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RESEARCH ARTICLE

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Extracellular vesicles (EVs): A promising therapeutic tool in the heart tissue regeneration

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Abstract

Mesenchymal stem cells (MSCs) treatment has been widely explored as a therapy for myocardial infarction, peripheral ischemic vascular diseases, dilated cardiomyopathy, and pulmonary hypertension. Latest in vitro studies suggest that MSCs can differentiate into contractile cardiomyocytes. One of the best-characterized MSCs products are MSCs-derived extracellular vesicles (EVs). EVs are crucial paracrine effectors of MSCs. Based on previous works, paracrine effects of MSCs play a primary role in the regenerative ability. Hence, in the current paper, we focused our attention on an alternative approach, exploiting products derived from human dental pulp stem cells (hDPSCs) rather than MSCs themselves, which may denote a cost-effective and safer approach. The focus has been on EVs and the bioactive molecules they contain to evaluate their ability to influence the differentiation process toward cardiomyogenic lineage. The expression of GATA4, ACTC1, CX43, and Nkx2.5 was evaluated using Immunofluorescence, real time-PCR, and Western blotting analyses. Furthermore, the expression profiling analysis of the microRNA hsa-miR-200c-3p, targeting the *GATA4* gene, was studied. The hsa-miR-200c-3p was found significantly down-regulated in both c-hDPSCs + EVs-hDPSCs and c-hDPSCs + EVs-HL-1 compared to untreated c-hDPSCs underlying a possible epigenetic mechanism behind the prevalent up-regulation of its targeted *GATA4* gene. The aim of the present work was to develop an in vitro model of hDPSCs able to differentiate into cardiomyocytes in order to investigate the role of EVs derived from hDPSCs and derived from HL-1 cardiomyocyte cell line in modulating the differentiation process toward cardiomyogenic lineage.

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KEYWORDS
cardiomyocyte, dental pulp stem cells, extracellular vesicles, oral mesenchymal stem cells

1 | INTRODUCTION

Mesenchymal stem cells (MSCs) are multipotent non-hematopoietic stem cells. Their key features are the ability to self-renew and differentiate into multiple lineages; in particular, they are able to differentiate into mesodermal, endodermal and ectodermal cell type. MSCs are distinguished by high expression of cluster differentiation (CD) 29, CD44, CD73, CD90, CD105, and lack the expression of CD14, CD34, CD45, and HLA (human leukocyte antigen)-DR. Although hMSCs were initially found in the bone marrow, to date other sources have been used to isolate hMSCs, adipose, placenta, endometrium, and dental tissues. The immunomodulatory properties of MSCs, characterized by the secretion of various soluble factors that interact with immune-receptors in the host tissue microenvironment, have been demonstrated in several studies. Thus, MSCs could be considered as an alternative approach in the treatment of several pathologies for their differentiation capacity, immunomodulatory properties and for the secretion of anti-inflammatory factors.[1,2] MSCs have emerged in a cardioprotective role due to their paracrine effects in the treatment of myocardial injury, as reported by Lai et al 2002.[3,4]

In addition, MSCs are able to differentiate into cardiomyocytes and express the of cardiomyocyte-specific factors other than contractile properties.

Fukuda et al. and Makino et al. reported that an in vitro exposure of MSCs to 5-azacytidine (5-Aza) lead to cell differentiation that showed a cardiomyocyte phenotype, and in the same time increased the expression of cardiac-specific markers, including GATA4 and Nkx2.5.[1] MSCs possess some biological properties that make potential therapeutic sources for cardiac tissue regeneration.[2]

5-Aza is a demethylating agent that leads the MSCs to the cardiomyogenic differentiation, several preclinical studies reported the use of MSCs as a promising tool to improve cardiac functionality.[3] Several pre-clinical studies stated that culture-expanded MSC transplantation could be a promising therapeutic approach for cardiac repair after myocardial infarction, ischemic cerebral vascular disease, dilated cardiomyopathy, and pulmonary hypertension.[4]

To date, oral tissues have been considered an easy and accessible source to collect MSCs, such as periodontal ligament, dental pulp, human exfoliated deciduous teeth, dental follicle, gingiva, and apical papilla.[5]

So far, 600 clinical trials worldwide are assessing the MSC-based cell treatment potentiality, but still this approach need further evaluations in order to be considered a safe clinical technology.[6,7] Furthermore, MSCs quality and safety aspects have not yet been entirely recognized. Based on this knowledge, we focused our attention on an alternative solution, using cell-free products derived by hDPSCs rather than MSCs themselves, which can be considered a safer and unexpansive tool. Extracellular vesicles (EVs) are one of the best characterized MSC-derived products.[8]

EVs are lipid bilayer-delimited particles that are released from almost all types of cells into the extracellular space. They can be classified into three principal classes based on their size, origin, and methods of isolation: (i) microvesicles (50–1000 nm size ranges, maturing from the plasma membrane and enriched in CD40), (ii) Apoptotic bodies (800–5000 nm size ranges, derived from fragments of dying cells and enriched in histones and DNA), and (iii) Exosomes, which are small membrane vesicles (∼30–120 nm) of endocytic source.[9]

The regenerative capacity of MSCs is mainly due to their paracrine effects.

The present research is focused on bioactive molecules found within EVs to evaluate their ability to influence the process of differentiation toward the cardiomyogenic lineage.

The risk factors associated with the application of stem cells themselves have led to the evaluation of cell-free strategies using conditioned medium or EVs. Recently, in the scientific community these alternative approaches have attracted considerable interest due to their wide range of potential applications including in the cardiovascular field. EVs enclose diverse cellular material including lipids, proteins, a pool of soluble cytokines, and nucleic acids such as mRNA and microRNA (miRNA). EVs, a novel axis of intercellular communication, bind to cell surface receptors and secrete their contents into target cells across the plasma membrane allowing interaction with target cells. Due to their properties, EVs denote a promising therapeutic tool in the cardiac tissue regeneration.[10]

Cardiomyocytes, endothelial cells, fibroblasts, and stem cells release EVs that play a vital part in pathological and physiological events, such as cardiac hypertrophy, cardiomyocytes survival, and apoptosis, cardiac fibrosis, and angiogenesis related to cardiovascular illness.

Growing evidence have demonstrated that EVs have significant regulatory properties in cardiovascular biology and illness.[11,12]

Based on the literature, exosomes derived from bone marrow stem cells (BMSC) were described as promising candidates to ameliorate myocardial function.

One of the transcription factors specific to cardiomyocytes is GATA4, which is a zinc finger transcription factor that controls differentiation, growth, and survival.[13,14]

It is well known that GATA4-expressing mouse BMSC exosomes may stimulate MSCs to differentiate into cardiomyocyte precursor cells, decrease programmed cell death of cardiomyocytes, and ameliorate heart functionality after myocardial infarction.[15]

The current study aimed at developing an hDPSCs in vitro model capable to differentiate versus cardiomyocytes in order to evaluate the role of EVs derived from hDPSCs and derived from HL-1 cells in controlling the differentiation event toward cardiomyogenic lineage.

2 | EXPERIMENTAL PROCEDURES

2.1 | Establishment and expansion of hDPSCs

This work was permitted by the G. d'Annunzio University Human Research Ethics Committee (No. 1711/13). The informed consent was signed by all patients as requested by rules of the Department of Medical, Oral and Biotechnological Sciences (ISO 9001:2015, RINA certified 32,031/15/S). hDPSCs were isolated as earlier reported by Diomede et al.[16] Samples were washed several times, cut into small pieces, and placed at 37°C to obtain adherent hDPSCs culture. Primary hDPSCs cultures were established and maintained in MSCs growth medium (MSCMB, Lonza, Basel, Switzerland). Cells were maintained in a humidified atmosphere 5% CO_2 at 37°C up to 80% of confluence and detached using Trypsin/EDTA solution (TripleSelect, Life Tech, Milan, Italy). Cells at second passage were used for experiments.

2.2 | Cellular characterization

MSCs were recognized as spindle-shaped cells in the bone marrow, characterized by their adherence to plastic in standard cultures and the potential for clonogenic proliferation. The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy, in 2006, reported the minimal criteria for MSCs: plastic adherence, capability to differentiate into three differentiated tissue lines (chondrocytic, osteoblastic, and adipocytes) in vitro, and the positivity to the specific surface markers, such as CD105, CD73, and CD90.[17] Based on these guidelines, the morphology and phenotype of cells acquired from dental pulp tissues were analyzed using light microscopy (Leica, DMIL, Milan, Italy) and cytofluorimetry. The cytofluorimetric analysis was executed through Fluorescence-Activated Cell Sorting (FACS) (Calibur, Becton-Dickinson [BD], San Jose, CA, USA). Briefly, cells were added to 0.1% trypsin-EDTA, harvested and suspended in PBS with 1:100 dilution of mouse monoclonal antibodies directed to the following human antigens, either conjugated with fluorescein isothiocyanate (FITC): HLA-DR, CD45, or with phycoerythrin (PE): CD90, CD105, CD73 (all from BD).

Osteogenic and adipogenic differentiation assays were also executed in order to assess the differentiation abilities of the cultured hDPSCs isolated.[18]

Human DPSCs were cultured under osteogenic and adipogenic conditions for 21 and 28 days, respectively. To evaluate the formation of lipid droplets and mineralized precipitates Adipo Oil Red and Alizarin Red S staining were accomplished on the undifferentiated and differentiated cells as previously described.[19]

2.3 | HL-1 mouse cell culture establishment

HL-1 is a mouse cardiac muscle cell line purchased from Sigma-Aldrich (cat. n. SCC065, Merck, Milan, Italy). HL-1 cells were manipulated as reported by manufacturer instructions. Cells were cultured in a Claycomb medium (cat. n. 51800C, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (cat. n. TMS-016, Sigma-Aldrich), 1% penicillin/streptomycin (cat. n. P4333, Sigma-Aldrich, St. Louis, MO, USA), Norepinephrine 10 mM (cat. n. A0937, Sigma-Aldrich, St. Louis, MO, USA), and L-glutamine 2 mM (cat. n.A7506, Sigma-Aldrich, St. Louis, MO, USA). Before culturing the cells, tissue culture flasks (T75, Corning, Merck) were coated with 3 mL of gelatin/fibronectin (cat. n. G9391/F1141, Sigma-Aldrich, St. Louis, MO, USA) for 1 h in a humidified atmosphere at 37°C and 5% CO_2.

2.4 | Induction cardiomyocytes differentiation

For cardiomyogenesis induction, second passage of hDPSCs were cultured in MSCBM supplemented with 5-azacytidine (5-aza) (10 μM), a well-known demethylating agent, ascorbic acid (AA) (50 μg/mL), and β-mercaptoethanol (0.1 mM) for 3 days. After 3 days, the

exposure of hDPSCs to 5-aza, AA, and β-mercaptoethanol (0.1 mM) lead to the development of embryoid bodies (EBs) (Figure 2). To form spheroids hDPSCs suspension will be mixed with the supplemented culture medium and placed in the Bioreactor (Syntechon-RCCS-4SCQ, Houston, TX, USA). After 3 days the EBs derived from hDPSCs were taken and placed into different plastic surface dishes and left to be attached in culture growth medium supplemented with 5-aza, AA, and β-mercaptoethanol. The cells migrated from the EBs (after 3 days) were treated with EVs derived from hDPSCs (hDPSCs-EVs) and EVs derived from HL-1 (HL-1-EVs) at 14 and 24 days.

2.5 | Experimental study design

All experiments were performed in triplicate. The study design was made as follows:

i. cardiomyocytes differentiated from hDPSCs untreated (c-hDPSCs) at 14 days;
ii. cardiomyocytes differentiated from hDPSCs treated with EVs derived from hDPSCs (hDPSCs-EVs) at 14 days;
iii. c-hDPSCs treated with EVs derived from HL-1(c-hDPSCs + HL-1-EVs) at 14 days;
iv. c-hDPSCs untreated at 24 days;
v. c-hDPSCs treated with EVs derived from hDPSCs (c-hDPSCs + hDPSCs-EVs) at 24 days;
vi. c-hDPSCs treated with EVs derived from HL-1 (c-hDPSCs + HL-1-EVs) at 24 days;

2.6 | Confocal laser scanning microscopy (CLSM)

EBs-hDPSCs were seeded on eight-well culture glass slides (Corning, Glendale, Arizona, USA), treated with EVs derived from hDPSCs or HL-1 at 14 and 24 days. Then, the samples were fixed for 1 h with 4% paraformaldehyde in 0.1 M of PBS (pH 7.4) (Lonza, Basel, Switzerland) at room temperature. Successively, several washes were performed. Then, the immunofluorescence assays on the specimens were performed as previously described.[20] Successively, the permeabilization of the samples was conducted with 0.5% Triton X-100 in PBS buffer (Lonza) for 10 min and blocked with 5% skimmed milk in PBS for 1 h.[21] The primary antibodies used in the study were as follows: anti-GATA4 (1:200) (sc-25310, Santa Cruz Biotechnology, Dallas, TX, USA), anti-ACTC1 (1:200) (TA308841, Origene, Rockville, MD, USA), anti-CX43 (1:200) (sc-13558, Santa Cruz Biotechnology), and anti-NKx2.5 (1:200) (sc-376565, Santa Cruz Biotechnology). The cells were incubated with primary antibodies overnight at 4°C. Afterward, specimens were incubated with secondary antibodies Alexa Fluor 568 red fluorescence-conjugated goat anti-rabbit (A11011, Molecular Probes, Invitrogen, Eugene, OR, USA) or Alexa Fluor 568 red fluorescence-conjugated goat anti-mouse (A11031, Molecular Probes) at a 1:200 dilution for 1 h at 37°C. To stain the cytoskeleton actin, the cells were incubated with Alexa Fluor 488 phalloidin green fluorescent conjugate (1:200) (A12379, Molecular Probes) for 1 h, and to stain the nuclei, HL-1 cells were treated with TOPRO (1:200) (T3605, Molecular Probes) for 1 h. Samples were observed using a Zeiss LSM800 confocal system (Carl Zeiss, Jena, Germany) connected to an inverted Zeiss Axiovert 200 microscope equipped with a Plan Neofluar oil-immersion objective (40×/1.3 NA).

Images were collected using an argon laser beam with excitation lines at 488 nm and a helium–neon source at 543 and 633 nm. The acquisition settings were maintained constant between specimens. The ImageJ software was used to analyze the captured images.

The quantification was based on 10 images randomly collected.

2.7 | EVs extraction from HL-1 cardiac muscle cells and hDPSCs

The conditioned medium (CM) was recovered from the HL-1 and hDPSCs cell culture (with a volume of 10 mL) after 5 days of incubation. The CM was centrifuged at 3000*g* for 15 min to eliminate cells suspension and debris. For EVs extraction, the ExoQuick TC commercial agglutinant (System Biosciences, EurocloneSpA, Milan, Italy) was used. Briefly, 2 mL of ExoQuick TC solution was added into 10 mL of CM. The mix was incubated overnight at 4°C without rotation; one centrifugation step was performed at 1500*g* for 30 min to sediment the EVs, and the pellets were re-suspended in 200 µL of PBS.[22] The detection of the whole homogenate proteins of the EVs was used as a confirmation of the presence of EV release in HL-1 and in hDPSCs.

2.8 | EVs characterization by flow cytometry

For each analysis, EVs were stained by a generic EV tracer (the Lipophilic Cationic Dye, LCD, 0.5 µL/test, BD Biosciences, San Jose, CA, USA, Custom kit Cat. 626,267) and the FITC (Fluorescein isothiocyanate)-conjugated phalloidin (0.5 µL/test, BD Biosciences, San Jose, CA,

USA, Custom kit Cat. 626,267), which stains damaged EVs. Samples were incubated for 40 min of staining (RT, in the dark) and 1×10^5 events/sample were acquired by flow cytometry (FASCVerse, BD Biosciences, San Jose, CA, USA), setting the trigger threshold at the APC channel, as already described.[23] Fluorescence Minus One (FMO) controls were used. Buffer-only, reagent-only and Triton X-100 1% control samples were acquired at the same settings used for analysis, measured using the same set time applied for the acquisition of stained samples. The antibody stock solutions were centrifuged before their use (21,000*g*, 12 min) to prevent the unspecific background linked to the antibody aggregation and the immune complex formation. Megamix-Plus beads (BioCytex, Marseille, France) were used to broadly setup scatter parameters for EV detection. Height (*H*) signals and logarithmic or bi-exponential modes were selected for all parameters. Instrument performances were daily checked by the Cytometer Setup and Tracking Module and further validated by the acquisition of Rainbow Beads (BD Biosciences, San Jose, CA, USA). Data were analyzed using FACSuite v 1.0.6.5230 (BD Biosciences, San Jose, CA, USA) and FlowJo v 10.0.7 (TreeStar, now Becton, Dickinson and Company, Ashland, OR, USA) software. Rosetta Calibration (Exometry, Amsterdam, The Netherlands) was used to establish the scatter to diameter relationship for the side scatter detector of the FACSVerse[24] As reported, we assumed a 4 mm phospholipid membrane, a lumen refractive index between 1.34 (water refractive index) and 1.40 and a shell refractive index between 1.46 (phospholipid) and 1.56 (phospholipid with proteins), for EVs.[25]

2.9 | Protein extraction from c-hDPSCs untreated or treated with EVs derived from hDPSCsor HL-1 at different time points for Western blot analysis

The total lysates (50 μg) of c-hDPSCs untreated or treated with EVs derived from hDPSCs or HL-1 at different time points underwent electrophoresis and were blotted to a polyvinylidenfluoride (PVDF) membrane. Successively, the membranes were blocked in 5% non-fat milk in PBS with 0.1% Tween-20, and then the blotted membranes were incubated overnight at 4°C with the following primary antibodies: anti-GATA4 (1:500) (sc-25310, Santa Cruz Biotechnology), anti-ACTC1 (1:500) (TA308841, Origene), anti-CX43 (1:500) (sc-13558 Santa Cruz Biotechnology), and anti-NKx2.5 (1:500) (sc-376565, Santa Cruz Biotechnology) and *β*-actin as loading control (1:750) (sc-47778, Santa Cruz Biotechnology). After five washes with 0.1% Tween-20 in PBS, the membranes were incubated for 1 h at room temperature with peroxidase-conjugated secondary antibody goat anti-mouse (A90-116P, Bethyl Laboratories Inc., Montgomery, TX, USA) andgoat anti-rabbit (A 120-101P) 1:5000 diluted in 1X PBS, 2.5% milk, and Tween-20 at 0.1%.[26,27] The levels of expression of the proteins were detected using the enhanced chemiluminescence exposure process (ECL) (Amersham Pharmacia Biotech, Milan, Italy) with an image documenter Alliance 2.7 (Uvitec, Cambridge, UK). The detected signals were analyzed by ECL enhancement and assessed through UVIband-1D gel analysis (Uvitec). The data obtained were normalized with values assessed by densitometric analysis of the *β*-actin protein.

2.10 | Calcium imaging

Intracellular Ca^{2+} variations were monitored in c-hDPSCs untreated or treated with EVs derived from hDPSCs or HL-1 by using the fluorescent calcium indicator dye Fluo4-acetoxymethyl ester (Fluo4/AM, Thermo Fisher Scientific). An upright Zeiss Axio Examiner microscope (Carl Zeiss) was used, using an 20× 1.0 NA water-immersion objectives connected by optical fiber to a 75 W Xenon lamp and wavelength controls was achieved by an OptoScan monochromator (Cairn Instrument, UK). Images were acquired using a back-illuminated camera (EMCCD, Evolve 512; Photometrics, Tucson, USA). The cells were incubated with 5 μM Fluo-4/AM in normal external solution (NES (in mM): 140 NaCl, 2.8 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 glucose, 10 Hepes, pH 7.3) supplemented with 1% (w/v) bovine serum albumin for 40 min at 37°C. Recordings on Fluo4-loaded cells were performed in NES. The fluorescence was acquired by setting excitation at 488 nm and images acquired at 20 Hz and stored on an interfaced computer for off-line analysis using Metafluor (Molecular Device, Sunnyvale, CA, USA). The temporal analysis was calculated as the mean fluorescence intensity signal in a selected cell area, as F/F_0, where F is the fluorescence emission of a single loaded cell acquired during a time lapse, and F_0 is the mean fluorescence intensity of the same cell calculated from the first images acquired.[28]

2.11 | RNA extraction from c-hDPSCs untreated or treated with EVs derived from hDPSCs or HL-1 at different time points and qRT-PCR for gene expression analysis

Total RNA was extracted from c-hDPSCs using the Nucleospin miRNA buffer set kit (Macherey-Nagel, Milan, Italy) according to the manufacturer's

instructions. Quantity and quality of total RNA were assessed by microvolume UV–vis spectrophotometer NanoPhotometer (Implen, GmbH, Munich, Germany). Expression analysis of *GATA4*, *ACTC1*, and *CX43* genes was performed in all samples by qRT-PCR on the QuantStudioTM 7 Pro Real-Time PCR detection system (Life Technologies, Carlsbad, CA, USA). One micro gram of total RNA was used for cDNA synthesis through the high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) under the following conditions: 25°C for 10 min, 37°C for 120 min, 85°C for 5 min and a final temperature of 4°C to cool down the reaction tubes. Amplification reaction was prepared in a total volume of 20 μL containing Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermo Fisher Scientific, Waltham, MA, USA), 1 μL of 100 ng target cDNA and 0.3 μM of each primer. *GAPDH* and *ACTB* (b-actin) were used as housekeeping genes. Each sample was run in triplicate. Specific primer pairs are reported in Table 1. The ΔΔCt method and *t* test were employed to assess the relative gene expression, considering data as significant when presenting a *p*-value <0.05.

2.12 | Highly sensitive stem-loop RT-PCR for miRNA analysis in c-hDPSCs untreated or treated with EVs derived from hDPSCs or HL-1 at two time periods

Quantification of miRNA hsa-miR-200c-3p (MIMAT 0000646; miRBase) was executed by highly sensitive stem-loop RT-PCR. miRNAs were reverted from 25 ng of total RNA by high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA), using 1 μM of specific stem-loop reverse primers (Table 2) as indicated by the following thermal protocol: 16°C for 30 min, followed by 60 cycles at 30°C for 30 s, 42°C for 30 s, and 50°C for 1 s. A final incubation at 85°C was also performed for 5 min in order to inactivate the reverse transcriptase. Amplification was carried out using the Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermo Fisher Scientific, Waltham, MA, USA) and PCR-specific 1 μM Forward Primer for each miRNA, as well as 1 μM of Universal Reverse Primer (Table 2), under the following PCR conditions: 95°C for 10 min, 40 cycles of 15 s at 95°C, and 40 s at 60°C. Upon completion of the reaction cycles, melt curves were obtained by heating the reactions from 60 to 95°C. Relative expression of miRNAs, normalized to endogenous reference RNU44 (Table 2), was determined by *t* test statistical analysis (*p*-value <0.05).

3 | RESULTS

3.1 | Human DPSCs exhibited the stemness markers expression and the adipogenic and osteogenic differentiation capacity in vitro

The cytofluorimetric analysis conducted on the hDPSCs established that hDPSCs were positive for CD73, CD90, CD105 and negative for CD14, CD34, and CD45 negative (Figure 1A). Moreover, the hDPSCs cultured on Petri dish were able to adhere on a plastic substrate with a spindle-shape morphology (Figure 1B). The hDPSCs had a multidirectional differentiation potential when maintained in vitro under osteogenic and adipogenic conditions, showing a positivity for histochemicaas reported using Oil red O and Alizarin Red S staining (Figure 1C,D).

TABLE 2 Stem-loop microRNA primer sequences.

microRNA	Primer sequences (5′–3′)
RT_miR-200c	GTTGGCTCTGGTGCAGGGTCCGAGG TATTCGCACCAGAGCCAACTCCATC
miR-200c_Forward	GCGGCGGTAATACTGCCGGGT
RT_RNU44	GTTGGCTCTGGTGCAGGGTCCGAGG TATTCGCACCAGAGCCAACAGTCA GTT
RNU44_Forward	GCGGCGGCCTGGATGATGATAG
Universal Reverse	GTGCAGGGTCCGAGGT

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
GATA4	GTGTCCCAGACGTTCTCAGTC	GGGAGACGCATAGCCTTGT
ACTC1	TCTTCCAGCCCTCCTTCATTG	AGCCAGAGCAGTGATTTCCTTC
CX43	CTTCATGCTGGTGGTGTCC	ACCACTGGTCGCATGGTAAG
GAPDH	AAGGTGAAGGTCGGAGTCAAC	GGGGTCATTGATGGCAACAATA
ACTB	GTTGTCGACGACGAGCG	GCACAGAGCCTCGCCTT

TABLE 1 qRT-PCR primer sequences.

(A)

hDPSCs	
Markers	**MFI Ratio ± SD**
CD73	96.9 ± 2.9%
CD90	103.2 ± 2.1%
CD105	113.1 ± 2.7%
CD14	ND
CD34	ND
CD45	ND

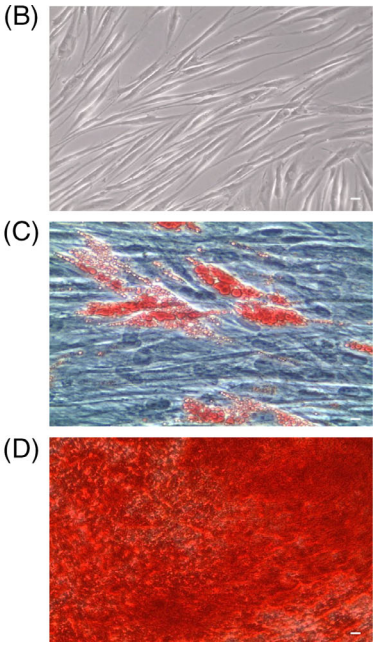

FIGURE 1 Characterization of hDPSCs. (A) Cytofluorimetric value of hDPSCs. (B) hDPSCs cultured in basal medium with a fibroblast-like morphology observed under inverted light microscopy. (C) Adipogenic differentiation evaluated using Oil Red O staining. (D) Osteogenic differentiation evaluated using Alizarin S Red staining. Scale bars: 20 μm.

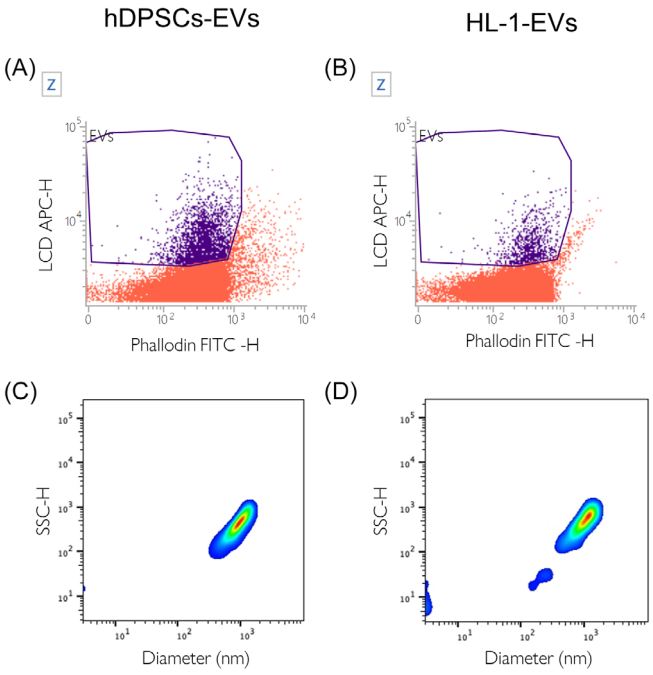

FIGURE 2 EVs identification by flow cytometry. EVs were identified on a phalloidin FITC-H/LCD APC-H dot-plot as LCD positive/phalloidin negative events (intact EVs), both for hDSPCs-derived (A) and HL-1 cells derived EVs (B). EVs diameters were also measured by the Rosetta bead system and representative SSC-H/diameters (nm) dot-plot for hDSPCs-derived (C) and HL-1 cells derived EVs (D) are shown.

3.2 | Extracellular vesicles characterization

As we already published, by applying a recently optimized flow cytometry method, EVs concentrations can be established by volumetric counts.[25] As shown in Figure 2A,B, EVs were identified as LCD positive/phalloidin negative events, and hDSPCs-derived EVs concentrations were higher than those obtained from HL-1 cells (19,607 EVs/μL and 7393.4 EVs/μL, respectively). Diameters of hDSPCs and HL-1 cells derived EVs were also measured. As shown in Figure 2C,D, and both types of EVs displayed a diameter of 900–1000 nm.

3.3 | Light microscopy

At 0 h hDPSCs were plastic-adherent and showed a fibroblast like morphology (Figure 3A). After 3 days, hDPSCs cultured with MSCBM supplemented with 5-aza (10 μM), AA (50 μg/mL) and β-mercaptoethanol (0.1 mM) to induce the differentiation toward cardiomyocytes formed a small cellular aggregate (EBs-hDPSCs) completely detached from the bottom well (Figure 3B), observed under inverted light microscopy. The cells were migrated at the periphery of EBs-hDPSCs as shown by light microscopy image (Figure 3C).

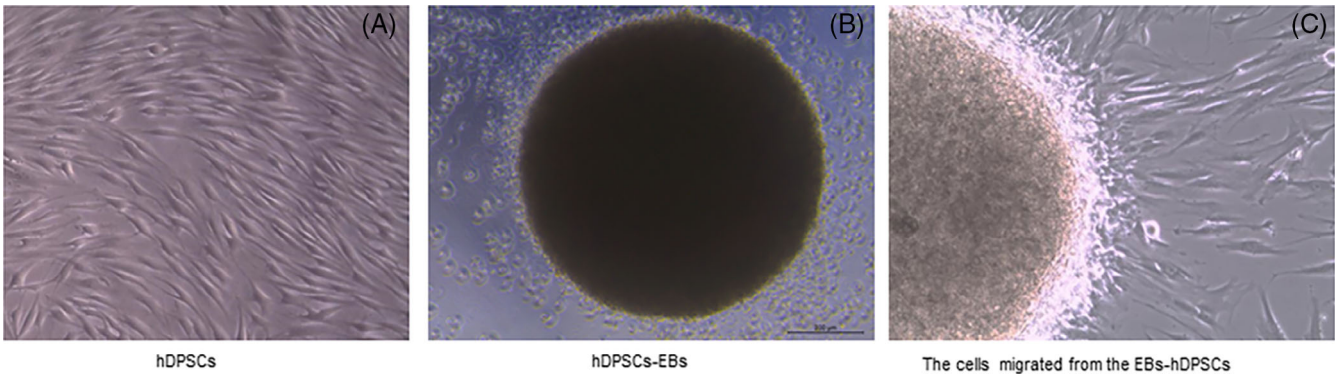

FIGURE 3 Inverted light microscope evaluation. (A) hDPSCs fibroblast-like morphology adherent to the plastic surface (B) hDPSCs cultured with MSCMB supplemented with 5-aza (10 μM), AA (50 μg/mL), and β-mercaptoethanol (0.1 mM) developed EBs-like structure after 3 days. (C) The cells were migrated at the periphery of EBs-hDPSCs as shown by light microscopy image. Mag: 10×.

3.4 | Expression levels of GATA4, ACTC1, CX43, NKx2.5 in cardiomyocytes derived from hDPSCs or HL-1 untreated or treated with EVs derived from hDPSCsor HL-1 at different time points

The immunofluorescence results showed that the GATA4, ACTC1, CX43, and NKx2.5 proteins were expressed significantly in c-hDPSCs + EVs-hDPSCs after 14 and 24 days compared to the untreated c-hDPSCs. Furthermore, c-hDPSCs + EVs-hDPSCs after 14 and 24 days evidenced higher level expression of GATA4, α-SMA, CX43, and NKx2.5 compared to c-hDPSCs + EVs-HL-1 (Figures 4 and 5), as also demonstrated by quantitative analysis represented as arbitrary unit of fluorescence per cell surface unit (F/μm^2) (Figure S1).

These results were comparable to the results obtained by Western blot analysis, where c-hDPSCs untreated at 14 days compared to c-hDPSCs +EVs-hDPSCs evidenced significant lower expression of GATA4 and CX43. Moreover, c-hDPSCs + EVs-hDPSCs at 24 days showed a significant higher expression of ACTC1 and GATA4, compared to c-hDPSCs untreated (Figure 6).

3.5 | Gene expression of *GATA4*, *ACTC1*, and *CX43* in cardiomyocytes derived from hDPSCs or HL-1 untreated or treated with EVs derived from hDPSCs or HL-1 at 14 and 24 days of culture

Gene expression analysis evidenced a significant up-regulation of *GATA4* and *CX43* genes in c-hDPSCs+EVs-hDPSCs compared to the untreated c-hDPSCs after 14 days of culture, in line with immunofluorescence and Western blot results (p-value <0.05). *ACTC1* was also found to be expressed significantly in c-hDPSCs + EVs-hDPSCs at the 14-day time point compared to the untreated controls as previously detected at a protein level, evidencing though a defined up-regulation pattern in terms of gene expression. At 24 days on the other hand, changes in the expression levels of *GATA4*, *CX43*, and *ACTC1* in the c-hDPSCs + EVs-hDPSCs samples were not found to be statistically significant compared to those of corresponding untreated c-hDPSCs controls, although they continued to show an up-regulation trend for both *GATA4* and *ACTC1*.

Nonetheless, at 14 days, c-hDPSCs + EVs-hDPSCs evidenced, overall, higher levels of *GATA4*, *CX43*, and *ACTC1* genes compared to c-hDPSCs + EVs-HL-1, as before. In addition, comparable to protein analyses results, c-hDPSCs + EVs-HL-1 also displayed a significant up-regulation in comparison with untreated controls at the 14-day timeframe for *CX43* (Figure 7).

3.6 | miRNA analysis of hsa-miR-200c-3p in c-hDPSCs + EVs-hDPSCs and c-hDPSCs + EVs-HL-1 compared to corresponding controls at 14 and 24-day time points

The expression profiling analysis of the miRNA hsa-miR-200c-3p, targeting the *GATA4* gene, was carried out in all samples. hsa-miR-200c-3p was found significantly down-regulated (t test, p-value <0.05) in both c-hDPSCs + EVs-hDPSCs and c-hDPSCs + EVs-HL-1 compared to untreated c-hDPSCs at both time points (Figure 8), indicating a possible underlying epigenetic mechanism behind the prevalent up-regulation of its targeted *GATA4* gene.

3.7 | Intracellular calcium dynamics

The differentiative potential of EVs secreted by hDPSCs and HL-1 cells were compared at 14 and 24 days by

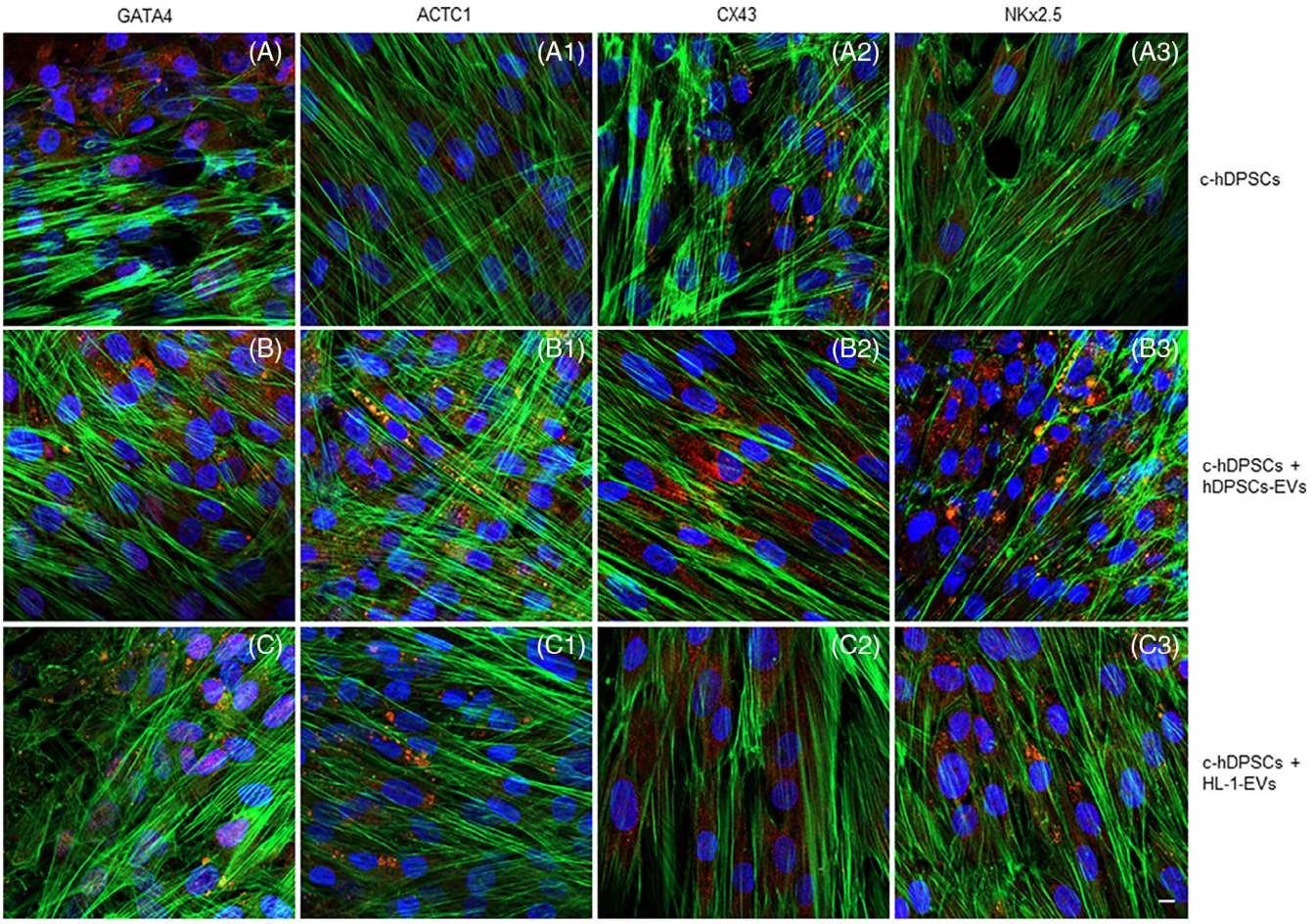

FIGURE 4 GATA4, ACTC1, CX43, and NKx2.5 cardiomyogenic markers and differences in the protein levels in cardiomyocytes derived from hDPSCs cell lines after 14 days of culture. Expression of GATA4, α-SMA, CX43, and NKx2.5, analyzed by confocal microscopy in c-hDPSCs (A–A3), c-hDPSCs + hDPSCs-EVs (B–B3), and c-hDPSCs + HL-1-EVs (C–C3). Green fluorescence: cytoskeleton actin. Red fluorescence: GATA4, ACTC1, CX43 and NKx2.5. Blue fluorescence: cell nuclei. Scale bar: 20 μm.

studying the spontaneous oscillations of intracellular calcium. At both experimental time points (14 or 24 days) in untreated cells none or a very little spontaneous intracellular calcium activities were recorded (Figure 9, A and D). Fourteen days treatment with EVs derived from hDPSCs time course analysis revealed the presence of spontaneous intracellular calcium oscillation in a similar manner of what was observed by the treatment of EVs derived from HL-1 cells (Figure 9B,C). However, eventhough a similar trend about the presence of the calcium oscillations were obtained in both the cells treated for 24 days, the dynamics appeared different in terms of time course, showing reduced spikes in amplitude, sustained during time.

4 | DISCUSSION

Oral MSCs are multipotent self-renewing cells isolated from various adult tissues, including those of the oral cavity such as hDPSCs, hSHEDs, hPDLSCs, hAPSCs, hDFSCs, and hGMSCs. Oral MSCs are capable to differentiate versus different cell lineages such as osteocytes, chondrocytes, adipocytes, neurocytes, and cardiomyocytes.

Based on the literature, oral MSCs have been widely used for tissue repair applications.[29]

In the current study, we evaluated the capability of hDPSCs to differentiate into cells with a cardiac phenotype and if the differentiation capability toward cardiomyogenic lineage was influenced by the treatment with EVs derived by hDPSCs or HL-1 cells.

A novel therapeutic perspective for ischemic heart disease is based on heart muscle restoration through the transplantation of cardiomyocytes differentiated in vitro from stem cells. MSCs have an exceptional potential to differentiate in vitro into cardiomyocytes.[30]

Scientists in the stem cells field acquired a particular interest in transcription factors that control lineage-

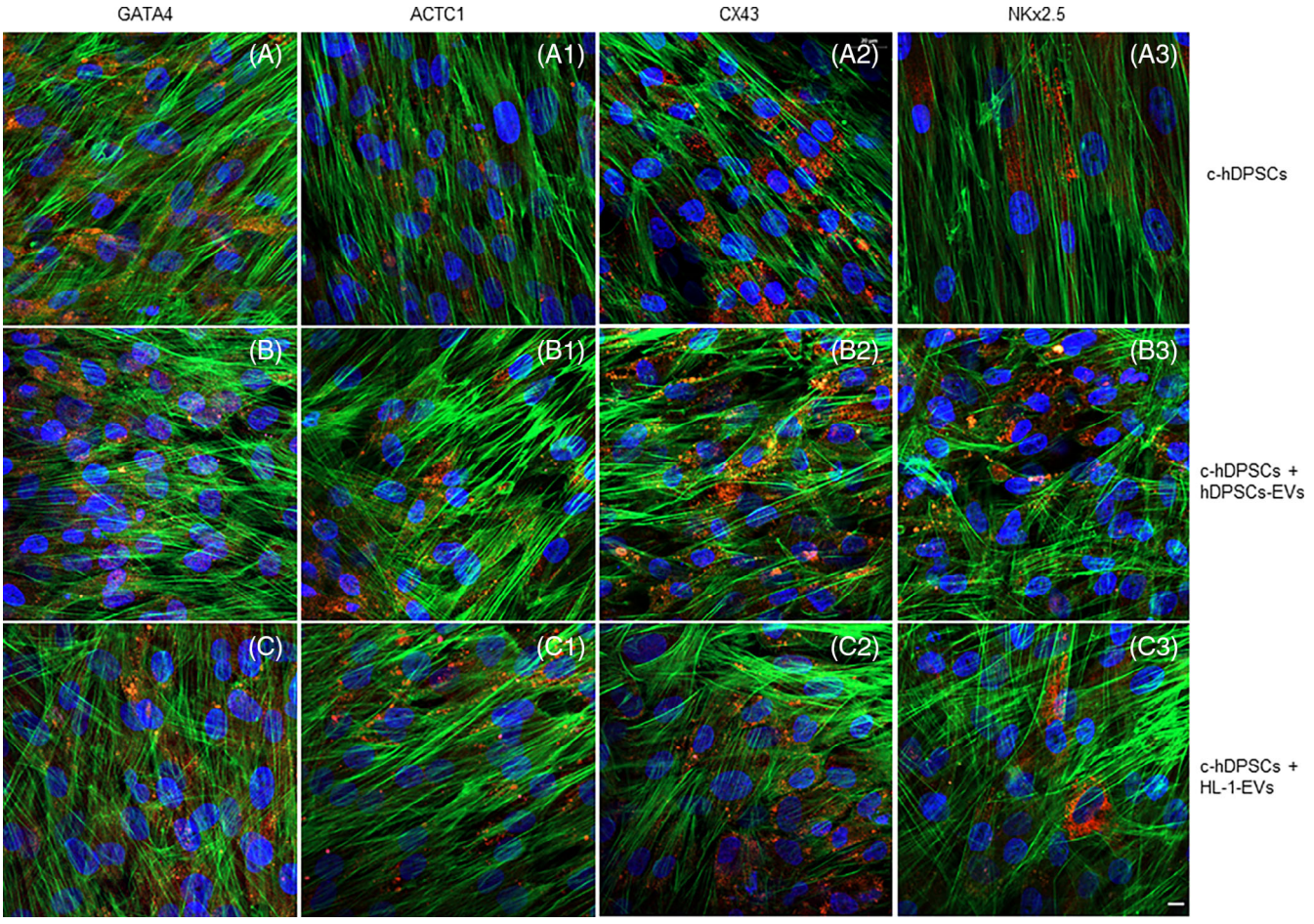

FIGURE 5 GATA4, ACTC1, CX43, and NKx2.5 cardiomiogenic markers and differences in the protein levels in cardiomyocytes derived from hDPSCs cell lines after 24 days of culture. Expression of GATA4, α-SMA, CX43, and NKx2.5 analyzed by confocal microscopy in c-hDPSCs (A–A3), c-hDPSCs + hDPSCs-EVs (B–B3), and c-hDPSCs + HL-1-EVs (C–C3). Green fluorescence: cytoskeleton actin. Red fluorescence: GATA4, ACTC1, CX43, and NKx2.5. Blue fluorescence: cell nuclei. Scale bar: 20 μm.

specific gene and protein expression during MSCs differentiation.

The upregulation of specific transcription factors involved in the cardiomyocytes differentiation and in cardiac tissue development has been evidenced in preclinical studies.[31]

Transcription factors specific for the cardiac tissue include Nkx2-5, GATA4, and GATA5. In particular, GATA4 interacts with Nkx2.5 increasing its transcriptional activity and activating chamber-specific genes such as those coding for atrial natriuretic peptide, connexin 40, and CX43. Li and Zhang demonstrated that rabbit BM-MSC transfected with Nkx2.5 or GATA4 may lead to a restoration of myocardial function through myocardial infarction induction in rabbits. Furthermore, it has been demonstrated that overexpression of GATA4 in rat BM-MSCs improves the differentiation of these cells toward cardiomyocytes. Overexpression of Nkx2.5 in human umbilical cord-derived MSCs has also been shown to induce differentiation of MSCs into cardiomyocytes.[32]

The modulation of gene expression is guaranteed not only by transcription factors, but also by miRNAs, fundamental for the post-transcriptional regulation of protein-coding genes. However, how miRNAs regulate gene expression of genes involved in cardiac development is not fully understood.[33] Among the various miRNAs related to cardiac tissue, miR-200c has attracted a lot of interest due to its implication in the contraction and development of the heart, as well as in the modulation of Ca^{2+} management. miR-200c represents one of the most important repressors of cardiomyogenesis.[34] The cooperative action of miR-200c and related targeted transcription factors, such as GATA4, may underlies processes and pathways associated with cardiomyocyte growth and function. Moreover, the upregulation of miR-200c in human embryonic stem cells (hESCs) may lead to a

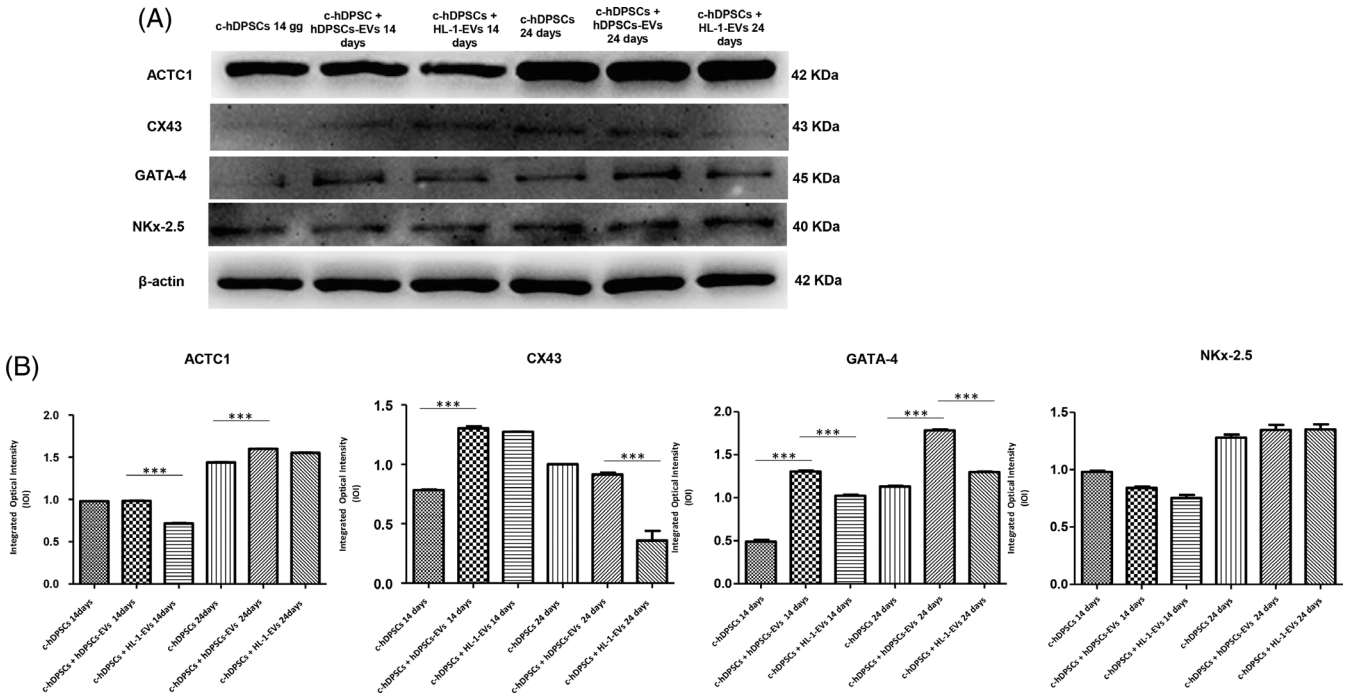

FIGURE 6 Western blotting analysis. (A) GATA4, ACTC1, CX43, and NKx2.5 protein expression in cardiomyocytes derived from hDPSCs untreated or treated with EVs derived by hDPSCs or HL-1 cells at different time points. Each membrane was probed with β-actin antibody to verify the loading consistency. Western blot image is the representative of three different experiments. (B) Histograms represent densitometric measurements of protein bands expressed as the integrated optical intensity (IOI) mean of three separate experiments. The error bars show the standard deviation (±SD). Densitometric values analyzed by ANOVA (post hoc application of Tukey's multiple comparison test) returned significant differences. ***$p < 0.001$.

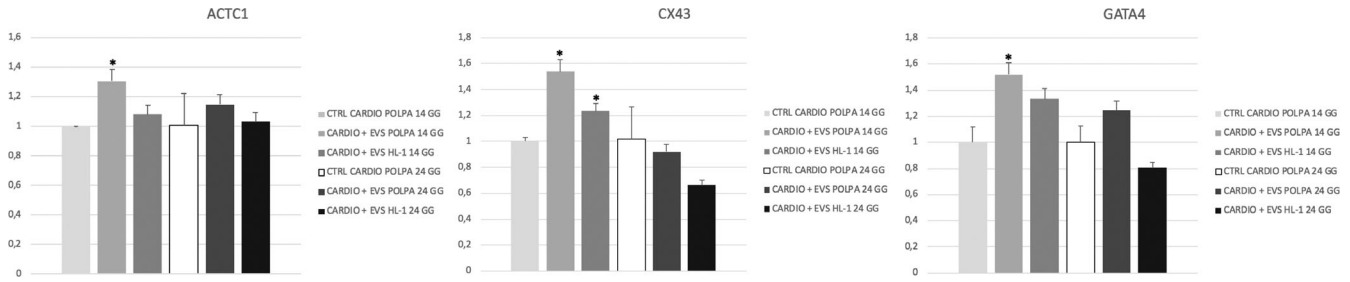

FIGURE 7 Real-time PCR assay for gene expression analysis. qRT-PCR bars show the significant (*$p < 0.05$) relative fold changes ± SD for *ACTC1*, *GATA4*, and *CX43* gene in c-hDPSCs + EVs-hDPSCs and c-hDPSCs + EVs-HL-1 samples compared to corresponding controls at 14 and 24 days.

significant reduction of GATA4 mRNA levels, suggesting an epigenetic regulation.[33]

It has already been extensively demonstrated that MSCs are capable of differentiating into cardiomyocytes, but the main effect of MSCs in the treatment of cardiovascular diseases depends on the paracrine effect.[35]

Based on this concept, the study aimed at evaluating EVs and the bioactive molecules within them, in order to exploit their ability to influence the differentiation process versus cardiomyogenic lineage.

EV-mediated bidirectional information exchange between progenitor cells and tissue-differentiated cells leads to phenotypic changes.[36]

The incredible biological properties of MSCs such as the capacity to differentiate into cardiac cells, the immunomodulation ability, and the capability to undergo neovasculogenesis, the production, and release of several factors stimulating tissue restoration make this type of cells a valid candidate for cell-based therapies.[35]

Besides the property of differentiating into cardiovascular progenitor cells, MSCs appear to have a

fundamental role in the cardiovascular frontier, due to their ability to produce EVs capable of modulating cellular properties.[37] In the present work, we have characterized EVs derived from both hDPSCs and HL-1 cells. hDPSCs are actively proliferating cells and release EVs at higher concentrations than HL-1 cells. Moreover, according to earlier studies, both hDPSCs and HL-1-derived EVs exhibited a size range typical of medium/large EV subsets.[38]

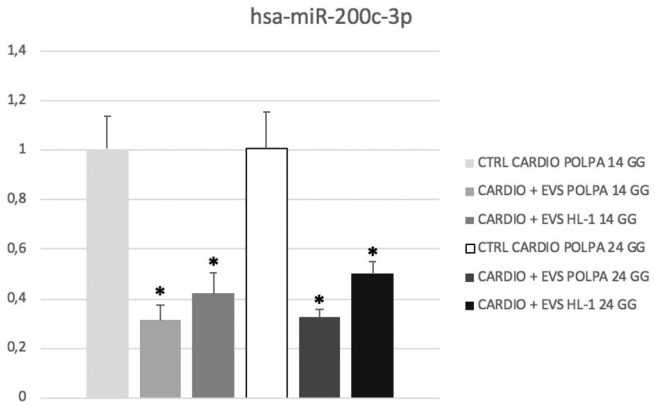

FIGURE 8 hsa-miR-200c-3p's expression. Fold change histogram of hsa-miR-200c-3p's expression levels in c-hDPSCs + EVs-hDPSCs and c-hDPSCs + EVs-HL-1 samples versus untreated c-hDPSCs at 14 and 24 days of cell culture (*$p < 0.05$) ± SD.

In the present study, the expression of two transcription factors GATA4 and Nkx2.5 and of the specific cardiac markers CX43 and ACTC1 was assessed in c-hDPSCs at different time points and in EV-treated c-hDPSCs derived from hDPSCs or HL cells −1.

Our work demonstrated that the cardiac transcription factors Nkx2-5 and GATA4 and specific cardiac markers ACTC1 and CX43 were upregulated in c-hDPSCs treated with EVs derived from hDPSCs in comparison with the untreated c-hDPSCs and c-hDPSCs treated with EVs derived from HL-1 cells. The data achieved by confocal microscopy were also confirmed by data obtained from Western blot and gene expression analysis. Intracellullar calcium signaling plays an essential role in the development of the cardiac system. For instance, the pro-survival outcome of intracellular calcium variations on anti-apoptotic gene expression has been established, underlining its part in the events linked to cell proliferation and differentiation in immature or progenitor cells.[39] In our experimental model, hDPSCs-EVs were capable of augmenting spontaneous intracellular calcium action similarly to that stimulated by HL-1-EVs, advancing the hypothesis that their secretome may contribute the mechanisms implicated in cardiac differentiation.

Treatment with EVs derived from hDPSCs evidences cardiomyogenic effects on c-hDPSCs leading to an increased expression of cardiomyogenic differentiation markers.

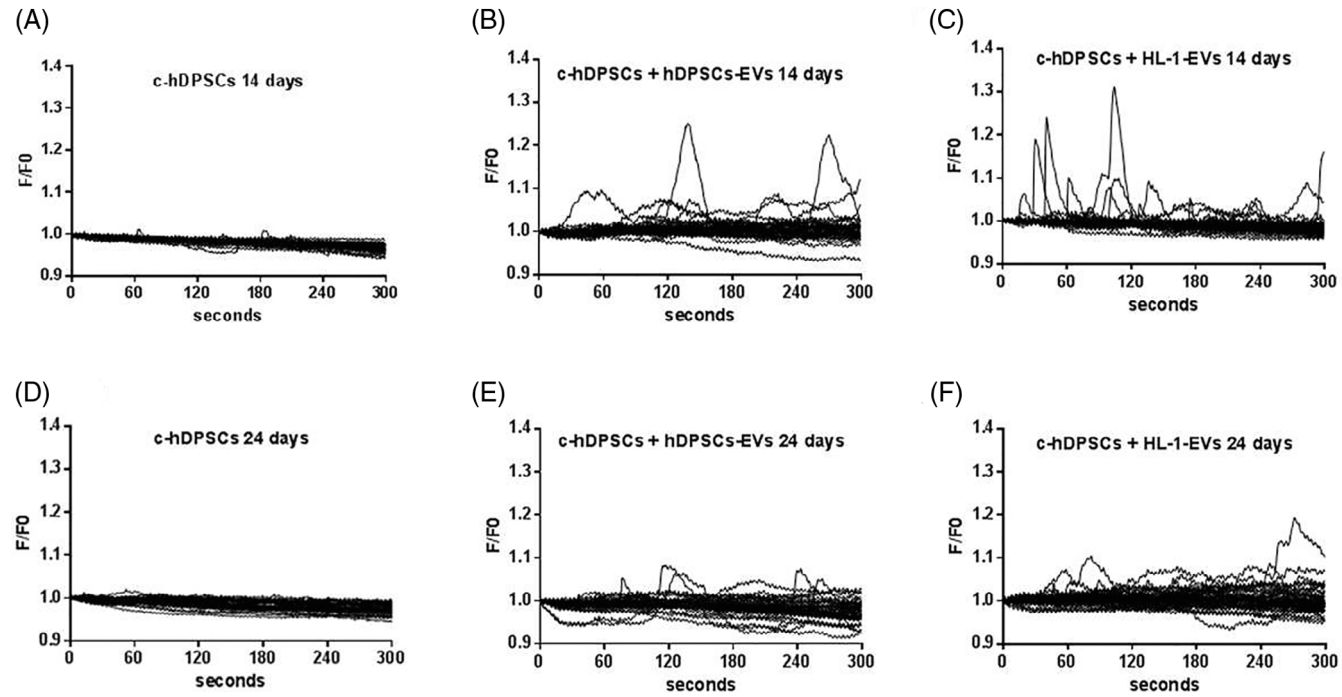

FIGURE 9 In vitro effects of EVs on intracellular Ca^{2+} spikes. (A–F) Representative of spontaneous intracellular Ca^{2+} oscillation hDPSCs recorded in FLUO-4 loaded cells, according to treatment groups.

Thus, EVs showed a key biological role in heart tissue homeostasis and repair and could represent a cell-free approach to lead an effective cardiac regeneration.

AUTHOR CONTRIBUTIONS

Conceptualization: Guya Diletta Marconi, Francesca Diomede, and Jacopo Pizzicannella. *Methodology*: Simone Guarnieri, Paola Lanuti, Fani Konstantinidou, and Francesca Diomede. *Software*: Simone Guarnieri, Paola Lanuti, Francesca Diomede, and Guya Diletta Marconi. *Validation*: Guya Diletta Marconi, Sante D. Pierdomenico, Oriana Trubiani, Jacopo Pizzicannella, Fani Konstantinidou, and Francesca Diomede. *Formal analysis*: Guya Diletta Marconi, Jacopo Pizzicannella, Francesca Diomede. Investigation: Guya Diletta Marconi, and Fani Konstantinidou. *Resources*: Guya Diletta Marconi, Jacopo Pizzicannella, and Francesca Diomede. *Data curation*: Guya Diletta Marconi, Simone Guarnieri, Paola Lanuti, Thangavelu Soundara Rajan, Jacopo Pizzicannella, and Francesca Diomede. *Writing—original draft preparation*: Guya Diletta Marconi. *Writing—review and editing*: Guya Diletta Marconi, Sante D. Pierdomenico, Thangavelu Soundara Rajan, Jacopo Pizzicannella, Valentina Gatta, and Francesca Diomede. *Visualization*: Guya Diletta Marconi, Thangavelu Soundara Rajan, Jacopo Pizzicannella, and Francesca Diomede. *Supervision*: Guya Diletta Marconi, Jacopo Pizzicannella, Francesca Diomede, Valentina Gatta, and Sante D. Pierdomenico. *Project administration*: Guya Diletta Marconi, Jacopo Pizzicannella, and Francesca Diomede. *Funding acquisition*: Guya Diletta Marconi, Oriana Trubiani, and Francesca Diomede. All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data are available to the corresponding author upon request.

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SUPPORTING INFORMATION

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