Cardiac Differentiation of Human Wharton`s Jelly Stem Cells – Experimental Comparison of Protocols

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Abstract: Cardiomyoplasty represents a promising approach for the repair of the injured heart, but is hampered by the availability of appropriate cells. Mesenchymal stem cells derived from the human umbilical cord tissue (UCMSC) can be obtained in large amounts without medical intervention, exhibit self renewal and immunological naivity as well as multipotency. In the present study, different published protocols of cardiac differentiation designed for different stem cell types were compared to differentiate UCMSC into cardiomyocyte-like cells (cUCMSC). Cardiac differentiation of UCMSC was driven by cell treatment with 5-azacytidine, oxytocin as well as by forming of "embryoid bodies". The morphological and immunocytochemical analysis of cUCMSC with an extensive panel of cardiac markers showed that oxytocin is a more potent inducer of cardiac differentiation than 5-azacytidine and the forming of "embryoid bodies". cUCMSC reveal a cardiomyocyte-like structure and the expression of cardiomyocyte associated proteins. The easy accessibility and the ability of UCMSC to differentiate into cells with characteristics of cardiomyocytes render UCMSC an attractive candidate for cell based therapies and cardiac tissue engineering.

Keywords: Cardiac differentiation, mesenchymal stem cells, umbilical cord, cardiac tissue engineering, regenerative medicine.

INTRODUCTION

Myocardial infarction is a severe cardiovascular disorder and the major cause of heart failure. Ischemic conditions result in an irreversible loss of functional cardiomyocytes which are gradually replaced by fibroblasts, forming non-contractile scar tissue [1]. In end stage heart failure allogeneic heart transplantation remains the last treatment option, but is limited due to donor organ shortage. According to the Eurotransplant International Foundation, in 2009 the demand for donor hearts was covered only to 37 % in Germany (www.eurotransplant.org). Cellular cardiomyoplasty represents a promising alternative approach for myocardial repair [2]. The transplanted cells may reduce post-infarction heart failures, limit scar expansion, support angiogenesis and stimulate interactions with resident cardiac progenitor cells. The main aim of cardiac cell therapy is the repopulation of the diseased myocardium with cells that can restore contractility [3-6]. Mesenchymal stem cells (MSC) are a potential cellular source for cardiac stem cell-based therapy, since they have the ability to differentiate into cardiomyocytes. In addition, MSC have been already tested clinically and do not raise any ethical concerns [7]. To date, human bone marrow (BM) represents the major source of MSC. However, the harvest of BM is an invasive procedure, the number as well as the differentiation potential and the maximum life span of human BM-derived autologous MSC significantly decline with donor age [8]. The umbilical cord tissue may be an attractive alternative source of MSC. Umbilical cord tissue-derived mesenchymal stem cells (UCMSC) are easily attainable, can be extensively expanded and maintained in culture even after cryopreservation [9-11]. Furthermore, UCMSC qualify for an allogeneic use due to their immunological naivity [12]. UCMSC also feature plastic adherence and multipotency [13-15]. Since undifferentiated MSC tend to spontaneously differentiate into multiple lineages when transplanted *in vivo*, it is possible that such uncommitted stem cells undergo maldifferentiation within the infarcted myocardium with potentially life-threatening consequences [16]. Therefore, it was postulated that a certain cardiac differentiation of stem cells prior to transplantation would result in enhanced myocardial regeneration and recovery of heart function [17]. In this context, initiating the transformation of stem cells into a cardiomyogenic lineage is accomplished by culturing them in defined culture conditions [18]. The DNA demethylating agent 5-azacytidine [19-21], oxytocic hormone [22], embryo-like aggregates [23] and several growth factors like transforming-growth-factor-β1 (TGF-β1), platelet-derived-growth factor (PDGF) and basic-fibroblast-growth-factor (bFGF) are used to induce myocyte differentiation of various stem cell types. The verification of cardiomyocyte induction is generally based on the expression of sarcomeric proteins. Sarcomeres are the contractile units of the heart and are composed of highly organized actin and myosin filaments, stabilized by α-actinin. Other muscle specific proteins such as troponins and tropomyosins also compose the contractile filaments and are involved in regulating contraction [2, 24]. The aim of this study was to investigate the cardiomyogenic potential of UCMSC by critically comparing previously published protocols which use differ-

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ent differentiation agents and procedures as well as morphological and immunocytochemical analyses.

MATERIALS AND METHODS

Isolation, Cell Culture, and Phenotypic Characterization of UCMSC

Umbilical cords were collected after birth and stored in Dulbecco`s Phosphate Buffered Saline (PBS; PAA Laboratories GmbH, Pasching, Austria) containing 100 U/ml penicillin/streptomycin (Invitrogen GmbH, Karlsruhe, Germany), at 4°C for 6 to 48 h before tissue processing. The mesenchymal stem cells were isolated as previously described [25]. Briefly, after removal of the two arteries, the cords were cut into small pieces and the fragments were digested enzymatically. Alternatively, UCMSC were collected by incubating arteries directly in medium to allow the outgrowth of perivascular mesenchymal stem cells (PVSC). As previously described [26], the obtained cell solution was cultured in complete serum- and xenofree MesenCult®-XF (StemCell Technologies SARL, Köln, Germany) medium. Enzymatically derived MSC and PVSC were collectively referred to as UCMSC. For phenotypic characterization, UCMSC were stained for human CD34, CD45, CD73, CD90, CD105 as well as HLA-DR and were analyzed by flow cytometry. As a functional positive control, UCMSC were differentiated into osteocytes and adipocytes and subsequently histochemically analyzed as previously described [27, 28] (data not shown).

Cardiac Differentiation of UCMSC (cUCMSC)

Cardiac differentiation of UCMSC from six different umbilical cords, were performed at passage p2 to p6 according to previously published protocols.

- Goumans *et al.,* [19]: An approximately 70 % subconfluent monolayer of UCMSC was treated with 5 µM 5-azacytidine (Sigma-Aldrich GmbH, Hamburg, Germany) for 72 h in differentiation medium Iscove`s Modified Dulbecco`s medium/Hams F12 (1:1) (IMDM; Invitrogen GmbH, Karlsruhe, Germany) supplemented with L-glutamine, 2 % horse serum (Sigma-Aldrich GmbH, Hamburg, Germany), non essential amino acids, 1% insulin-transferrin-selenium (Invitrogen GmbH, Karlsruhe, Germany) and 10^{-4} M ascorbic acid (Sigma Aldrich, Hamburg, Germany). After induction, cells were maintained in medium consisting of Dulbecco`s modified eagle medium (DMEM; Invitrogen GmbH, Karlsruhe, Germany) and 1 ng/ml TGF-β1 (R&D Systems GmbH, Wiesbaden, Germany).

- Matsuura *et al.,* [22]: 1,2 x 10^4 UCMSC/cm² were cultured in IMDM (Invitrogen GmbH, Karlsruhe, Germany), 10 % FBS (PAA Laboratories GmbH, Pasching, Austria) and 1 % Penicillin/Streptomycin (Invitrogen GmbH, Karlsruhe, Germany). 24 h after seeding, the cells were either treated with 10 µM 5-azacytidine (Sigma-Aldrich GmbH, Hamburg, Germany) for 24 h, referred to as "Matsuura protocol I", or 10 nM Oxytocin (Wako Chemicals GmbH, Neuss, Germany), referred to as "Matsuura protocol II", for initial 72 h.

- Maltsev *et al.,* [23]: UCMSC were cultured in hanging drops to form "embryoid bodies" in DMEM con-taining 20 % FBS (PAA Laboratories GmbH, Pas-ching, Austria), 2 mM L-glutamine, 5x10^{-5} M β-Mercapotethanol and 1 % non-essential amino acids (Invitrogen GmbH, Karlsruhe, Germany). 10^3 UCMSC in 20 µl culture medium were placed in drops on the lids of petri dishes filled with PBS. After 2 d of culture in hanging drops the embryoid bodies were harvested and incubated in bacteriological petri dishes for further 5 d in suspension. The 7 d old embryoid bodies were plated in 0.2 % gelatine-coated (Merck KGaA, Darmstadt, Germany) petri dishes for cell outgrowth.

- Wang *et al.,* [20]: An approximately 70 % subconfluent monolayer of UCMSC was treated with 3 µM 5-azacytidine (Sigma-Aldrich GmbH, Hamburg, Germany) in serum-free DMEM (Invitrogen GmbH, Karlsruhe, Germany) for 24 h. After this treatment, cells were washed with PBS and maintained in DMEM (Invitrogen GmbH, Karlsruhe, Germany) containing 10 % FBS (PAA Laboratories GmbH, Pasching, Austria).

- Wu *et al.,* [21]: An approximately 70 % subconfluent monolayer of UCMSC was incubated for 24 h in DMEM-F12 (Invitrogen GmbH, Karlsruhe, Germany) containing 5 µM 5-azacytidine (Sigma-Aldrich GmbH, Hamburg, Germany). Then the cells were washed with PBS and replaced with fresh DMEM-F12 containing 5 % FBS (PAA Laboratories GmbH, Pasching, Austria), 10 ng/ml bFGF (R&D Systems, Wiesbaden, Germany), 10 ng/ml PDGF (R&D Systems GmbH, Wiesbaden, Germany).

In all cases, medium was changed every 3 days. cUCMSC were investigated by immunocytochemistry staining after 4 to 5 weeks of differentiation.

Immunocytochemistry of UCMSC / cUCMSC

For a confluent culture, 5 x 10^3 cells/well were seeded in 18 well-flat µ-slides (Ibidi GmbH, Martinsried, Germany). The next day, cells were washed with PBS and fixed with 3.7 % Formaldehyd (Roth GmbH & Co. KG, Karlsruhe, Germany) for 10 min at room temperature. For intracellular staining, cells were permeabilized with 0.1 % Triton X-100 (Sigma-Aldrich GmbH, Hamburg, Germany) in PBS for 15 min. Cells were incubated overnight at 4 °C with primary antibodies against human cardiac actin (10 µg/ml; Sigma-Aldrich GmbH, Hamburg, Germany), cardiac actinin (2 µg/ml; Sigma-Aldrich GmbH, Hamburg, Germany), cardiac troponin C, cardiac troponin T (2.5 µg/ml; Abcam plc, Cambridge, UK), connexin 43 (10 µg/ml; Millipore GmbH, Schwalbach, Germany), GATA4 (2 µg/ml; Becton Dickinson GmbH, Heidelberg, Germany), myosin heavy chain (1 µg/ml; Santa Cruz Biotechnology Inc., Heidelberg, Germany), sarcomeric actin (1 µg/ml; Sigma-Aldrich GmbH, Hamburg, Germany), sarcomeric actinin (1:25 dilution according to manufacturer´s instructions (no concentration provided; Zytomed Systems GmbH, Berlin, Germany), and slow myosin (1 µg/ml; Sigma-Aldrich GmbH, Hamburg, Germany). Primary antibodies were diluted in PBS/

0.1%Triton/1%BSA. After washing with PBS/0.1% Triton, cells were incubated 2 h at 37° C with secondary antibody goat anti mouse IgG, Alexa Fluor® 488 conjugate (5 µg/ml; Invitrogen GmbH, Karlsruhe, Germany) and goat anti rabbit IgG, Fluoresceinisothiocyanat (FITC) conjugate (22 µg/ml; Sigma-Aldrich GmbH, Hamburg, Germany), respectively. Antibodies were diluted in PBS/0.1%Triton/1%BSA. After washing with PBS, cell nuclei were stained with 1µg/ml diamidinophenylindole (DAPI; Roche GmbH, Mannheim, Germany) in PBS for 20 min at room temperature. For fluorescence microscopy, cells were covered with mounting medium (Ibidi GmbH, Munich, Germany).

Undifferentiated UCMSC served as a negative control. Controls for nonspecific binding of secondary antibodies were performed by excluding primary antibodies. Based on previous experience isotype controls were generally not necessary.

Immunofluorescence Microscopy of UCMSC / cUCMSC

Fluorescence signals were detected using computer-guided fluorescence microscopy (Axio Observer and AxioVision Rel. 4.8; Zeiss MicroImaging GmbH, Jena, Germany). The excitation and emission of Alexa Fluor® 488 conjugated antibodies was 495 nm and 519 nm, respectively. The excitation and emission of FITC conjugated antibodies was 495 nm and 517 nm, respectively. Sample and control of nonspecific binding of secondary antibodies were measured at identical exposure times.

Statistical Analysis

Statistical significance was assessed by the two-sided student`s t-test.

RESULTS

In this study, six previously published protocols for cardiac differentiation of human cardiomyocyte progenitor cells [19], murine heart cells [22], murine embryonic stem cells [23], and umbilical cord cells [20,21] were systematically tested for its potential to differentiate human umbilical cord tissue-derived mesenchymal stem cells (UCMSC)

into cells of cardiomyogenic lineage. Cardiac differentiation procedures and immunocytochemical stainings were performed with umbilical cord samples from six different donors.

Morphological Analysis

UCMSC were isolated, phenotypically characterized and cultured in xeno- and serum-free medium as previously described [26]. Cardiac differentiation of UCMSC was performed as given in material & methods. Undifferentiated UCMSC show the typical elongated spindle shaped morphology as demonstrated in Fig. (**1A**). In contrast, cardiac differentiated UCMSC (cUCMSC) according to Matsuura protocol II [22], using oxytocin, grow flat with short cytoplasmic extensions and reveal a distinct striate pattern indicating fibrillar structure (Fig. **1B**).

Immunocytochemical Analysis

Expression of Cardiac Markers Varies among Differentiation Protocols and Donors

UCMSC were cardiac-differentiated to cUCMSC according to six previously published protocols (for experimental details see *materials and methods*). For immunocytochemical analysis after different differentiation procedures, cUCMSC were stained for cardiac and skeletal proteins. Table **1** summarizes the whole analysis by highlighting positive stainings in green and negative stainings in red. Some stainings after differentiation of Goumans *et al.*, Maltsev *et al.,* and Wang *et al.,* were not evaluable due to insufficient cell numbers and were indicated as "not available (n. a.)".

Matsuura *et al*., protocol II [22] exposed Sca-1+ cells from adult murine heart to oxytocin for cardiac differentiaton. This procedure proved to be superior in cardiac specific protein expression of cUCMSC compared to other tested protocols. Goumans *et al.,* [19], Matsuura *et al*., protocol I [22], Wang *et al*., [20], as well as Wu *et al*., [21] described the cardiac differentiation of human cardiomyocyte progenitor cells, Sca-1+ cells from adult murine heart and human umbilical cord cells, respectively by the use of 5-azacytidine. Treatment of UCMSC with 5-azacytidine and
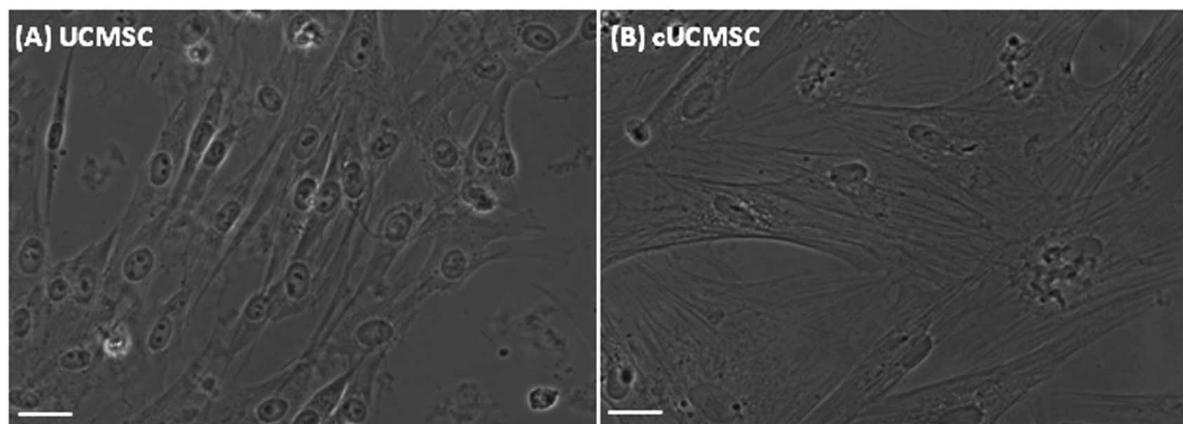
Fig. (1). Morphological analysis of undifferentiated (UCMSC) and cardiac-differentiated UCMSC (cUCMSC) by phase contrast microscopy. UCMSC display the characteristic fibroblastoid morphology (**A**) in contrast to the outspread appearance of cUCMSC (differentiation according to Matsuura protocol II using oxytocin) with a striped cytoplasma indicating filaments (**B**). These are representatives of six independent experiments with UCMSC and cUCMSC from six different umbilical cords. Scale bars = 50 µm.

Table 1.   Overview of Immunocytological Results of Cardiac-Differentiated UCMSC (cUCMSC) after Differentiation According to Previously Published Protocols. Cardiac Specific Protein Expression of cUCMSC Depends on Differentiation Procedure and Donor Umbilical Cords. Protocols according to Goumans *et al.,*[1] [19], Matsuura *et al.,* protocol I[2] [22], Wang *et al.,*[5] [20]. and Wu *et al.,*[6] [21] use 5-azacytidine for cardiac differentiation of different cell types. Protocol of Matsuura *et al.,* II[3] [22] describes oxytocin in cardiac differentiation of adult Sca-1+ murine heart cells. Cardiac differentiation of murine embryonal stem cells was performed by Maltsev *et al.,*[4] [23] using "embryoid bodies". Fields highlighted in green distinguish positve staininings from red-shaded areas, with negative staining. n.a: not applicable = cUCMSC were generated in an insufficiant level for immunocytochemical analysis (Goumans *et al.*, Maltsev *et al.*) or cUCMSC staining was not evaluable (Wang *et al.*). These are total data of six independent experiments with cUCMSC from six different umbilical cords (UC).

Differentiation according to	UC	cA	cAn	cTnC	cTnT	Cx43	GATA4	MYH	sA	sAn	sM
Goumans *et al.*[1]	1	+	+	-	-	+	-	-	-	+	-
	2	+	-	-	-	+	-	-	+	+	-
	3	+	+	-	-	-	-	-	-	+	-
	4	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.
	5	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.
	6	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.
Matsuura *et al.* I[2]	1	+	+	-	-	+	-	-	+	+	-
	2	+	+	-	-	+	-	-	+	+	-
	3	+	+	-	+	+	-	-	+	+	-
	4	+	-	-	+	+	-	-	-	+	-
	5	+	-	-	-	+	-	-	-	-	-
	6	+	-	-	-	-	-	+	-	-	-
Matsuura *et al.* II[3]	1	+	+	-	-	+	-	+	+	+	-
	2	+	+	-	+	+	-	-	+	+	-
	3	+	+	-	+	+	-	+	+	+	-
	4	+	+	-	+	+	-	+	+	+	-
	5	+	+	-	-	+	-	-	+	+	-
	6	+	+	-	+	+	-	-	+	+	-
Maltsev *et al.*[4]	1	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	-	n. a.	n. a.	n. a.
	2	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	-	n. a.	n. a.	n. a.
	3	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	-	n. a.	n. a.	n. a.
	4	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	-	n. a.	n. a.	n. a.
	5	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	-	n. a.	n. a.	n. a.
	6	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	-	n. a.	n. a.	n. a.
Wang *et al.*[5]	1	+	+	-	-	+	-	-	-	+	-
	2	+	+	-	-	+	-	-	-	+	-
	3	+	+	-	-	+	-	-	-	+	-
	4	-	-	n. a.	-	+	-	-	+	-	-
	5	+	-	-	-	+	-	-	+	-	-
	6	+	-	-	-	+	-	-	+	-	-
Wu *et al.*[6]	1	+	+	-	-	-	-	-	-	-	-
	2	+	-	-	-	+	-	-	+	+	-
	3	+	+	-	-	+	-	+	+	+	-
	4	+	+	-	-	-	-	-	+	+	-
	5	+	-	-	-	+	-	+	+	-	-
	6	+	-	-	-	-	-	-	+	+	-

cA = cardiac actin	cTnT = cardiac Troponin T	MYH = myosin heavy chain	sM = slow myosin	+ = positive staining
cAn = cardiac actinin	Cx43 = connexin 43	sA = sarcomeric actin	- = negative staining	
cTnC = cardiac Troponin C	GATA4 = GATA4	sAn = sarcomeric actinin	n. a. = not available	

the formation of "embryoid bodies", performed by Maltsev *et al.,* [23] for cardiac differentiation of murine embryonal stem cells, are less effective in generating cUCMSC.

The cardiomyocyte-associated proteins cardiac actin, cardiac actinin, sarcomeric actin, sarcomeric actinin, and connexin 43 were expressed with differentiation procedures,
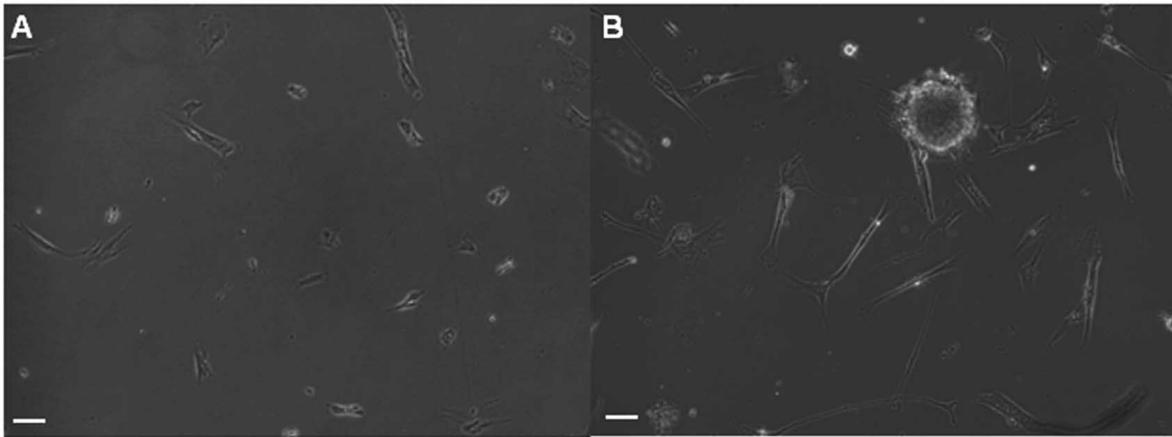
Fig. (2). Demonstration of low cell density of cardiac-differentiated UCMSC (cUCMSC) by phase contrast microscopy. Cardiac differentiation of UCMSC according to Goumans *et al.*, [19] (**A**) and Maltsev *et al.,* [23] (**B**) leads to insufficient cell numbers for immunocytochemistry. These are representatives of six independent experiments with cUCMSC from six different umbilical cords. Scale bars = 100µm.

but not in all umbilical cord samples. cUCMSC differentiated according to Wu *et al.*, additionally express cardiac myosin heavy chain that interacts with actin to generate the force for cellular movements. Besides the expression of aforementioned proteins, differentiation according to Matsuura protocol. I with 5-azacytidine and differentiation according to Matsuura protocol II with oxytocin results in the expression of cardiac troponin T, a subunit of the troponin complex which regulates muscle contraction. However, cardiac differentiation of UCMSC induced by oxytocin (Matsuura *et al.,* protocol II) leads to a higher frequency of cardiac specific protein expression than the treatment with 5-azycytidine (Matsuura *et al.,* protocol I). Independent of the differentiation protocol, cardiac troponin C, GATA4 and slow myosin were not detected. Cardiac protein expression of cUCMSC varies between umbilical cords indicating donor-specific differences in the differentiation potential. Protocols according to Goumans *et al.*, [19] (Fig. **2A)** and Maltsev *et al.,* [23] (Fig. **2B**) did not generate sufficient numbers of cUCMSC for an extensive immunocytochemical analysis.

Oxytocin Treatment Results in Most Cardiac Specific Protein Expression

As shown in Table **1**, cardiac differentiation of UCMSC with the oxytocin protocol published by Matsuura *et al.,* [22] (Matsuura protocol II) gave rise to the highest number of cardiac markers and was, therefore, considered to be the most efficient differentiation procedure. These cUCMSC stained positive for cardiac actin, cardiac actinin, cardiac troponin C, cardiac troponin T, connexin 43, sarcomeric actin, sarcomeric actinin, slow myosin, as well as myosin heavy chain (Fig. **3 E-H, L-M**). Staining of cUCMSC for the contractile proteins cardiac actin, cardiac actinin, sarcomeriac actin, sarcomeric actinin, cardiac troponin T and myosin heavy chain reveal a fine striate pattern, indicating sarcomere formation. Furthermore, cUCMSC express connexin 43, suggestive of their potential to electrically couple to each other (Fig. **3 N-O**). As a control, undifferentiated UCMSC display negligible staining for all cardiac antigens (Fig. **3 A-D, I-K**).

As far as changes in the expression of mesenchymal markers during the differentiation process are concerned, undifferentiated and cUCMSC differentiated according to Matsuura *et al.,* protocol II were tested for the expression of CD73, CD90, and CD105 by flow cytometry. All surface proteins significantly decreased upon differentiation, as indicated by the mean fluorescence intensity (CD73: 3929 ± 753 in UCMSC *vs*. 846 ± 28 in cUCMSC (p = 0.001), CD90: 1310 ± 296 in UCMSC *vs*. 263 ± 43 in cUCMSC (p = 0.002), CD105: 370 ± 145 in UCMSC *vs*. 80 ± 6 in cUCMSC (p = 0.01); data not illustrated).

The protocol of Matsuura et al. II also termed out to be best in terms of the differentiation rate. About 70.4 % of cUCMSC showed cardiomyocyte markers, whereas in all other protocols differentiation varied between 33.6 % and 48.8 %.

DISCUSSION

Despite advances in the management of myocardial infarction (MI), current pharmacologic and interventional strategies fail to regenerate damaged myocardium [29]. Resident cardiac progenitor cells can be found in transplanted human hearts, and evidence of myocyte proliferation in the human heart exists, however, this proliferation is not sufficient to compensate for the up to 1 billion cardiomyocytes that are lost after MI [30]. The transfer of MSC to the ischaemic region is a promising treatment to improve left ventricular function after myocardial injury, mainly due to the high proliferation capacity, plasticity and homing potential of MSC [31]. Here, we provide evidence that a novel source of MSC derived from the umbilical cord tissue (UCMSC) is an attractive candidate for cardiac tissue engineering which is in line with recently published reports [10,12,14]. With regard to future clinical trials, we successfully managed to grow UCMSC under GMP-compliant culture conditions, while retaining their phenotype and functional properties [26]. We also demonstrated that titanium-coated, clinically approved cardiovascular patches enhance retention of UCMSC and thus offers a potential cell delivery system for repair of damaged myocardium [32]. In order to improve engraftment efficiency by directing UCMSC toward
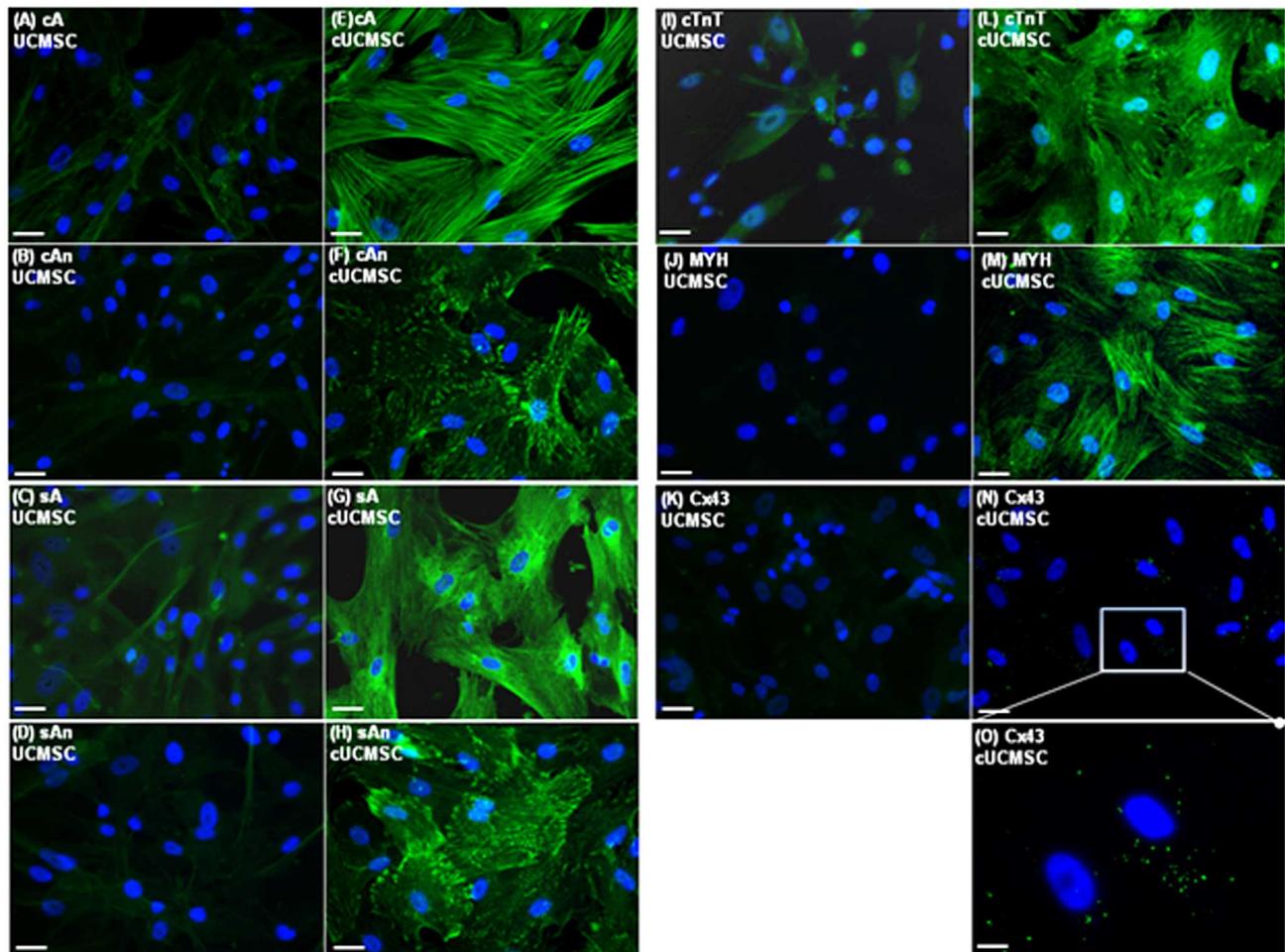
Fig. (3). Immunocytochemical analysis of cUCMSC differentiated according to Matsuura *et al.,* [22] protocol II using oxytocin. cUCMSC express the contractile proteins cardiac actin (cA), cardiac actinin (cAn), sarcomeric actin (sA), sarcomeric actinin (sAn), cardiac troponin T (cTnT), myosin heavy chain (MYH) (E-H, L-M;) as well as the gap junctional protein connexin 43 (Cx43) (N,O; all in green fluorescence) for electrical cell-to-cell coupling. Undifferentiated UCMSC did not express any cardiac specific proteins (A-D, I-K). Cell nuclei were stained by DAPI (A-O; blue). These are representatives of six independent experiments with UCMSC and cUCMSC from six different umbilical cords. Scale bars: A-N = 50 μm, O = 25 μm.

the cardiomyocyte lineage prior to transplantation and prevent maldifferentiation *in vivo*, cardiac differentiation of UCMSC *in vitro* seems necessary. Cardiac differentiation of MSC induced by the demethylating agent 5-azacytidine is controversially discussed. Martin-Rendon and colleagues report that 5-azacytidin treated human mesenchymal stem cells derived from umbilical cord and bone marrow do not generate cardiomyocytes *in vitro* at high frequencies [33]. In contrast, results of Antonitsis *et al.,* and Pereira *et al.,* [31] indicate that adult human bone marrow MSC [34] and MSC from umbilical cord [31] can differentiate towards a cardiomyogenic lineage after 5-azacytidine treatment. These discrepancies might be explained by the variability in culture conditions [35] or by different specification criteria for what makes a cell a cardiomyocyte. Xu *et al.,* stated that bFGF is necessary during the differentiation process because of its capability to develop the myogenic phenotype and promote the formation of myotubes [36]. However, our studies showed that UCMSC exposed to 5-azacytidine convert into cells changing their morphology and expressing cardiac-specific proteins irrespective of the presence of bFGF. cUCMSC differentiated according to Wu *et al.,* [21], using 5

μM 5-azacytidine for 24 h and bFGF containing culture medium, increase in size with striate pattern and express cardiac actin, cardiac actinin, sarcomeric actin, sarcomeric actinin, myosin heavy chain as well as connexin 43 after 5 weeks of culture. cUCMSC treated with 3 μM 5-azacytidine for 24 h according to Wang *et al.,* [20] and 10 μM 5-azacytidine for 72 h according to Matsuura protocol I [22] also change their morphology and express these cardiac specific proteins known for regulating contraction and gap-junctional communication without supplemented bFGF. TGF-β1 in combination with 5-azacytidine have been found to promote differentiation of human cardiomyocyte progenitor cells [19]. However, in our hands, the combination of 5-azacytidine and TGF-β1 stimulation of UCMSC leads to a flattened appearance and the expression of cardiac actin, cardiac actinin, sarcomeric actin, sarcomeric actinin as well as connexin 43 after 5 weeks of culture, but cUCMSC do not express any troponins or myosins necessary for contraction. In addition, during the differentiation process, cell numbers dramatically decreased to some extent insufficient for extensive immunocytochemical analysis. Maltsev *et al.,* demonstrated the expression of cardio-specific genes, proteins, and

action potentials in cells differentiated from murine embryonal stem cells by cultivation in hanging drops as "embryoid bodies" [23]. Using this differentiation system, UCMSC form aggregates but cellular outspread is not sufficient for performing extensive immunocytochemical analysis. Failure of cell outgrowth may be explainable due to the dependence of this method from the initial cell number differentiating in the aggregates. Another strategy of cardiac differentiation is the exposure of cells to oxytocin. This female reproductive hormone is necessary for uterine contractions during ovulation and parturition. The expression levels of oxytocin are higher in developing hearts than in adult hearts suggesting that oxytocin may be involved in cardiomyocyte differentiation [37]. Data from Matsuura *et al.,* indicate that oxytocin is a more potent inducer of cardiac differentiation of Sca-1+ adult murine heart cells than 5-azacytidine [22]. This is in line with our results demonstrating that human UCMSC exposed to 10 nM oxytocin for 72 h express the cardiomyocytes associated proteins including cardiac actin, cardiac actinin, sarcomeric actin, sarcomeric actinin, myosin heavy chain, connexin 43 and cardiac troponin T in significantly higher frequencies than after 5-azacytidine (10 µM, 72 h) treatment.

Of note, neither UCMSC nor cUCMSC expressed GATA4 which is in contrast to the results of La Rocca *et al.*, showing a strong expression of GATA4 in Wharton`s jelly cells [38]. The reason for this discrepancy could be differences in the isolation procedure (enzymatic *vs.* non-enzymatic). However, in our hands also MSC outgrowing from the perivascular region of the umbilical arteries did not show any GATA4 expression, irrespective of the differentiation status. Another possible explanation could lie in the use of different culture media. It would be interesting to assess whether the presents of e.g. serum might influence the expression pattern of GATA4 and/or other cardiac markers.

In conclusion, comparative immunocytochemical analyses revealed that UCMSC can be differentiated into cardiomyocyte-like cells with oxytocin being the most efficient differentiation agent. It is currently under progress in our laboratory to perform functional analyses of oxytocin-differentiated cUCMSC, such as to monitor action potentials by the patch-clamp technique and measurements of intracellular calcium flux.

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CONFLICT OF INTEREST

None declared.

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