camera DN100, Nikon). The experiments were done in triplicates.

Preparation of embryonic ventricular slices

All animal experiments were carried out according to EU legislation in force that sets the legal framework for the use of animals in scientific research. Mice were supra-ovulated, time-mated and the embryos harvested between days 16.5 and 17.5 of gestation. Isolated hearts were processed as previously reported [34], kept in ice-cold (4°C) Ca²⁺-free Tyrode's solution under continuous oxygenation, then embedded in 4% low-melting agarose (Roth, Karlsruhe, Germany). Embedded ventricles were sectioned into 300- μ m-thick slices along the short axis with a vibratome (Leica VT1000S, Leica Microsystems, Wetzlar, Germany). Ventricular slices were then kept for 30 min. in Tyrode's solution supplemented with 0.9 mmol/l Ca²⁺, and transferred in an incubator under normoxic conditions (37°C, 5% CO₂, 21% O₂) in IMDM. To mimic myocardial infarction [37], oxygen and glucose deprivation (OGD) was induced by incubating 300- μ m-thick ventricular slices for 1 hr in IMDM, washed with 1xPBS and then transferred in a custom-made steel hypoxia chamber filled with Tyrode's solution, in which D-glucose was substituted for an equimolar concentration of 10 mmol/l 2-deoxyglucose, and placed in a water-bath maintained at 37°C; oxygen tension was reduced by constant bubbling with pure nitrogen to induce hypoxia. After 48 hrs of OGD, slices were washed twice in 1xPBS, transferred in IMDM supplemented with 20% FBS, and stored in the incubator under normoxic conditions until coculturing with WJ-derived cells.

Generation of cocultures

Custom-made wells containing a small funnel-shaped cavity at its bottom to prevent nMSCs from being washed away from the ventricular slices were used for cocultures. nMSCs (1×10^5) were added onto the top of either living or ischemic ventricular slices placed into the funnel-shaped cavities of the coculture wells (see Fig. 1B describing the experimental design). Two nMSC populations, derived from independent subjects and characterized as described above, were used in the coculture experiments. Cocultures were maintained in the incubator under normoxic conditions between 6 and 10 days in IMDM supplemented with 20% FBS, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 50 μ g/ml neomycin; medium was changed twice a week. The controls consisted of either living or ischemic ventricular slices exposed to the same culture conditions, in the absence of nMSCs. The coculture experiments were done on three separate occasions, with 6 samples per each experimental group. At the completion of the experiments (day 6 and day 10 of coculture for the ischemic and living slice preparations, respectively), ventricular slice preparations were washed twice with 1xPBS and fixed either in 100% ice-chilled methanol, for 20 min. at -20° C, or in 4% paraformaldehyde, for 4 hrs at room temperature. The samples were then embedded in O.C.T compound (Tissue-Tek, Sakura Finetek) and kept at -70°C until further processing.

Evaluation of cocultures

Cocultures were evaluated daily by light microscopy. During examination, pictures and movies were taken to monitor cocultures development in a chronological manner, using an inverted microscope (Axiovert 10, Carl Zeiss MicroImaging GmbH, Germany) and a digital camera system for imaging (Digital Interface, DFW-x700, Sony Electronics Inc., Köln, Germany).

Adenosine triphosphate assays

Adenosine triphosphate (ATP) assays were performed on both living embryonic ventricular tissue slices, at different postoperative days, and OGD-treated slices (n=3), using a protocol previously described [35]. Briefly, ATP was quantified by a luciferase-driven bioluminescence assay (ATP-Bioluminescence Assay Kit CLS II, Roche, Germany) using a luminometer (Sirius Luminometer V2.2, Berthold Detection Systems, Germany). Protein quantification was performed according to Bradford method (Roti-Quant, Roth).

Immunocytochemistry and immunohistochemistry analyses

For immunocytochemistry, nMSCs were cultured on glass cover slips, washed twice with 1xPBS, dried at room temperature, and fixed for 20 min. with ice-chilled 100% methanol. Immunohistochemistry was performed on 8- μ m-thick serial cryosections, situated 24 μ m apart, generated from living and ischemic coculture preparations (LEICA 3050 Cryostat, Leica Microsystems GmbH). Cell and tissue preparations were treated for 30 min., at room temperature, with a blocking buffer consisting of 4% bovine serum albumin (BSA) in 1xPBS. The preparations were then incubated overnight, at 4°C, with the

following primary antibodies: monoclonal mouse anti-human nuclei (1:200, Millipore, Billerica, MA, USA) and polyclonal rabbit anti-human pan-cadherin (1:800, Sigma-Aldrich). The slides were washed three times with 1xPBS, for 10 min., and then incubated for 2 hrs at room temperature, in the dark, with the secondary antibodies (all from Sigma-Aldrich): goat anti-rabbit polyclonal Alexa Fluor 488 (1:1000) and either goat anti-mouse Alexa Fluor 555 (1:2000) or goat anti-mouse Alexa Fluor 647 (1:1000); cell nuclei were stained with Hoechst (1:1000, Sigma-Aldrich). The slides were washed three times with 1xPBS, for 10 min., in the dark, and mounted in Vectashield mounting media for fluorescence (Vector laboratories, Burlingame, CA, USA). The pictures were taken using an inverted epifluorescence microscope with an incorporated digital camera system for imaging (Axiovert 200M ApoTome, Carl Zeiss MicroImaging GmbH), and the AxioVision software (Carl Zeiss MicroImaging GmbH). The AxioVision software was also used to assess the percentage of areas with immunohistochemically stained, integrated nMSCs at the level of living ventricular slice preparations (n=3 / day of coculture). This was done every other day, from day 1 to day 7 of coculture, on serial cryosections (n=9 / living ventricular slice preparation / day of coculture), by measuring the areas with integrated cells, at the level of each cryosection. For data analysis, the average of the areas with integrated cells was reported to the average of total areas of all evaluated cryosections, per day of coculture. Data was represented as percentage of the average of all cryosections total areas, which was considered 100%.

Statistical analysis

Statistical analysis was performed by a one-way analysis of variance (ANOVA) using the OriginPro (version 7.5) and SigmaPlot 2002 (version 8.0, SPSS Inc.) software. The results were presented as mean $\pm$ standard error of the mean (SEM). Differences were considered statistically significant when p value < 0.05.

Results

Characterization of nMSCs

Adherent cells with a mesenchymal-like phenotype started growing out from the explants, on average, 10 days after the initiation of tissue cultures. The cells were first passaged, on average, after 14 days of cultivation and proliferated in colonies (Fig. 2A). Electron microscopy examination showed that the cells grew in monolayers. The nuclei contained a uniformly distributed chromatin and a double nuclear membrane. Mitochondria, rough endoplasmic reticulum, and Golgi apparatus were very well represented, indicating an active, secretory phenotype with intense protein synthesis; Golgi apparatus had many parallel cisternae and a high number of secretory vesicles; few autophagic vacuoles with membraneous content and plasmalemmal vesicles were also visible (Fig. 2B). The *in*

Fig. 2. Light- and electron microscopy images of adherent, mesenchymal-like cells derived from WJ under specific cell culture conditions. A. Light microscopy images of (a) primary culture - mesenchymal-like colony growing out of the explants, x20; (b) and (c) cells at passage 1, x10 and x20, respectively; B. Electron microscopy images of WJ-derived nMSCs: N-nucleus; mit-mitochondria; RER-rough endoplasmic reticulum; v-plasmalemmal vesicle; av-autophagic vacuole; Go-Golgi apparatus; C. *In vitro* differentiation of WJ-derived nMSCs: The cultured nMSCs showed robust chondrogenic (a, alcian-blue staining), moderate osteogenic (c, von Kossa staining; d, negative control for von Kossa staining), but not adipogenic (b, oil red O staining) differentiation.

vitro differentiation assays revealed that, under specific culture conditions, the isolated cells presented robust chondrogenic, low osteogenic and no adipogenic differentiation potential, as compared to controls (Fig. 2C).

Flow cytometry analyses showed that nMSCs were markedly positive for CD13, CD73, CD90, CD105, and CD146, moderately positive for CD31, and negative for CD14, CD34, CD45, CD117, CD133, and HLA-DR (Fig. 3A). MTS cell proliferation assay revealed that nMSCs

Fig. 3. A. Flow cytometry analyses on WJ-derived nMSCs; B. MTS assay for the assessment of proliferative activity of WJ-derived nMSC populations (n = 5).

showed a high proliferation rate, twice higher than that exhibited by HUVECs (Fig. 3B). Furthermore, molecular analyses revealed mRNA expression for transcription factors that regulate cell fate determination [41–43], such as GATA2, GATA3, and GATA4 (Fig. 4A). Expression of VEGFR1, which controls endothelial cell proliferation and differentiation [44], and chemokine receptors VEGFR2, CXCR4, Tie-2, and RAGE – the receptor for High-Mobility Group Box 1 (HMGB1) chemokine, important in stem/progenitor cells homing [45, 46] – was documented at gene and/or protein levels by reverse transcription polymerase chain reaction (Fig. 4A) and Western blotting analysis (Fig. 4B). Chemotaxis assays

showed that nMSCs were significantly chemoattracted in response to Tie-2, CXCR4, and VEGFR receptor ligands represented by Ang1, SDF-1 α/β , and VEGF, respectively (Fig. 4C).

Evaluation of cocultures

After the slicing procedure, the majority of the living slices contracted spontaneously. Furthermore, approx. 40% of the living coculture preparations continued to contract in tissue culture conditions, on average, as far as day 4 of coculture. The OGD procedure resulted in non-contractile, non-vital slices, reproducing the pathological setting of the *in vivo* myocardial infarction

Fig. 4. Reverse transcription polymerase chain reaction (A) and Western blotting (B) analysis for the assessment of mRNA and protein expression of molecules involved in WJ-derived nMSCs proliferation, differentiation, chemotaxis, and survival, as compared to HUVECs; (C) Chemotaxis assay for the evaluation of nMSCs chemotactic activity in response to various chemokines. Results are represented as mean $\pm$ SEM; n = 3; * p<0.05 vs. control.

Fig. 5. A. ATP protein measurements of vital and OGD-treated embryonic slices; ATP concentration of living embryonic ventricular tissue slices was assessed at different postoperative days, as compared to OGD-treated slices (n=3). Results are expressed as mean $\pm$ SEM; #, p<0.05 vs. OGD (48h); *, p<0.05 vs. postoperative day 1. B. Assessment of the dynamics of nMSCs integration into the living ventricular slice preparations (n=3) during the first week of coculture, by measurement of areas with immunohistochemically stained nMSCs at the level of serial cryosections (n=9). Results are represented as mean $\pm$ SEM; *, p<0.05 vs. postoperative day 1.

[37]. The ATP assays performed on cultured embryonic ventricular slices at different postoperative days (from day 1 to day 14) revealed that the ATP protein concentration was significantly higher until postoperative day 7, as compared to OGD-treated slices; when compared to postoperative day 1, the ATP protein level was significantly decreased starting from the postoperative day 7 (Fig. 5A). In order to assess nMSCs capacity to integrate into the cardiac tissue, we added 1×10^5 nMSCs onto the top of either living or ischemic ventricular slices. At one hr after initiation of cocultures (referred to as *day 0*), the nMSCs were strongly chemoattracted towards both living (Fig. 6 - day 0) and ischemic (Fig. 7Aa) ventricular slices. On *day one* of coculture, in both living and ischemic ventricular preparations (Fig. 6A - day 1 and Fig. 7C - day 1, respectively

Fig. 6. Representative light microscopy images for chronological assessment (day 1 to day 10) of human WJ-derived nMSCs cocultured with a living murine embryonic ventricular slice (A), as compared to control - murine ventricular slice only (B).

vely), the nMSCs appeared as organized in sphere-like structures. From *day 2* of coculture, nMSCs started proliferating and populated the whole surface of the coculture dishes. They embedded robustly into both living (Fig. 6A) and ischemic ventricular slices (Fig. 7B-C), filling fissures and cavities on the surface of the slices. At this time, even rigorous washing procedures could not detach the nMSC layer from the ventricular slices. In spontaneously beating ventricular slice cocultures, the contraction force induced excitation spreading throughout the nMSCs layer surrounding the slice. Compared to the living slice cocultures, the control living slices showed modest growth of the endogenous epithelial-like cells migrating out of the murine cardiac tissue (Fig. 6B). No endogenous cell growth was observed in any of the control ischemic slices, at any time-point of the experiments (Fig. 7Ab&c).

Immunohistochemical staining of both living and ischemic ventricular slice preparations with a highly specific anti-human nuclei antibody and a pan-cadherin antibody, which stained for both human and murine cellular adhesion junctions, confirmed that nMSCs attached on both living and ischemic ventricular slices, forming a wide layer around them. Furthermore, immunohistochemistry performed on serial cryosections documented the robust integration of nMSCs into the depth of both living (Fig. 8A-G & Fig. 10.1) and ischemic (Fig. 9A-G & Fig. 10.2) cardiac tissue. The immunohistochemical staining was also used to assess the dynamics of nMSCs integration into the living ventricular slices during the first week of coculture. The nMSCs started integrating into the living cardiac tissue from day 1 of coculture, the percentage of

Fig. 7. Representative light microscopy images for coculture preparations consisting of human WJ-derived nMSCs and ischemic murine embryonic ventricular slices, as compared to control - murine ventricular slice only (A.b and A.c). A.a and B - chronological assessment (day 1 to day 6) of WJ-MNCs cocultured with an ischemic ventricular slice; C. Higher magnification of different coculture preparations.

areas with integrated cells being 4%; 48 hrs latter, the percentage of areas with integrated cells was 7 folds higher (28%), level which has been maintained until day 5 of coculture, declining afterwards on day 7 of coculture (Fig. 5B).

Fig. 8. Immunohistochemistry analyses of 10-day cocultures of murine embryonic ventricular living slices (a) with WJ-derived nMSCs (b). A, B, C, D, E, F, G. Different levels of integration of WJ-derived nMSCs into the murine embryonic ventricular living slices; higher magnification details of the preceding image are pointed by arrows; H. Negative control - murine ventricular slice only; I. Negative control - secondary antibodies only. Polyclonal rabbit anti-human pan-cadherin - Alexa Fluor 488 (green); Monoclonal IgG1 mouse anti-human nuclei - Alexa Fluor 555 (red); Nuclei - Hoechst (blue).

Fig. 9. Immunohistochemistry analyses of 6-day cocultures of murine embryonic ventricular ischemic slices (a) with WJ-derived nMSCs (b). A, B, C, D, E, F, G. Different levels of integration of WJ-derived nMSCs into the murine embryonic ventricular ischemic slices; higher magnification details of the preceding image are pointed by arrow; H. Negative control - murine ventricular slice only; I. Negative control - secondary antibodies only. Polyclonal rabbit anti-human pan-cadherin - Alexa Fluor 488 (green); Monoclonal IgG1 mouse anti-human nuclei - Alexa Fluor 647 (red); Nuclei - Hoechst (blue).

Discussion

In this study, we report that under appropriate culture conditions, cells that share MSC, early endothelial colony-forming cell and ESC characteristics could be isolated from the matrix of the human umbilical cord; furthermore, the ability of these cells to integrate into living and ischemic cardiac tissue was validated *in vitro*. On one hand, the cells were isolated on fibronectine substrate using endothelial differentiation cell culture conditions, which led to moderate expression of the endothelial marker CD31, but not of other endothelial specific markers, such

as VE-cadherin or VWF. On the other hand, the cells exhibited a mesenchymal-like morphology, markedly expressed the classical MSC markers CD73, CD90, CD105 [47], and were able to robustly differentiate into the chondrogenic lineage. The lack of plasticity of the nMSC towards the adipogenic and especially the very weak differentiation capacity towards the osteogenic lineage are beneficial since this overcomes a difficult problem encountered *in vivo* – the spontaneous differentiation of MSCs towards unwanted lineages [10]. In addition, these cells markedly expressed the CD146 surface molecule, which has been identified not only as an endothelial cell marker, but also as an early marker of MSCs [48], as well as of multipotent mesenchymal stromal

Fig. 10. Representative immunohistochemistry images of a 10-day (1) and a 6-day (2) coculture of WJ-derived nMSCs with either a living (1) or an ischemic (2) murine embryonic ventricular slice. A. Bright field; B. Monoclonal IgG1 mouse anti-human nuclei – coupled with either Alexa Fluor 555 (red, for picture panel 1) or Alexa Fluor 647 (red, for picture panel 2); C. Nuclei - Hoechst (blue); D. Polyclonal rabbit anti-human pan-cadherin - Alexa Fluor 488 (green); E. Merged image of B & C; F. Merged image of B, C & D; x5; v = ventricular cavities.

cells isolated from human embryonic tissues [49]. It has been reported by others that the CD146 molecule was also expressed by MSCs derived from the periodontal ligament, which demonstrated multi-lineage differentiation capacity, including chondrogenic potential, and were suggested not only for regeneration of the periodontium, but also of other tissues [48]. Furthermore, nMSCs expressed transcription factors of nonhematopoietic ESCs, such as GATA2, which promotes angiogenesis [41], GATA3, which regulates critical steps of differentiation during embryonic development [42], and GATA4 that regulates genes involved in embryogenesis and myocardial differentiation and function [43]. NMSCs also expressed VEGFR1 with role in stem cell recruitment and angiogenesis [44] and the chemokine receptors Tie-2, CXCR4, VEGFR2, and RAGE, at mRNA and/or protein levels. The interactions between these receptors and their corresponding ligands, such as Ang1, SDF-1 α , SDF-1 β , VEGF, and HMGB1, which are important players in stem and progenitor cells homing, might be responsible for nMSCs high chemotaxis towards the ventricular slices in response to the chemokines secreted by the living/ischemic myocardium [50, 51].

Nevertheless, the fact that these cells did not fully differentiate into endothelial phenotypes may be due to cell isolation procedure. In our study we employed WJ explants, instead of cell suspensions generated from enzymatically-dissociated WJ, as used by others [19, 20] for the generation of cells with endothelial characteristics at morphological, gene, protein, and functional levels. We hypothesize that growth factors contained in the endothelial differentiation medium may

have interfered with the innate signaling pathways of key paracrine factors secreted by the cardiac cells/matrix, which regulate differentiation processes. Thus, in the presence of cardiac tissue, the culture milieu might have been markedly modified, thus impairing the isolation of cells with full endothelial potential. However, the exposure of WJ-derived explants to endothelial promoting factors might have led to the isolation of a unique cell population with potential superior properties to repopulate the infarcted myocardium, as compared to other cell types proposed so far for cardiovascular repair [6, 52-54]. Therefore, the WJ-derived cells described here deserve to be further characterized for their ability to contribute to cardiovascular repair *in vivo*.

Interestingly, the ATP assays revealed that the living embryonic ventricular slices started losing their viability after postoperative day 7, data consistent with the observation that nMSCs integration at the level of living slices declined after day 7 of coculture, potentially due to cytotoxic activity exerted by the apoptotic/necrotic factors released by the degrading cardiac tissue. During the early *in vitro* assessment of nMSCs integration into living ventricular preparations, we observed that the excitation produced by the spontaneously contracting ventricular tissue was spread throughout the wide nMSCs layer surrounding the slices. The resistance of nMSC layer to the repeated contraction force stimulation validated the presence of robust adhesion junctions between nMSCs and the murine tissue, which were also evidenced by pan-cadherin staining. Therefore, these cells could be valuable to treat patients with cardiovascular diseases associated

with decreased ventricular stiffness, such as ischemic dilated cardiomyopathy for which the currently available therapies are the palliative medication or surgical ventricular restoration [55]. Through incorporation into the ventricular tissue and subsequent secretion of cardiac extracellular matrix (ECM) components, these cells may increase ventricular stiffness, therefore improving left ventricular ejection fraction. Furthermore, it is well known that increased collagen deposition is a prerequisite for preventing dilatation of the infarcted area [56]. Therefore, nMSCs could prove valuable for the treatment of patients seeking cardiac ECM remodeling following myocardial infarction, either by ECM synthesis or degradation. After integration into the ischemic cardiac tissue, these cells could provide structural proteins, such as fibrillar collagens, and matricial proteins that modulate cell:ECM interaction, to restore the milieu in which myocytes, endothelial cells and fibroblasts could communicate and function [57]. Nevertheless, the nMSCs might be able to also improve contraction of the fibrotic area by cell-cell and cell-matrix interactions, aspects that deserve to be investigated in animal models of myocardial infarction.

Although the *in vitro* transplantation models described here have been already effectively used by our group for the investigation of integration properties and contraction forces that could be attributed solely to ESCs as candidates for cardiac cell replacement therapy [37], the current study is the first which has addressed the use of these *in vitro* models to assess cardiac integration of fetal/adult stem cells. These models are more relevant to the clinical situation than the artificial ECM [58] or other multicellular *in vitro* models [30, 31], and present also the advantage to overcome the rejection and inter-individual variability issues raised by heterologous or even xenogeneic cell transplantation into immunodeficient animals. They could facilitate the conduction of both short- and long-term investigations of fetal/adult stem cell homing, integration, proliferation, differentiation, and survival, pharmacological studies, as well as studies of functional cross-talk between the cardiac tissue and different stem cell types proposed for cell therapy. Furthermore, it has been indicated that embryonic tissue has unique properties to influence survival and differentiation [59]. Therefore, the use of embryonic cardiac slices might be advantageous for priming fetal/adult stem and progenitor cells in order to study the mechanisms of their differentiation into

endothelial/cardiomyocyte phenotypes, by modifying their fate according to the angiogenic/cardiomyogenic programs dictated by the embryonic cardiac environment. Further experiments conducted on cocultures treated with inhibitors of molecules with key rolls in degradation of ECM proteins, such as matrix metalloproteinases and integrins, or on slices generated from mice with genetically-induced cardiac ECM modifications would also be helpful to elucidate the molecular mechanisms of WJ-derived nMSCs integration into the cardiac tissue.

Research studies such as those described here hold premises to contribute to optimization of cell therapy approaches, advancing the field of stem cell research from bench to bedside. The WJ could be easily stored at birth, like the umbilical cord blood units, in tissue banks, and specific clinical-grade cell populations isolated afterwards, as needed. Although clinical trials involving the use of WJ-derived stem/progenitor cells have not been yet undertaken, these cells are considered to be a safer source for allogeneic cell therapy than bone marrow-derived stem cells, due to their low immunogenicity and high immunomodulatory properties [21, 60]. Therefore, the investigation of WJ-derived cells interaction with the cardiac niche, which may regulate their integration, differentiation, and survival, deserves further consideration.

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