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Study of Potential Differentiation of Wharton's Jelly-Derived Umbilical Cord-Mesenchymal Stem Cells into Renal Tissues

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Abstract: Bone Marrow, yet the gold standard in Mesenchymal Stem cells (MSCs) isolation, is an invasive procedure. Herein, an attempt to identify potential alternative MSCs sources for therapeutic applications, we studied MSCs derived from Wharton's Jelly of Umbilical Cord (WJ-UC) being easy accessible source. We tried to explore their potency for in - vitro expansion and differentiation into renal cells by comparing usage of different cocktails as nephrogenic growth factors and basal medium as control. In current study, MSCs were isolated from 6 human UCs and further immunophenotyping using immunocytochemistry and CD29 expression flow cytometry, then in-vitro expansion and differentiation of these isolated cells into renal progenitor stem cells by adding separately 5 different nephrogenic growth factors cocktails and basal medium, then assessing and comparing the outcomes in term of differentiation capacities. The WJ-UCs isolated MSCs, showed significant positive expression of CD29, MSCs surface marker. After expansion and induction of differentiation, results revealed that using nephrogenic growth factors cocktail containing combination of (Retinoic acid + Activin-A) then adding bone morphogenetic proteins (BMP-7) later, gave highest significant positive expression of renal progenitor stem cell surface markers CD24 and CD133 using immunocytochemistry and flow cytometry. WJ-UC seems to be a promising source alternative to BM-MSCs, since they can be easily available, without any ethical concerns and considered as medical waste. After *in-vitro* expansion and differentiation, using appropriate studied growth factors cocktail, newly formed renal progenitor stem cells may be further utilized, opening new perspectives for cell-based therapies in patients suffering from renal diseases.

Key words: Mscs · Wharton's Jelly Of The Umbilical Cord (WJ-UC) · Renal Progenitor Stem Cells · CD29 · CD24 & CD133

INTRODUCTION

Mesenchymal Stem cells (MSCs) are multipotent adult stem cells present in all tissues, as a part of the perivascular population. They have long-term ex vivo proliferation, as multilineage potential cells, which can be differentiated into different tissues originating from mesoderm ranging from bone and cartilage to cardiac muscle.

Other several studies described the isolation of MSCs from fetal tissues such as umbilical cord blood [1], amniotic fluid [2], placenta [3], amniotic membrane [4]

Wharton's Jelly of Umbilical Cord (WJ-UC) [5].

Contrary to bone marrow, isolation of UCMSCs is non-complicated without any risk of contamination, ethical issues and invasive methods.

The UC matrix is considered to be a rich, non-controversial and inexhaustible source of primitive MSCs [6]. Human umbilical cord Wharton's jelly-derived MSCs (WJMSCs) could be differentiated in vitro into adipocytes, chondrocytes, osteocytes, neurons and myogenic cells and they did not express major histocompatibility complex class II antigens [7].

Apart from general characteristic features of bone marrow-derived MSCs, Wharton jelly-derived MSCs (WJ-MSCs) have the ability to maintain phenotypic attributes, cell growth kinetics, cell cycle pattern, *in vitro*

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multi lineage differentiation plasticity, apoptotic pattern and normal karyotype-like intrinsic mesenchymal stem cell properties in long-term in vitro cultures. Therefore, WJ-MSCs could become a useful alternative source of MSCs for cell therapy and tissue repair in the field of regenerative medicine. WJ-MSCs contribute to tubular epithelial-mesenchymal transition (EMT) delay and the alleviation of renal fibrosis [8].

MSCs are an excellent candidate for cell therapy because they are easily accessible, their isolation is straight forward, they can be bio-preserved with minimal loss of potency and having also immunomodulatory properties. Therefore, these cells were explored to regenerate damaged tissues and treat inflammation, resulting from cardiovascular disease and myocardial infarction (MI), brain and spinal cord injury, stroke, diabetes, cartilage and bone injury, Crohn's disease and graft versus host disease (GVHD), also utilization of MSCs in the repair of kidney, muscle and lung. The cells were also found to promote angiogenesis and were used in chronic skin wound treatment [9].

MSCs obtained from the WJ of UC were used in the present study, it gained much attention over the last years as a result of easy isolation, without any ethical concerns, from a tissue which is discarded after birth. Furthermore WJ-MSCs represent a more primitive population than their adult counterparts, opening new perspectives for cell-based therapies.

We aimed in this work to study the effectiveness of isolation of MSCs from WJ-UC and to assess the potential effect of various nephrogenic growth factors cocktails on the in-vitro differentiation of these MSCs into renal tissue. This study was performed to give rise to a new hope of treatment in the future among patients suffering from renal diseases either acute or chronic and end stage renal diseases.

MATERIALS AND METHODS

Collection of Human Ucs: Six fresh human UCs that are usually discarded after delivery, were retrieved with the written consents from healthy full-term women by elective cesarean section deliveries, to avoid contamination during normal labor, at the Department of Obstetrics and Gynecology, Cairo University, Kasr-El Aini hospital.

Isolation and Expansion Cultures of Human Wharton's Jelly MSCs: WJ-MSCs were prepared and identified as previously described by Seshareddy *et al.* [10]. Briefly, within 24 hours of collection, UCs were cut into approximately 3cm long segments, handled in laminar flow aseptically and embedded in 70% alcohol. Then they were thoroughly rinsed with sterile PBS to remove as much blood as possible, following incising the cord segments lengthwise.

Cord pieces were then stuck into 160 ml flasks, 10 ml of culture medium were added in each flask, consisting of alpha Modified Eagle Medium (α-MEM), containing 10% fetal bovine serum and 2 mmol/ l L-glutamine and 100 U/ml penicillin, 100 ìg/ml streptomycin and 500 µl fungizone.

Flasks were incubated at 37°C, 5% CO2 and 95% humidity for 2 weeks. On every 3rd day, half of the old culture media was replaced by fresh media, with removal of cord pieces and non adherent cells.

By the end of 4th week, adherent MSCs to the culture flasks, were then detached by subjecting the confluent flasks with 2% Trypsin-EDTA for 10 minutes, then the cells were washed with normal saline, centrifuged for 10 minutes at 1200 rpm, the supernatant was completely discarded.

Morphologically, mesenchymal and renal tissues in entire sections were examined in ten consecutive fields under light microscope at magnifications x 400 with the highest expression was calculated from their mean. Meanwhile, a negative staining was defined as the absence of cells expressing the marker as (Zero).

Immunocytochemistry (ICC) staining and flowcytometry (FC) using anti CD 29 were performed on cells suspended in 1ml normal saline and counted using hemocytometer (20 µl sample + 180 µl acetic acid).

MSCs Differentiation Cultures into Renal Tissues: The expanded MSCs with the concentration of 1000, 000 / ml were plated onto 96-well plates coated with polyamine, 200µl (Basal medium) in each well were added. Further 6 cocktails were added sequentially to the plate in which each cocktail was in 2 columns as follow (Table 1).

Immunophenotypic Analysis: Cells were identified by ICC staining of CD 29, CD 24 and CD 133 and FC for the specific markers of the undifferentiated MSCs and renal tissue after induction of differentiation.

Statistical Analysis: It was done by using software package (SPSS PC, Chicago, IL) version 18.The difference between groups (More than 3 groups) were analyzed for statistical significance by one way ANOVA test. All tests were two tailed considered statistically significant when p value < 0.05.

Table 1: The 5 different growth factors cocktails (2 to 6) added to the 1st protocol containing only expansion media as control at the studied interval times

	Protocols					
Days	1	2	3	4	5	6
0 - 3	basal medium with no added growth factors.	basal medium + 10 ng /ml Retinoic a.	basal medium + 10 ng /ml Activin.	basal medium + 10 ng /ml Activin + 10 µg /ml EGF.	basal medium + 10 ng /ml Activin + 10 µg /ml EGF + 10 ng /ml Retinoic a.	basal medium + 10 ng /ml Activin + 10 ng /ml Retinoic a.
3 - 6		+ 10 ng /ml Activin	+ 10 ng /ml Retinoic a.	+ 10 ng /ml Retinoic a.	+ 10 µg /ml FGF + 10 ng /ml Retinoic a.	+ 10 ng /ml Retinoic a.
6 - 10		+ 50 ng /ml BMP-7.	+ 50 ng /ml BMP-7.	+ 50 ng /ml BMP-7.	+ 50 ng /ml BMP-7.	+ 50 ng /ml BMP-7.

RESULTS

The isolation of MSCs from WJ -UCs was successfully done as shown in Fig.1, where cells were stained with Leishman & Giemsa. MSCs were identified morphologically, by being spindle-shaped with large, round nucleus and prominent nucleolus. They adhere to tissue culture glass or plastic.

The cultured cells were identified by ICC staining and FC analysis for the specific markers of the undifferentiated MSCs (CD29) before the addition of growth factors cocktails as shown in Fig.2 and 3 respectively. Meanwhile, they were negative for CD 24 & CD133 (Renal cell progenitor's markers).

After expansion of MSCs while plated into 96 well-plates and growth factors cocktails were added in the form of 6 different protocols, yet the first one consisting of only basal expansion medium as control with no added growth, revealed negative expression of CD29 except using the 1st protocol, however varying and significant positive expression of CD24 and CD133 by ICC, that indicated the varying ability of the added nephrogenic growth factors cocktails to induce differentiation of MSCs into renal cells, results shown in Table (2). Positive cells for CD29, CD24 and CD133 by ICC staining showed cytoplasmic staining with brown color as shown in Fig. 2.

Comparing obtained results after addition the studied cocktails showed that the CD24 and CD133 expressions in protocol 6, were the highest statistically significant compared to other studied protocols as shown in table (2).

From the current study, we noticed that protocols 5 & 6 are giving high positive results in the expression of CD24 & CD133 by ICC, in relation to all used differentiation cocktails (Table 3), also protocol 6 shows positive expression of CD24 & CD133 by FC as shown in Table 4. Both protocols are characterized by the combination of Retinoic acid and Activin-A growth factors at the beginning of differentiation procedure followed by addition of BMP-7.

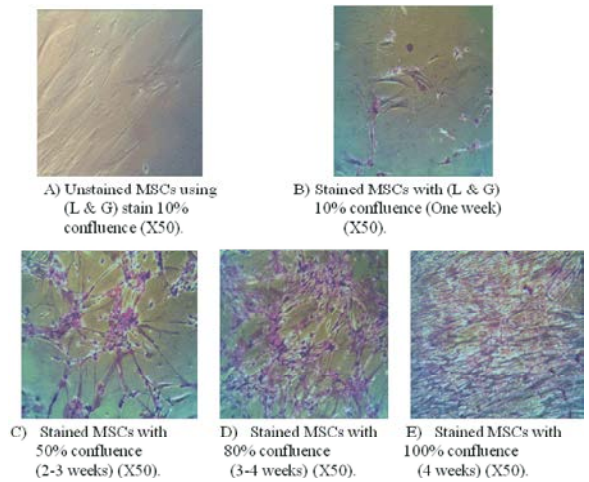


Fig. 1: Undifferentiated MSCs, stained with Leishman & Giemsa (L & G) stain at different culture interval times

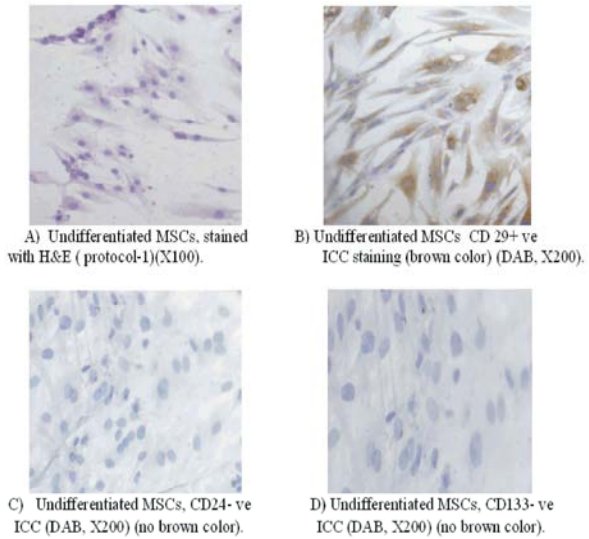


Fig. 2: (A) Undifferentiated MSCs, stained with H&E (protocol-1) X100 , (B) typical ICC staining CD 29 +ve, (C) &(D) –ve for CD24 & CD133, respectively.

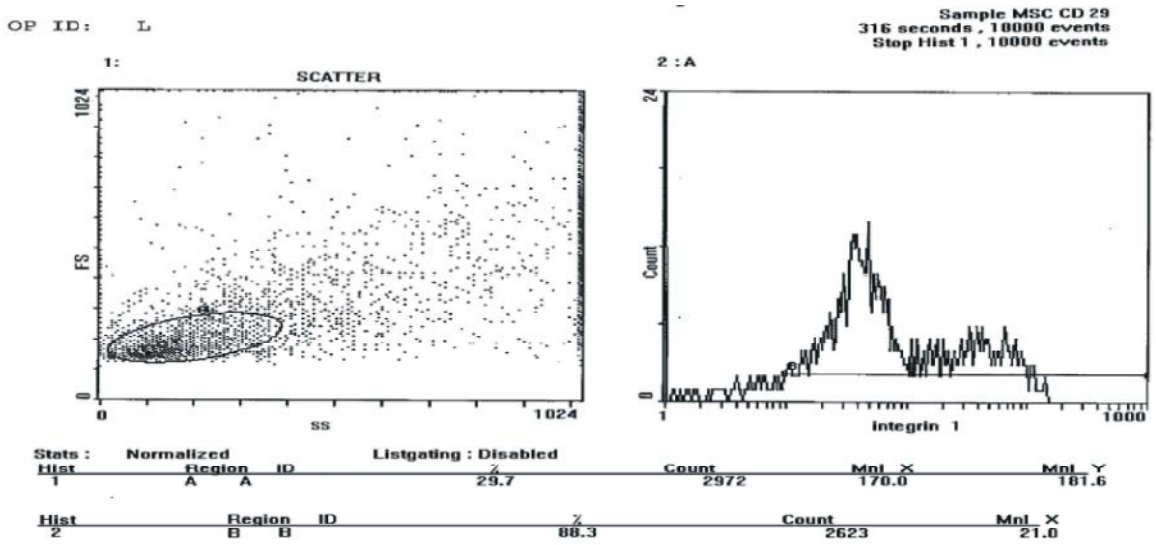


Fig. 3: Flow cytometric chart for CD29 (Undifferentiated MSCs surface marker), showing expression of CD29 in 88.3 %

Table 2: Statistical comparison of results of CD29, CD24 and CD133 identified by ICC after MSCs expansion and differentiation using the 6 protocols

	CDs		
Protocols	CD29	CD24	CD133
Protocol 1	73.5±2.4	0	0
Protocol 2	0	5.4±0.9	5.4±0.8
Protocol 3	0	6.1±1.3	9.1±2.2[a]
Protocol 4	0	9.3±1.6[a,c]	9.6±2.6[b]
Protocol 5	0	18.7±1.8[b,d,e]	20.7±3.4[b,d,e]
Protocol 6	0	76.1±5.2[b,d,e,f]	72.8±3.9[b,d,e,f]

Data are presented as: Mean ± Standard deviation where p value < 0. 05 is considered statistically significant.

a $p < 0.05$, b $p < 0.01$ compared to protocol 2.

c $p < 0.05$, d $p < 0.01$ compared to protocol 3.

e $p < 0.01$ compared to protocol 4.

f $p < 0.01$ compared to protocol 5.

Table 3: Comparison of results of CD24 and CD133 identified by ICC after MSCs expansion and differentiation using 5th & 6th protocols

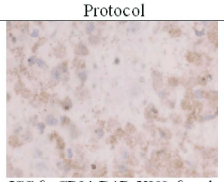
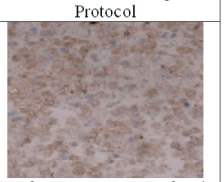
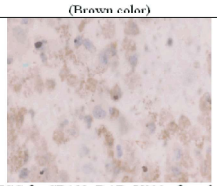
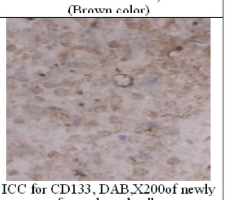
Cell Markers	ICC results after using 5 th Protocol	ICC results after using 6 th Protocol
CD 24	 ICC for CD24, DAB, X200of newly formed renal cells (Brown color)	 ICC for CD24, DAB, X200 of newly formed renal cells) (Brown color)
CD 133	 ICC for CD133, DAB, X200 of newly formed renal cells (Brown color)	 ICC for CD133, DAB,X200of newly formed renal cells (Brown color)

Table 4: Comparison of FC results between the usage of protocols 1 versus 6

	Protocol	
CD	1	6
CD29 (MSCs)	88.3%	0%
CD24 (Renal stem cells)	0%	81.2%
CD133 (Renal stem cells)	0%	81.0%

DISCUSSION

Kidney injury carries severe consequences and has limited treatment options [11]. Considerable morbidity from long-term dialysis treatment and the ever increasing organ transplant waiting lists, operative risks, infection, using of immunosuppressant drugs, complications of general anesthesia and post transplantation organ rejection indicated clearly a need for new renal

replacement therapies [12]. These patients are traditionally treated by either peritoneal or hemodialysis which expose the patients to many risks as blood born infections like HBV, HCV and HIV.

In the last few years, reports of bone marrow cell conversion into various types of renal cells have emerged. MSCs have also been shown to provide renal protection by paracrine effects. The diverse developmental potential of bone marrow stem cells as well as the easy access to Haematopoietic stem cells (HSCs) and expandable nature of MSCs in cultures opens the possibility of stem cell-based therapy for kidney diseases [11].

Umbilical cord is promising to serve as the first choice for harvesting MSCs owing to its wide source and no ethical concerns [13]. WJ-MSCs preserve renal and hepatic function [14].

In the present study MSCs were isolated by culturing of small pieces of the UCs in culture flasks and subsequently special expansion medium was added. According to Troyer and Weiss [15] cells can be isolated either by enzymatic digestion of umbilical cord tissue or by culture expansion of small pieces of tissue, known as the explants method.

The present work showed positive expression of CD 29 marker of the un-differentiated MSCs using ICC staining and FC after expansion. CD29 is a specific surface marker for the MSCs in addition to their homogenous fibroblast like morphology. This finding is in agreement with those of Koliakos *et al*. [5] and Dominici *et al*. [16]. They isolated the MSCs and cell surface marker expression was determined by FC and found that cells consistently expressed CD29 in addition to CD105 and CD90 at high percentages. The expression of these markers is one criterion for defining multipotent mesenchymal stromal cells.

Obtained Data from this study revealed growth of cells expressing CD24 and CD133 surface markers from differentiation of MSCs derived from WJ of the UC in growth factors cocktails that were used in the trial to differentiate MSCs into any type of renal cells or tissues.

In the current work CD24, which is a marker of renal progenitor stem cells was detected by ICC staining and FC analysis of cells present in cultures containing growth factors cocktails. This result was proved by Challen *et al*. [17] who demonstrated CD24 as a character of the molecular phenotype of renal progenitor cells in the developing mouse kidneys.

Other studies also confirmed the same result [18, 19] identified multipotent progenitors and/or SCs in adult human kidney. They reported the presence of CD24, a surface molecule that has been used to identify different types of human SC and also is expressed by uninduced metanephric mesenchyme during renal embryogenesis. Immunostaining of human fetal kidney showed widespread expression and localized CD24 to mature tubules (Renal stroma was devoid of CD24) [20]. The authors documented that CD24 surface marker is specific for human renal progenitor stem cells.

In the current study the expanded MSCs that were cultured in the presence of the nephrogenic growth factors cocktails differentiated to renal progenitor cells expressing CD133. The results demonstrated weak expression of CD133 (20.7±3.4) by ICC staining in protocol 5 compared to protocol 6 which showed expression of CD133 surface marker in (72.8±3.9) by ICC staining. This may be explained by using of FGF as one of growth factors in the 5th differentiation cocktail in addition to RA and Activin combination. As some authors examined the ability of CD133+ve cells for epithelial differentiation, cells were grown in the presence of FGF. After 10 days of culture, cells lost CD133 expression [21].

It was documented that among adult organs, the kidney had expressed large numbers of CD133 +ve cells [22].

Coincided with the present data, other authors reported that renal progenitor cells were obtained from the normal portion of cortex obtained from surgically removed kidneys. After dissection and passage through a graded series of meshes, CD133+ cells were isolated from the tubular fraction by magnetic cell sorting, using the MACS system (Miltenyi Biotec, Auburn, CA) [21].

Results of the present work are supported by the study concerned with analysis of cell surface marker expression in the human adult kidney using FC revealed increased expression levels for CD105, CD90, CD133 and CD24. Moreover, CD24+ve CD133 +ve cells represent a large cell fraction in the human adult kidney, comprising 64.26 ±10.15 % of the total cells [23].

Data obtained from the present study regarding differentiation of WJ MSCs into renal progenitors under certain culture conditions are confirmed by several studies. It was found that CD24 and CD133 surface markers were co-expressed by a subset of parietal epithelial cells (PEC) in the Bowman's capsule of the adult normal kidney. Once isolated, CD24+ve, CD133+ve PEC were found to lack lineage-specific markers; to express transcription factors Oct-4 and BmI-1, that are characteristics of multipotent stem cells and to exhibit self-renewal, high clonogenic efficiency and multi-differentiation potential. When CD24+ve, CD133+ve PEC

were injected intravenously in SCID mice that had ARF, they regenerated tubular structures in different portions of the nephron and also reduced the morphologic and functional kidney damage [24].

It was also demonstrated that CD133+, CD24+ and CD106+ cells were localized at the urinary pole of Bowman's capsule, they exhibited a high proliferative rate and could differentiate toward the podocyte as well as the tubular lineage. Also they showed higher resistance to injurious agents in comparison to all other differentiated cells of the kidney. Once they injected in SCID mice affected by acute tubular injury, they displayed the capacity to engraft within the kidney, generate novel tubular cells and improve renal function. These properties were not shared by other tubular cells of the adult kidney. The data suggest that CD133+ & CD24+ cells represent tubular-committed progenitors that display resistance to apoptotic stimuli and exert regenerative potential for injured tubular tissue [25].

The findings of the present study revealed that the growth factors cocktails number 5 and 6 gave rise to high positive results among all added differentiation cocktails in the form of positive expression of CD24 and CD133 which are surface markers of renal progenitor stem cells. Both cocktails contained Activin and retinoic Acid (RA) in combination from the beginning. Previous experiments revealed that the addition of RA and Activin in combination generated the highest levels of Pax2 protein gene which is one of the earliest markers of the intermediate mesoderm, from which the renal epithelial cells arise [26]. These results were also found by Ren *et al*. [27] who reported that the combination of RA and Activin effectively induced the expression of the intermediate mesodermal markers, Pax2, in embryonic bodies (EBs).

Also the current results are in consistent with the finding of Bruce *et al*. [28] and Morizane *et al*. [29] who found that both RA and Activin can induce pronephric markers.

Other growth factors cocktails were tried by other researchers. Embryonic stem (ES cells) can differentiate into renal lineage via embryonic bodies (EBs) in-vitro by Activin, RA and BMP7, when added in combination to mouse embryonic stem cell culture elevate Pax2 and WT-1 gene expression were detected. Pax2 and WT-1 are the earliest markers of the intermediate mesoderm, from which the renal epithelial cells arise and improve embryonic renal tubule cell integration compared with undifferentiated mouse ESs [26, 28, 30].

The present study revealed successful generation of renal progenitor stem cells having CD24 +ve and CD133 +ve surface markers from differentiation of MSCs derived from WJ of the umbilical cord which is an easy accessible source with no ethical problems. These results together with those previous studies constitute important implications for the development of regenerative medicine in patients suffering from renal diseases.

CONCLUSIONS

To sum up, WJ of the UC is an easy accessible source for MSCs isolation. Undifferentiated MSCs that were isolated from WJ of the UC had the ability to produce primitive renal cells depending on growth factors cocktails that were added. These primitive renal cells may be further utilized by infusion or injection into patients with acute or chronic renal diseases for possible improvement of their condition, this improvement can be followed up by monitoring the patients clinical signs and symptoms in addition to their renal function tests.

Conflict of Interest: The authors have not disclosed any potential conflicts of interest.

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