ORIGINAL RESEARCH PAPER

Obstetrics and Gynaecology

CHARACTERIZATION OF AMNIOTIC, UMBILICAL CORD, ADIPOSE AND BONE MARROW MESENCHYMAL STROMAL CELLS AND A COMPARISON

KEY WORDS: mesenchymal stromal cells, characterization, isolation

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ABSTRACT

Four types of mesenchymal stromal cells: amniotic, umbilical cord, adipose and bone marrow were isolated and characterized. The cell yield per gram tissue is the lowest with umbilical cord. Bone marrow cells showed a higher expression of SSEA-4 cell surface marker and for umbilical cord cells CD164, a marker for homing. All 4 types of MSCs expressed the pluripotency genes, Sox-2, Oct-4 and Nanog. However, both umbilical cord and adipose cells showed a lesser expression of Sox-2 and Rex-1. All 4 types of MSCs showed the capability of in-vitro differentiation and colony formation on soft agar assay.

Introduction

Mesenchymal stromal cells (MSCs) were initially identified as a subgroup of bone marrow cells with osteogenic potential. (Friedenstein, Petrakova, Kurolesova, & Frolova, 1968) It was later noted that these cells possessed other properties including plastic adherent and capability to differentiate into mesodermal(Caplan, 1991) and other cell lineages(Pittinger et al., 1999). MSCs has since been applied in various areas of clinical medicine.(Abdallah & Kassem, 2008)

MSCs have been isolated from a variety of human sources, including but not limited to bone marrow, placenta(Parolini et al., 2008), dental pulp(Alipour et al., 2010), amniotic fluid(Zheng et al., 2008), umbilical cord tissue(Penolazzi et al., 2009), umbilical cord blood(Cicuttini, Welch, & Boyd, 1994; Flynn, Barry, & O'Brien, 2007), adipose tissue(Baglioni et al., 2009), etc.(Keating, 2012). Bone marrow MSCs have been studied and used therapeutically for the longest time. However, donors are required for a continuous supply. Amniotic, umbilical cord and adipose MSCs have been studied and used widely recently, because of the readily availability and relatively unlimited sources. Amniotic and umbilical cord MSCs are fetal in origin, closer to embryonic stem cells and yet devoid of the ethical issues associated with the latter. However, differences in characteristics have been reported with different studies utilizing various types of MSCs. The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy had therefore proposed minimal criteria to define human MSC. The criteria are firstly, plastic adherent property in standard culture conditions; Secondly, the expression (≥95%) of CD105, CD73 and CD90, and lacking (≤2%) CD45, CD34, CD14 or CD11b, CD79□, or CD19 and HLA-DR surface molecules; and thirdly, capability of in-vitro differentiation to osteoblasts, adipocytes and chondroblasts.(Dominici et al., 2006)

In this study, we characterize and compare human amniotic, umbilical cord, adipose and bone marrow MSCs using the same culture and experimental conditions.

Methods and material:

A) Isolation and growth

The amniotic membrane, umbilical cord and subcutaneous adipose tissue were obtained from pregnant women undergoing elective Caesarean sections with ethics approval from the Institutional Review Board of the University of Hong Kong / Hospital Authority Hong Kong West Cluster (HKU/HA HKW IRB). The tissues were washed thoroughly with phosphate buffered saline (PBS) and dissected free of contamination from other tissues as far as possible (including umbilical vessels and the amniotic covering in the case of umbilical cord, and overlying epidermal and dermal tissues and intermingling blood vessels in the case of subcutaneous adipose tissue). The dissected tissues were weighed, divided into fine pieces and grown in xeno-free Stempro culture medium supplemented with 2.5% human platelet lysate and 1% glutamine (Thermo Fisher Scientific U.S.A.). Bone marrow samples were retrieved from stored frozen samples collected from donors. For each type of cells, at least 3 samples were collected. The cells were incubated at 37°C in 5% carbon dioxide (CO_2). The cells were passaged at 70-80% confluency with TrypLE Select (Thermo Fisher Scientific, U.S.A.). Cells in passage 1 to passage 4 were used for characterization, and up to Passage 9 for cell morphology (Axiovert 25, Zeiss, Germany).

B) In-vitro differentiation

Amniotic, umbilical cord, adipose and bone marrow MSCs were seeded onto 6 wells plates at a density of 2×10^3 per cm^2 in complete Stempro medium. The culture medium was changed to commercially available complete adipogenic or osteogenic culture medium (Thermofisher Scientific, U.S.A.) on the following day and subsequently every 2-3 days. The cells were fixed on Day 14 and stained with Oil Red O for intracytoplasmic lipid granules in adipogenesis and Alizarin Red for calcium deposition in osteogenesis respectively (Merck Millipore, Germany). For chondrogenesis, 2.5×10^5 respective cells were pelleted and cultured in complete chondrogenic culture medium (Thermo Fisher Scientific, U.S.A.). The cell pellets were collected after 2-3 weeks, sectioned and the cartilage formed was stained with Alcian Blue (Merck Millipore, Germany).

C) Immunophenotyping

Amniotic, umbilical cord, adipose and bone marrow MSCs were cultured in complete Stempro culture medium prior to analysis. The cells were labelled with the following anti-human antibodies: CD14, CD19, CD34, CD38, CD44, CD45, CD73, CD135, CD164, SSEA-4 (BD, Becton, Dickinson and Company, U.S.A.), CD90, CD105 and HLA-DR (Thermo Fisher Scientific). At least 5×10^5 respective cells were analysed for cell surface markers with Attune

flow cytometer (Thermo Fisher Scientific), using mouse isotypes antibodies (BD, Becton, Dickinson and Company, U.S.A.) for control.

D) RNA isolation and gene expression

The amniotic, umbilical cord, adipose and bone marrow MSCs at Passage 3 were grown to 80% confluency and at least 5 x 10^5 cells were harvested respectively. RNA was isolated with TRIzol and PureLink RNA Mini Kit, and purified with On-column PureLink DAase treatment according to the manufacturer's protocol (Thermo Fisher Scientific, U.S.A.).

500 ng RNA of each respective cell type was reverse transcribed and amplified with primers for GAPDH, Sox-2, Oct-4, Rex-1, FGF-4, and Nanog (GeneAmp PCR System 9800, Thermo Fisher Scientific, U.S.A.), followed by electrophoresis with 2% Agarose E-Gel (Thermo Fisher Scientific, U.S.A.). The conditions for polymerase chain reaction (PCR) was based on the paper by Kita et.al. (2010)(Kita, Gauglitz, Phan, Herndon, & Jeschke, 2010)

E) Tumorigenicity assessment with soft agar assay

The methodology was adopted and modified from the protocol published by Borowicz et,al. (2014).(Borowicz et al., 2014) An equal volume of respective mesenchymal stem cells in complete Stempro culture medium and 0.6% Agar solution were mixed (giving a final concentration of 5000 cells per well) and layered on top of a layer of equal volume of 1% Agar solution and 2x DMEM cell culture medium (Thermo Fisher Scientific, U.S.A.), and triplicated in a 6-well plate. The plate was then incubated at 37°C in 5% CO_2 for 21 days. The cells were then stained in the plate with 0.1% Crystal violet solution. The appearance of colonies were viewed under inverted microscope (Axiovert 25, Zeiss). A Z-stack image was taken from each well at 20µm interval with EVOS FL Auto (Thermo Fisher Scientific, U.S.A.), and analyzed with Image J software (by filtering off the background till only the stellate colonies remaining) for the total number, the total area and the average size of and the percentage of area being occupied by colonies.

Statistical analysis

Statistical analysis was performed with Statistical Package for the Social Sciences version 21.0 (SPSS Inc., Chicago, IL, USA). A two-sided probability (p) value of <0.05 was considered statistically significant.

Results:

A) Isolation and growth

The cell yield per gram (g) of dissected amniotic, umbilical cord and adipose tissue was calculated and shown in Fig. 1 and charted in Table 1. As no raw bone marrow sample was received during the study period, the cell yield of amniotic and umbilical cord was compared to adipose tissue. For umbilical cord, the mean cell yield appeared to be only half of that of adipose (p = 0.6), and one-fifth of that of amniotic tissue (p=0.07), although the difference did not reach statistical significance probably due to the sample number (Table 1).

Fig. 1: The cell yield per gram of dissected amniotic, umbilical cord and adipose tissue

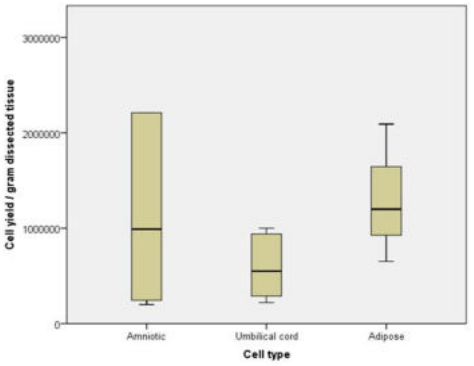


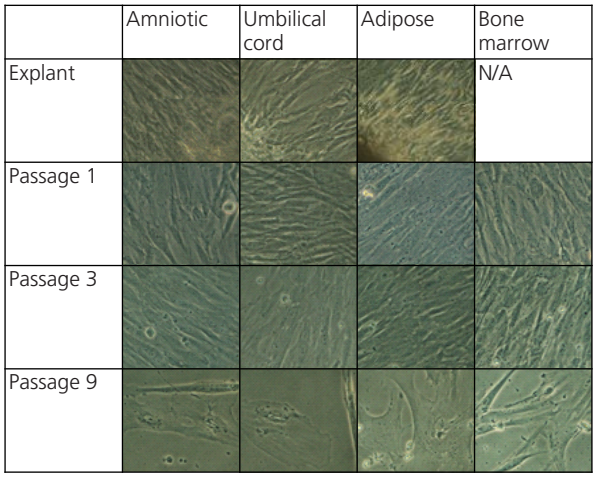
Table 1: The mean cell yield per gram of dissected amniotic and umbilical cord compared to adipose tissue

Cell type	Mean cell yield per gram of dissected tissue ± S.D.	p value†
Amniotic	2.5 x 10^6 ± 3.6x 10^6	0.6
Umbilical cord	5.9 x 10^5 ± 3.2 x 10^5	0.07
Adipose	1.3 x 10^6 ± 0.7 x 10^6	1

S.D., standard deviation; †, independent sample student t test, 2-tailed; p, probability, equal variance assumed; *, statistical significant, p < 0.05

The cell morphology from the explant, Passage 1, 3 and 9 respectively are shown in Fig. 2. The appearance of the cells appeared to be similar for the 4 types of MSCs, being spindle in shape. The cell proliferation slowed down with later passages and the cells appeared scanty and bizarre at passage 9.

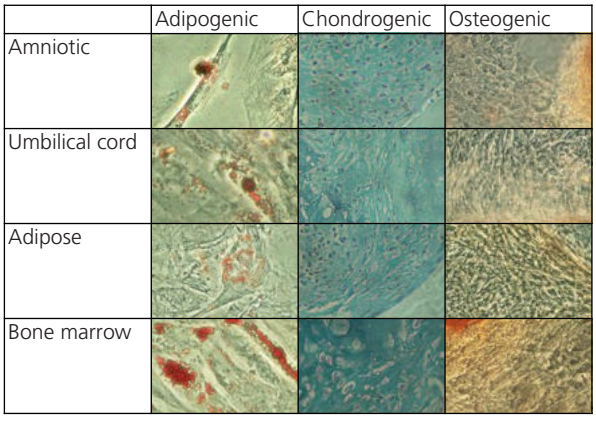
Fig. 2: The microscopic appearance of the amniotic, umbilical cord, adipose and bone marrow MSCs in culture



B) In-vitro differentiation

All 4 types of MSCs showed in-vitro differentiation under the adipogenic, osteogenic and chondrogenic conditions, confirming multipotency (Fig. 3).

Fig. 3: Multipotency of amniotic, umbilical cord, adipose and bone marrow MSCs: in-vitro differentiation towards adipogenic, chondrogenic and osteogenic lineages.

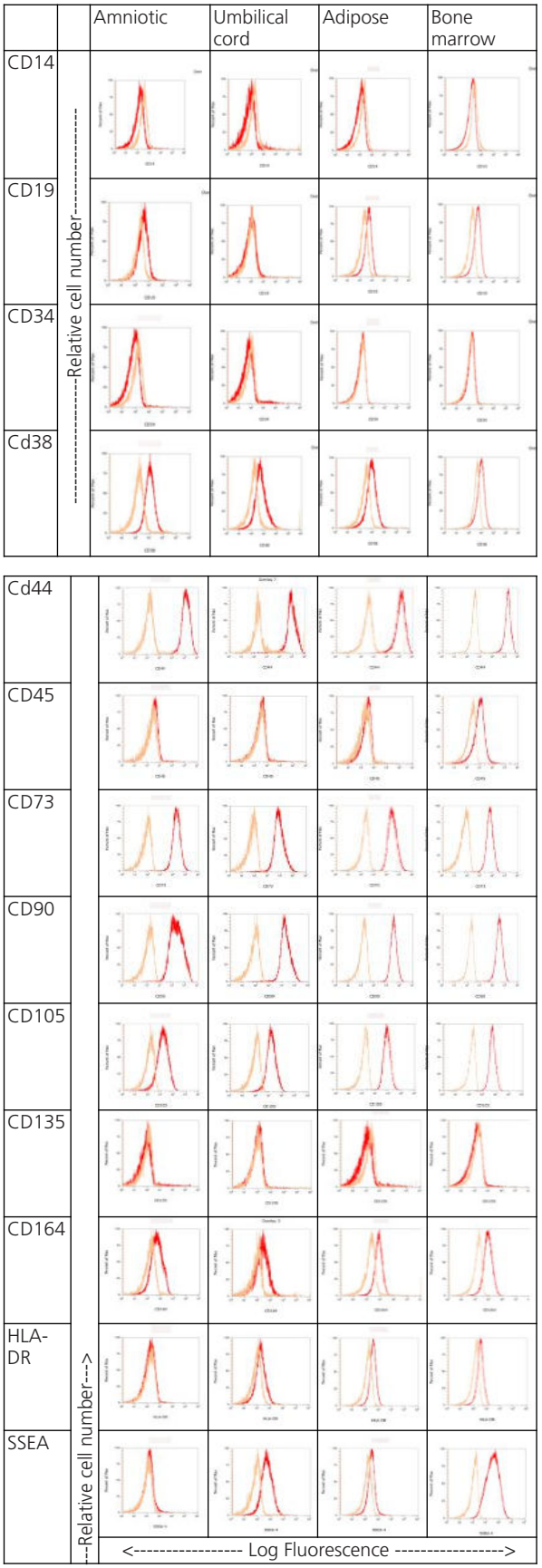


The intracytoplasmic lipid granules were stained red with Oil Red O. The calcium mineral deposition on osteogenesis were stained brownish-red with Alizarin Red, and the cartilage formed on chondrogenesis were stained blue with Alcian Blue.

C) Immunophenotyping

The cell surface markers expressed by the 4 types of MSCs were presented in Fig. 4 and Table 2.

Fig. 4: Cell surface markers of amniotic, umbilical cord, adipose and bone marrow MSCs demonstrated on flow cytometry



Beige, isotype control; Red, conjugated with antibodies

Table 2: A comparison of percentage of cells expressing surface markers in amniotic, umbilical cord and adipose MSCs against bone marrow MSCs

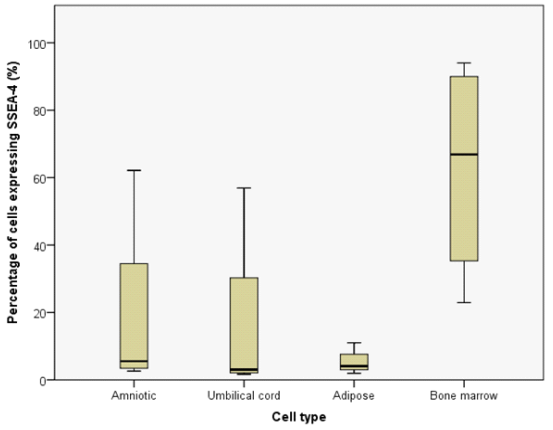
Cell surface markers	Percentage of cells expressing cell surface markers						
	Amniotic		Umbilical cord		Adipose		Bone marrow
	Mean ± S.D.	p†	Mean ± S.D.	p†	Mean ± S.D.	p†	Mean ± S.D.
CD14	0.7 ± 0.7	0.2	0.8 ± 0.4	0.02*	0.3 ± 0.3	0.3	0.1 ± 0.2
CD19	5.5 ± 9.0	0.3	32.5 ± 28.0	0.4	3.7 ± 3.4	0.2	16.7 ± 19.2
CD34	1.2 ± 1.3	0.4	0.7 ± 0.2	0.5	0.4 ± 0.3	0.4	0.6 ± 0.3
CD38	38.8 ± 17.3	0.6	64.0 ± 29.2	0.1	37.0 ± 16.8	0.7	30.8 ± 25.0
CD44	98.9 ± 1.0	0.2	99.6 ± 0.5	0.6	99.6 ± 0.3	0.5	99.7 ± 0.3
CD45	1.9 ± 1.0	0.4	2.1 ± 0.5	0.4	2.3 ± 1.9	0.4	5.6 ± 7.8
CD73	98.6 ± 1.9	0.3	99.5 ± 0.4	0.3	99.7 ± 0.3	1.0	99.7 ± 0.3
CD90	97.0 ± 4.7	0.4	99.5 ± 0.3	0.6	98.7 ± 1.4	0.6	99.2 ± 1.0
CD105	53.0 ± 34.7	0.2	93.3 ± 9.0	0.6	86.0 ± 22.2	1.0	85.4 ± 26.8
CD135	3.9 ± 6.0	0.8	0.7 ± 0.7	0.3	1.4 ± 1.1	0.4	3.0 ± 3.4
CD164	19.3 ± 13.5	0.8	52.3 ± 31.2	0.3	19.7 ± 14.9	0.9	24.0 ± 35.6
HLA-DR	5.8 ± 4.1	0.8	16.7 ± 14.7	0.3	3.9 ± 3.1	0.5	6.9 ± 7.4
SSEA-4	19.0 ± 28.8	0.09	16.2 ± 27.2	0.07	5.3 ± 3.9	0.01*	62.7 ± 33.3

S.D., standard deviation; p, probability; †, independent sample student t test, 2-tailed; *, statistical significance, equal variances assumed, with reference to bone marrow cells.

Overall, the 4 types of MSCs were similar in their expression of the surface markers. They were positive for CD90, CD105 and CD73 and expressing a low level of CD14, CD34 and Cd45.

The expression of SSEA-4 (stage-specific embryonic antigen) was statistically significantly higher in bone marrow MSCs compared to adipose (p=0.01), and there was a similar trend when compared to amniotic (p=0.09) and umbilical cord MSCs (p=0.07) (Table 2, Fig. 5).

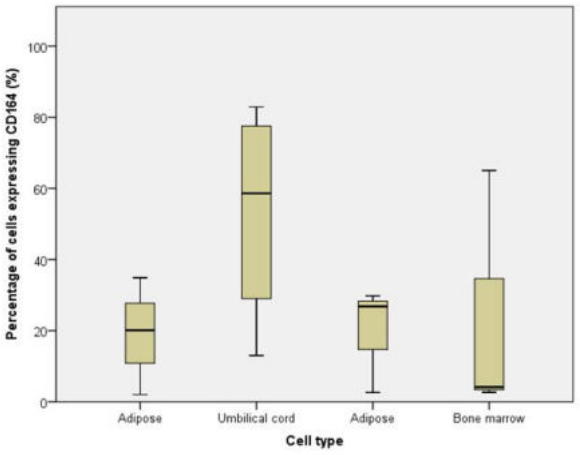
Fig. 5: The expression of SSEA-4 in amniotic, umbilical cord, adipose and bone marrow MSCs



Cd164 was expressed at a higher level in umbilical cord cells (mean = 52.3%) compared with amniotic, adipose and bone marrow cells (mean = 19.3, 19.7 and 24.0 respectively) although statistical

significance was not reached (p = 0.8, 0.9 and 0.3 respectively) (Table 2, Fig 6).

Fig. 6: The expression of CD164 in amniotic, umbilical cord, adipose and bone marrow MSCs



D) Gene expression

The primers and PCR conditions used were shown in Table 3.

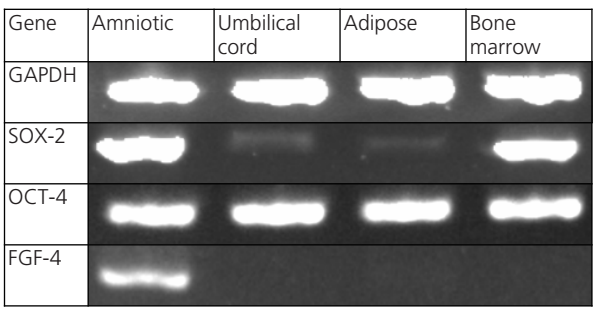
Table 3: The Primer pairs and conditions designed for PCR

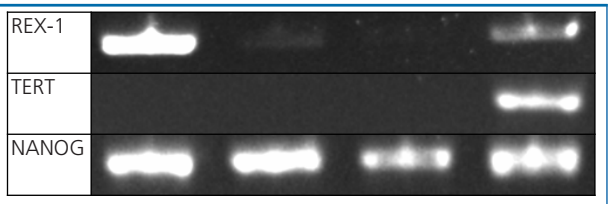
Primers	Primer sequence	PCR condition
GADPH-F	5'-GTCAGTGGTGGACCTGACCT-3'	35 cycles, 57oC, 255bp
GADPH-R	5'-CACCACCCTGTTGCTGTAGC-3'	
Sox-2-F	5'-GCCGAGTGGAAACTTTTGTC-3'	35 cycles, 57oC, 264bp
Sox-2-R	5'-GTTCATGTGCGCGTAACTGT-3'	
Oct-4-F	5'-GAGGAGTCCCAGGACATCAA-3'	45 cycles, 57oC, 151bp
Oct-4-R	5'-GTCGTTTGGCTGAATACCTT-3'	
Rex-1-F	5'-CAGATCCTAAACAGCTCGCA GAAT-3'	45 cycles, 57oC, 306bp
Rex-1-R	5'-GCGTACGCAAATTAAAGTC CAGA-3'	
FGF-4-F	5'-CTACAACGCCTACGAGTCC TACA-3'	45 cycles, 57oC, 370bp
FGF-4-R	5'-GTTGCACCAGAAAAGTCAGA GTTG-3'	
TERT-F	5'-AGAGTGTCTGGAGCAAGTTGC-3'	45 cycles, 57oC, 185bp
TERT-R	5'-CGTAGTCCATGTTCACAATCG-3'	
Nanog-F	5'-AGAAGGCCTCAGCACCTAC-3'	35 cycles, 51oC, 205bp
Nanog-R	5'-GGCCTGATTGTTCCAGGATT-3'	

RT, real time; PCR, polymerase chain reaction; F, forward; R, reverse

The results of electrophoresis were shown in Fig. 7.

Fig. 7: Gene expression of amniotic, umbilical cord, adipose and bone marrow MSCs at Passage 3





The umbilical cord and adipose MSCs showed a low level of Sox-2 and Rex-1 (Fig. 7, Table 4).

Among the 4 types of MSCs, the expression of TERT could only be demonstrated in bone marrow MSCs, and FGF-4 only in amniotic MSCs.

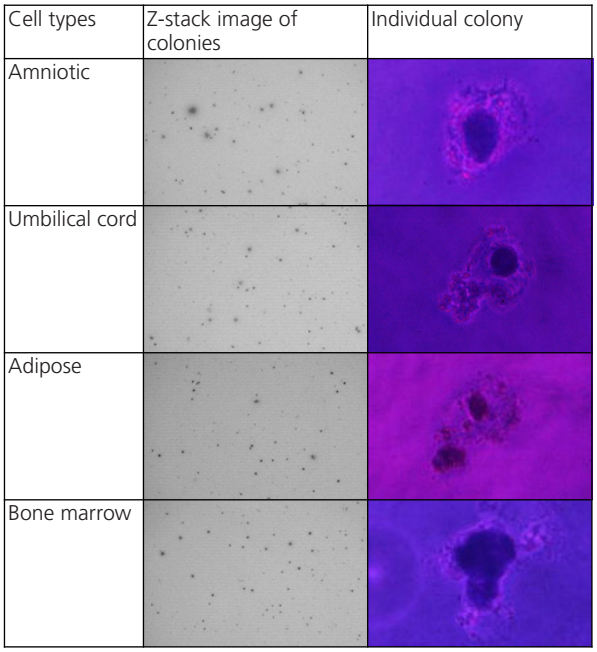
Table 4: Summary of gene expression of amniotic, umbilical cord, adipose and bone marrow MSCs

	SOX-2	OCT-4	REX-1	FGF-4	TERT	NANOG
Amniotic	++	++	++	++	-	++
Umbilical cord	+	++	+	-	-	++
Adipose	+	++	±	-	-	++
Bone marrow	++	++	+	+	++	++

5) Tumorigenicity assessment with soft agar assay

The appearance of the colonies of 4 types of MSCs in soft agar assay were shown in Fig. 8 and an analysis of their colonies in terms of the total number, the total area, the average size and the percentage of area being occupied by colonies was shown in Table 5. All 4 types of MSCs demonstrated the ability to form colonies in soft agar assay and no statistically significant difference in the properties investigated.

Fig 8: The appearance of the colonies of amniotic, umbilical cord, adipose and bone marrow MSCs on soft agar assay under phase contrast microscopy



Discussion:

In our study, the 4 types of MSCs share some common features in terms of cell morphology, in-vitro differentiation, flow cytometry, gene expression and tumorigenicity. However, there are some differences that may be noteworthy.

Umbilical cord MSCs have raised some interests in recent years due to the reported immunomodulatory effect.(Li et al., 2014) However, the isolation is labour intense and the yield is relatively poor compared with the other cell types as demonstrated in our study.

SSEA-4 (stage-specific embryonic antigen) is an early embryonic glycolipid antigen and has been commonly used as a marker for pluripotency. Its high level in bone marrow cells in the present study is consistent with other publication.(Gang, Bosnakovski, Figueiredo, Visser, & Perlingeiro, 2007) The percentage of MSCs expressing SSEA-4 was statistically significantly higher when compared to adipose cells, and a less marked trend was observed when compared to amniotic and umbilical cord MSCs. Whether this finding may have any clinical significance is not certain.

Cd164 is a marker for homing. Its high level in umbilical cord cells may have clinical implications although statistical significance has not been reached in this study.

In this study, the 4 types of MSCs showed gene expression for pluripotency, including Sox-2, Oct-4 and Nanog. However, the expression of Sox 2 and Rex-1 appeared to be less in umbilical cord and adipose MSCs (Fig. 7, Table 4). Rex-1 is a known marker for undifferentiated embryonic stem cells. It is known that Nanog transactivates Rex-1 and Sox-2 co-operates with Nanog in up-regulating Rex-1(Shi, Wang, G.;, Geng, & Pei, 2006). Therefore the low level of Rex-1 in umbilical cord and adipose MSCs may be related to the level of Sox-2 in these cells.

In this study, only bone marrow MSCs demonstrated the expression of TERT (telomerase reverse transcriptase). Telomerase plays an important role in cellular senescence and chromosomal repair. However, alternatively spliced variants encoding different isoforms of TERT are known to exist and the full length sequence of some variants has not yet been determined.(NCBI) It is therefore difficult to compare the results of gene expression for TERT.

The soft agar assay is a well-established semi-quantitative method for characterizing anchorage-independent growth, a hallmark of carcinogenesis.(Borowicz et al., 2014) All the 4 types of MSCs in this study showed colonies formation.

Conclusion:

Amniotic, umbilical cord, adipose and bone marrow MSCs satisfy the minimal criteria for multipotent mesenchymal stromal cells. There are certain differences in their characteristics, in terms of the cell surface markers and gene expression.

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