EVALUATION OF HUMAN UMBILICAL CORD BLOOD AS A SOURCE OF EMBRYONIC STEM CELLS

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Abstract

Human umbilical cord blood (HUCB) has been poorly characterised as a source of embryonic stem cells (ESCs). The aim of this study, therefore, was to evaluate HUCB as source of mesenchymal stem cells (MSCs) with embryonic characteristics. HUCB was collected from consenting women undergoing elective caesarean sections. HUCB was meticulously explanted into MesenCult media and incubated. Qualitative and quantitative immunophenotyping of cells was achieved using fluorescein isothiocyanate (FITC) labelled antibodies (CD34, CD45, CD29, CD44, CD73 and CD105) phenotypic markers. Immunocytochemistry was carried out for the human ESC markers CD9, stage-specific embryonic antigen-1 and 4 (SSEA-1 and SSEA-4), E-cadherin, Podocalyxin (PODXL), sex-determining region Y-box 2 (SOX2), NANOG and Octamer (OCT3/4). MSCs were cultured to induce differentiation into adipogenic, osteogenic, chondrogenic and neurogenic cells. Immunocytochemistry was used to identify fatty acid binding protein-4 (FABP-4), osteocalcin, aggrecan, SOX2 and oligodendrocyte-4 (Olig-4) markers. The cells were strongly positive for the MSC markers CD29, CD44, CD73 and CD105; these cells also expressed the ESC markers CD9, SSEA-1 and SSEA-4, E-cadherin, PODXL, SOX2, NANOG and OCT3/4. Additionally, the MSCs expressed the adipogenic FABP-4, osteogenic osteocalcin, chondrogenic aggrecan and neural Olig-4 and SOX2 markers after differentiation. Therefore, HUCB is a rich source for MSCs with embryonic characteristics.

Keywords: Human umbilical cord blood (HUCB), embryonic stem cells (ESCs), mesenchymal stem cells (MSCs), embryonic stem cell markers

Introduction

Human umbilical cord blood (HUCB) has been poorly characterised as a source of embryonic stem cells (ESCs). Allogeneic graft transplantation of Haematopoietic stem cells (HSCs) derived from HUCB has been successfully used in clinical practice for the last three decades. MSCs have been isolated from a variety of human tissues, e.g., bone marrow (BM) (Lee et al., 2004). Previous studies have reported the successful isolation of MSCs from the umbilical cord vein, blood and both mature and progenitor foetal cells were found in the umbilical vein blood (Malgieri et al., 2010). The annual rate of 100 million human births globally suggests that HUCB could be a large and readily accessible stem cell source that could be acquired ethically for research (McGuckin et al., 2005).

Currently, scientists have found evidence that MSCs are present in some embryonic and extra-embryonic tissues. The foetal BM, blood and liver have been analysed for the presence of MSCs. First trimester foetal BM and liver generated colony-forming unit fibroblasts (CFU-F) at similar frequencies. The CFU-F frequency in the first trimester blood was slightly lower, although the MSCs could not be isolated from the second or third trimester blood and demonstrated that these MSCs could differentiate into different cell lineages (Lee et al., 2004).

ESCs originate from embryos from the inner cell mass (ICM) of the human blastocyst in 4-5 day old embryos, which were created using *in vitro* fertilisation (Rogers et al., 2004). Within this time-scale, the dividing cells reach the 8/16-cell stage; thus, in theory, eight single cells can be isolated from the eight-cell embryo that are capable of further generating independent embryos if implanted in a woman's uterus at that stage (Borge, 2004). ESCs are derived from totipotent cells, in the early mammalian embryo, with unlimited proliferation *in vitro* (Evans et al., 1981). ESCs are pluripotent, i.e., they can differentiate into all cell types, including all three foetal germ layers (Tuch, 2006). Murine ESCs were first isolated in 1981, and human ESCs were isolated in 1998 (Evans et al., 1981; Thomson & Marshall, 1998). Both of these cell types preserve their self-renewal and potential for multilineage differentiation (Austin, 2001).

A number of human ESCs markers have been well defined. These markers are expressed in undifferentiated human ESCs, and their expression is downgraded after differentiation. Some genes and cell surface protein markers have been selected as potential universal stem cell markers for determining ‘stemness’. The surface markers that are used to define ESCs and the level of stemness in a given cell population are: CD90, which is found on thymocytes,

and CD34+ from prothymocytes (Cai et al., 2004); the zinc-finger protein Rex1 (zfp42); teratocarcinoma-derived growth factor 1 (TDGF1); SSEA-1, SSEA-3 and SSEA-4; lacto-series glycolipid antigen (Shevinsky et al.,1982); tumour rejection antigen (TRA-1-60, TRA1-81) (Thomson & Marshall, 1998; Scotland et al., 2009); and PODXL-like protein (Kerosuo et al, 2004). Other markers include the CD9 transmembrane-4 superfamily proteins, which are characterised by the presence of four hydrophobic domains and play an integral role in sperm egg fusion (Kaji et al., 2002), and the cadherin transmembrane-1 family proteins, which are necessary for cell adhesion and cell signalling (Bryan & Stow, 2004).

In addition, three transcription factors are used to identify the ESCs. The expression of OCT-3/4 is critical to maintain embryonic-like stem cell self-renewal and pluripotency through an octamer motif containing a cis-element responsible for gene transcription (Ellisd et al, 2004; McGuckin et al, 2005). NANOG is expressed in the ICM of the blastocyst, morula and ESCs. SOX2 is a transcription factor expressed in the ICM and is involved in developing the central nervous system (Brivanlo et al., 2003; Mitsui et al., 2003; Eckfeldt et al., 2005; Ulloa-Montoya et al., 2005).

Schulz *et al.* (2007) identified proteins expressed by human ESCs while conducting a large-scale proteomic analysis of human ESCs using Kinexus and PowerBlot assays and subgrouped these proteins into eighteen categories based on their function. These protein molecules could potentially be beneficial for cell sorting procedures, with receptor expression leading to data on the self-renewal or differentiation of human ESCs through the identification of growth factors or bioactive peptides (Schulz et al., 2007). Human ESCs can become differentiated without limits and have been used in transplantation medicine, developmental biology and drug trials (Thomson & Marshall, 1998; Mitsui et al., 2003).

Materıals And Methods

A research laboratory safety protocol was followed. To avoid contamination of the cells, the culture work was conducted within a Class II Microbiological Safety Cabinet, and sterile conditions were maintained. All used items were correctly discarded in accordance with the laboratory safety protocol. The Derbyshire Research Committee provided ethical approval for this study.

Collection of Umbilical Cord Blood

HUCB samples were collected from healthy women undergoing elective caesarean section at the maternity units of Lincoln County and Pilgrim hospitals (UK). Signed informed consent forms were obtained from all participants. Midwives collected specimens from the detached umbilical cord after the delivery and separation of the baby. An *in utero* collection method,

prior to placental delivery, was used. Once the collection was complete, the blood was transferred to plain tubes.

Isolation of Cord Blood Cells (Clot Spot) Primary Culture

HUCB clot spot was meticulously explanted into MesenCult complete media and incubated in a 5% CO_2 incubator at 37°C (Hussain et al., 2012).

Identification of MSCs

Immunofluoresence for MSCs markers

Cytospin preparations were produced (Thermo Shandon, Runcorn) by centrifugation at 114 g for 5 minutes. The adherent cells on the slides were then fixed with 0.3% H_2O_2 in 99.7% methanol (Sigma-Aldrich, Gillingham) for 10 minutes. The slides were incubated with primary monoclonal antibodies (MoAs) against human antigen CD34, CD45 (Novocastra, Milton Keynes, UK), CD29, CD44 (Caltag Medsystems, Claydon, UK) and CD105 (Immunostep, Salamanca, Spain) for 30 minutes, followed by washing with phosphate buffered saline (PBS). The cells were then incubated with fluorescein-polyclonal rabbit anti-mouse IgG secondary antibody (1:300) for 30 minutes in the dark. After incubation, the slides were washed with PBS, and the cover slips were applied and examined under the fluorescence microscope (Nikon ECLIPSE E800, Digital Camera DN100, UK)

Immunostaining (CD9, SSEA-1, SSEA-4, E-Cadherin PODXL, SOX2, NANOG and OCT3/4) of cells

Following the 1st passage, HUCB MSCs were seeded and cultured in 24-well plates. When the cells reached approximately 60% confluence, the cells were fixed in 0.5 mL of 4% paraformaldehyde in PBS for 20 minutes at room temperature and were incubated in permeabilisation and blocking buffer (0.5 mL of 0.3% Triton X-100 (Sigma, UK)), 1% bovine serum albumin (BSA), and 10% of the appropriate serum at room temperature for 45 minutes. The primary antibodies were diluted (1:50) in PBS containing 1% BSA and 10% of the appropriate serum. The cells were then incubated overnight at 2-8°C in 300 µL/wells and subsequently washed three times for 5 minutes with PBS containing 1% BSA. The secondary antibodies were diluted (1:100) in PBS and 2% serum (the same serum used for preparing the blocking solution) and added to the cells at 300 µl/well in the dark for 60 minutes at room temperature. Ten microlitres of mounting medium (Calibochem, UK) was placed on a clean glass cover slip, and then the immunostaining was visualised under a fluorescence microscope with appropriate filters for each fluorophore used.

Immunophenotyping Cell Surface Proteins (CD34, CD45, CD29, CD44 CD73 CD105, CD9, SSEA-1, SSEA-4)

To detect the expression of the CDs (except for CD73; R&D system, UK), primary FITC-labelled MoAs were used to immunophenotype the cells. The CD73 surface marker was assessed using phycoerythrin (PE)-conjugated

MoA (eBioscience, San Diego, USA). For analysis, the cells were trypsinised, resuspended in ice cold PBS containing 2% foetal bovine serum (FBS), pelleted in 5 ml polystyrene round-bottom tubes and dissociated into single cell suspensions. Approximately 50 $x10^3$ cells/ml were transferred into a 5 ml tube, and the cells washed in 2 ml of 3% BSA/PBS followed by the addition of 10 µl of primary antibody (FITC-labelled) to the cell suspensions and incubation for 30 minutes at 2-8ºC. Following incubation, the samples were washed twice in 3 ml of fluorescence activated cell sorting (FACS) buffer and centrifuged for 3 minutes at 800 g. The supernatants were removed, and the cells were resuspended in 400 µl of FACS buffer for analysis using flow cytometry.

Adipogenic Differentiation

For adipogenic differentiation, MSCs at passage 3 were plated in 24-well plates (1.76 cm^2) at 3.7 x 10^4 cells/well, cultured in complete MesenCult media and incubated overnight in a 37 °C and 5% CO2 incubator. When the cells reached confluence, the complete MesenCult medium was replaced with 0.5 ml of adipogenic differentiation medium in each well. The medium was changed carefully every 3 days. The cells were examined for differentiation from 7 to 14 days.

Osteogenesis Differentiation

MSCs at the 3rd passage were plated in 24-well plates (1.76 cm^2) at a concentration of 7.4 x 10^3 cells/well and cultured in complete MesenCult media overnight at 37°C in a 5% CO2 and 95% air culture incubator. When the cells reached 50-70% confluence, the complete MesenCult medium was replaced with 0.5 ml of osteogenic differentiation media in each well. The media were changed every three days, and cells were examined for differentiation from 14 - 21 days.

Chondrogenic Differentiation

MSCs from cord blood at the 3rd Passage (250 $x10^3$ cells/ml) were transferred in their existing medium (complete MesenCult medium) to 15 ml conical tubes. The cells were centrifuged at 200 g for 5 minute at room temperature, the supernatants were aspirated and the cells were resuspended in 1.0 ml of D-MEM/F-12 basal medium (Gibco, UK). Subsequently, 0.5 ml of chondrogenic differentiation medium was added to the cell pellet and centrifuged at 200 g for 5 minutes at room temperature and incubated at 37°C in a 5% CO2 and 95% air culture incubator. The caps of tubes were loosed to allow gas exchange. The cells pellet was not attached to the tube, and the media were changed carefully every 3 days. The cell pellet was prepared for frozen sectioning within 14-21 days.

Neurogenic Differentiation

MSCs at passage 3 were trypsinised and resuspended in complete Neurocult differentiation medium (Stem Cell Technologies, UK), and $0.8x10^5$

cells/ml were plated onto 8-well culture slides in complete neurocult differentiation medium and incubated in a 37 °C and 5% CO2 incubator. Approximately 350 µL of medium was removed daily and replaced with fresh complete Neurocult differentiation medium. The cells were examined after 5-10 days using an inverted microscope (Nikon, UK).

Immunocytochemistry

The cells were fixed in 0.5 mL of 4% paraformaldehyde in PBS for 20 minutes at room temperature and incubated in permeabilisation and blocking buffer (0.5 mL of 0.3% Triton X-100 (Sigma, UK)), 1% BSA, and 10% of the appropriate serum at room temperature for 45 minutes. The slides and wells were incubated with goat anti-mouse primary antibody against FABP-4 (1:50), mouse anti-human osteocalcin MoA (1:50), goat anti-human aggrecan polyclonal antibody (1:50) and mouse primary antibodies against human SOX2 and OLIG-4 (1:50) (R and D system, UK) overnight at 2 - 8°C followed by incubation with donkey anti-goat IgG rhodamine red and donkey anti-mouse IgG. Rhodamine red and donkey anti-mouse IgM rhodamine red were used as the secondary antibodies for 1 hour at room temperature. The fat cells were fixed with 4% formaldehyde and stained with oil-red O (Sigma-Aldrich) for 10 minutes. The bone cells were stained with Alizarin red (Sigma-Aldrich, UK).

Results

Primary Cell Culture

The cells appeared to be spindle-like in their morphology when viewed under an inverted light microscope. After 7 days, the cells spread out from the clots of cord blood and formed clusters. Seven days later, the cells were heterogeneous in morphology, appearing as fibroblast-like cells (Figure 1).

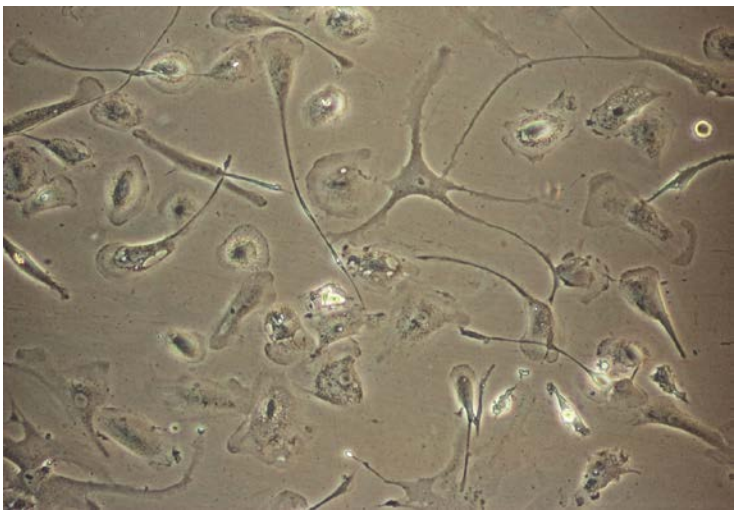


Figure 1: HUCB-derived MSCs with fibroblast-like morphology after 14 days of culture (20X).

Immunofluorescence

The characterisation of MSCs was conducted using a fluorescent microscope after labelling the extracted cells with known MSC CD markers conjugated with FITC. The HSC markers CD34 and CD45 were used as negative controls, and cells did not fluoresce when these antibodies were used.

Immunostaining for Embryonic Stem Cell Markers

I mmunocytochemistry was performed following the 1st passage to confirm the quantitative results and revealed that the MSCs from the HUCB expressed the ESC markers CD9, "SSEA-1 and SSEA-4" transmembrane markers, E-cadherin and PODXL; and the intracellular markers SOX2, NANOG and OCT-3/4 (Figures 2 A, B, C, D, E, F, G and H).

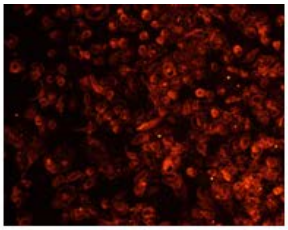
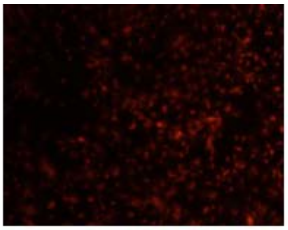


Figure 2 A



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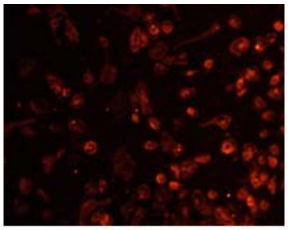

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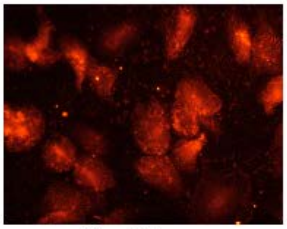
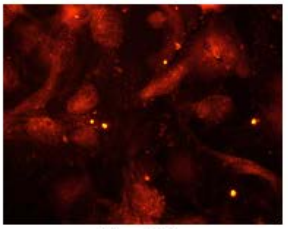


Figure 2 D



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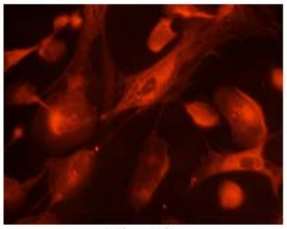

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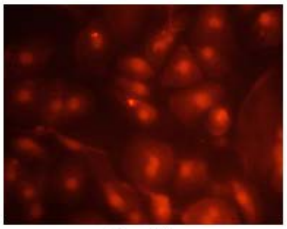

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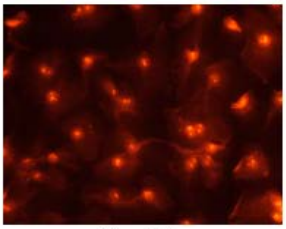


Figure 2 H

Figure 2 (A): Expression of CD9 embryonic stem cell surface marker in MSCs isolated from HUCB using the immunocytochemical staining, following the 1st passage; mouse anti-CD9 MoAb bound to the specific antigens and fluoresced subsequent to the application of donkey anti-mouse IgG rhodamine red X-conjugated secondary antibody (10X), **(B)** The expression of SSEA-4 embryonic stem cell surface markers in MSCs isolated from HUCB using immunocytochemical staining following the 1st passage; mouse anti-SSEA-4 MoAb bound to the specific antigens and fluoresced following the use of donkey anti-mouse IgG rhodamine red X-conjugated secondary antibody (10X), **(C)** Expression of SSEA-1 embryonic stem cell surface marker in MSCs isolated from HUCB using immunocytochemical staining following the 1st passage; mouse anti-SSEA-1 MoAb bound to the specific antigens and fluoresced following the use of donkey anti-mouse IgM rhodamine red X-conjugated secondary antibody 10X, **(D)** The expression of E-cadherin embryonic stem cell transmembrane marker in MSCs isolated from HUCB using the immunocytochemical technique following the 1st passage; mouse anti-E-cadherin MoAb bound to the specific antigens and fluoresced following the use of donkey anti-mouse IgG rhodamine red X-conjugated secondary antibody (40X), **(E)** Expression of PODXL embryonic stem cell transmembrane marker in MSCs isolated from HUCB using immunocytochemical staining following the 1st passage; mouse anti-PODXL MoAb bound to the specific antigens and fluoresced following the use of donkey anti-mouse IgG rhodamine red X-conjugated secondary antibody (40X), **(F)** Expression of SOX2 embryonic stem cell intracellular marker in MSCs isolated from HUCB using immunocytochemical staining following the 1st passage; mouse anti-SOX2 MoAb bound to the specific antigens and fluoresced following the use of donkey anti-mouse IgG rhodamine red X-conjugated secondary antibody 40X, **(G)** Expression of NANOG- embryonic stem cell intracellular marker in MSCs isolated from HUCB using immunocytochemical staining following the 1st passage; goat anti-NANOG polyclonal antibody bound to the specific antigens and fluoresced following the use of donkey anti-goat IgG rhodamine red X-conjugated secondary antibody 40X, **(H)** expression of OCT-3/4 embryonic stem cell intracellular marker in MSCs isolated from HUCB using immunocytochemical staining following the 1st passage; goat anti-OCT-3/4 polyclonal antibody bound to the specific antigens and fluoresced following the use of donkey anti-goat IgG rhodamine red X-conjugated secondary antibody 40X.

Analysis by Flow Cytometry

The characterisation of MSCs using flow cytometry revealed that the cells isolated by the previously described methods lacked expression of CD34 and CD45. Cells were found to express CD29 (97%), CD44 (95%), CD73 (95%) and CD105 (97%) (endoglin). MSCs also expressed the ESC markers CD9 (90%), SSEA-4 (90%), E-cadherin (84%), and PODXL (40%) (Figures 3 A, B, C and D).

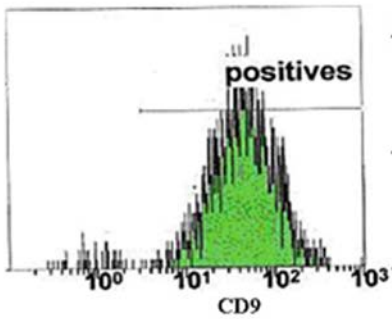
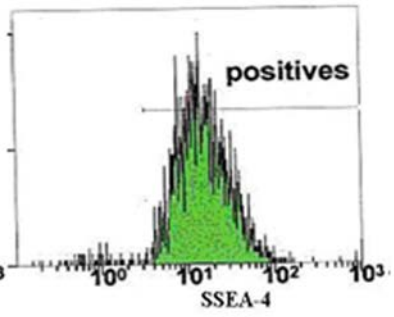


Figure 3 A



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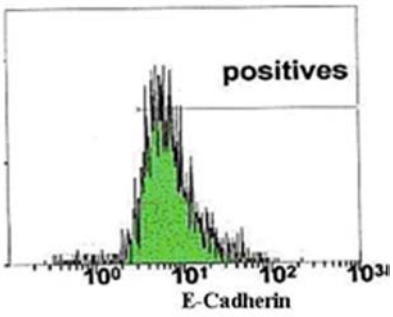


Figure 3C

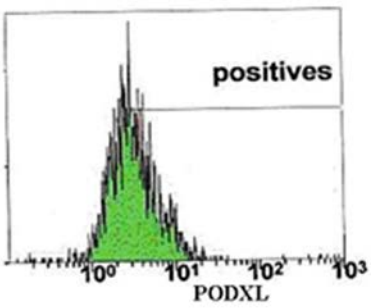


Figure 3D

Figure 3 (A): Immunophenotyping of MSCs derived from HUCB. The cells were labelled following the 1st passage using mouse MoAb against the CD9 marker; rabbit anti-mouse IgG polyclonal secondary antibody bound to the specific antigen, and flow cytometric analysis was performed. Approximately 91% of the cells expressed CD9. Data are presented as the mean ±SE of the 25 samples studied, **(B)** Immunophenotyping of MSCs-derived from HUCB. The cells were labelled following the 1st passage using mouse MoAb against the SSEA-4 marker; rabbit anti-mouse IgG polyclonal secondary antibody bound to the specific antigen, and flow cytometric analysis was performed. Approximately 90% of the cells expressed the SSEA-4. Data are presented as the mean ±SE of the 25 samples studied, **(C)** Immunophenotyping of MSCs derived from HUCB. The cells were labelled following the 1st passage using mouse MoAb against E-cadherin; rabbit anti-mouse IgG polyclonal secondary antibody bound to the specific antigen, and flow cytometric analysis was performed. Approximately 84% of the cells expressed the E-cadherin. Data are presented as the mean ±SE of the 25 samples studied, **(D)** Immunophenotyping of MSC- derived from HUCB. The cells were labelled following the 1st passage using MoAb against PODXL; rabbit anti-mouse IgG polyclonal secondary antibody bound to the specific antigen, and flow cytometric analysis was performed. Approximately 40% of the cells expressed the PODXL. Data are presented as the mean ±SE of the 25 samples studied.

Mesenchymal Stem cell Differentiation

The differentiation capacities of the MSCs derived from HUCB were exposed *in vitro* to differentiation medium to induce adipogenic,

chondrogenic, osteogenic or neurogenic differentiation. Adipocytic differentiation was evidenced by the formation of lipid vacuoles by oil-red O staining and the expression of fatty acid binding protein-4 marker (Figures 4 A and B). In chondrogenic differentiation; the three-dimensional pellet cultures of MSCs produced aggrecan, which is characteristic of *in vitro* cartilage. These pellets did not possess the organised structure of hyaline cartilage, but a disorganised distribution of the chondrocyte within the cartilage-like extracellular matrix was observed (Figure 4 C). The cultured MSCs were differentiated into osteocytes; the cell morphology started to change by day 15, and Alizarin red staining and osteocalcin labelling was observed by day 21 (Figure 4 D). Neurogenic differentiation and the expression of oligodendrocyte-4 and SOX2 markers were evidenced after 10 days of induction (Figures 4 E and F).

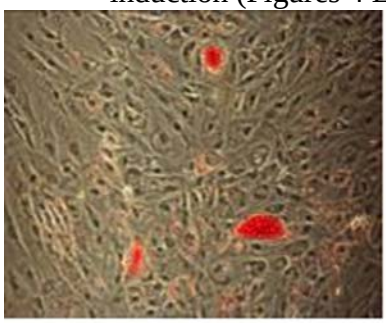


Figure 4 A

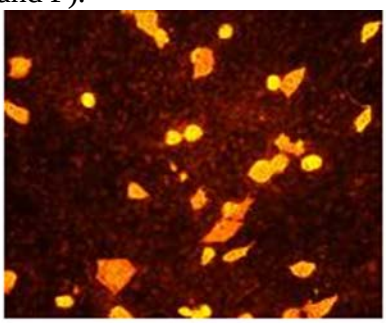


Figure 4 B

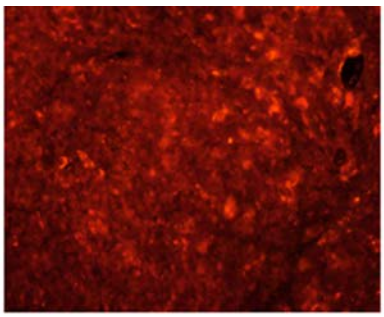


Figure 4 C

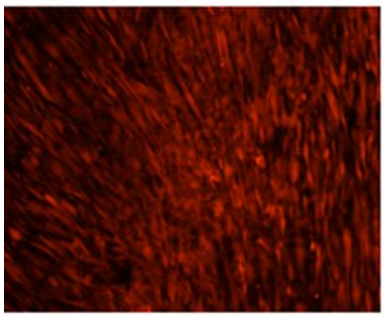
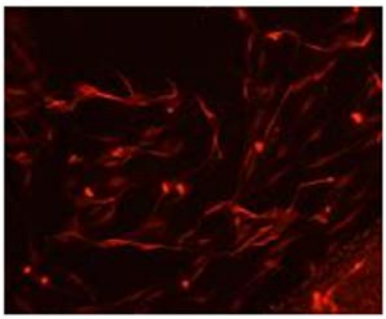


Figure 4 D



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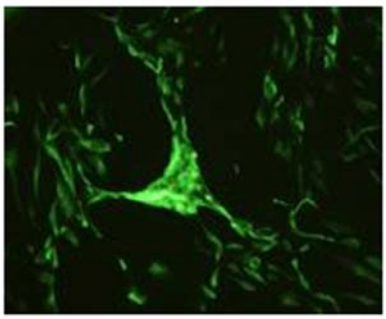

Figure 4 F

Figure 4: (A) MSCs formed lipid vacuoles during adipogenic differentiation, detected using oil red staining (10X). **(B)** Immunocytochemical staining for the FABP-4 adipogenic differentiation marker (10X). **(C)** Immunocytochemical staining for the aggrecan chondrogenic differentiation marker (10X). **(D)** Immunocytochemical staining for the osteocalcin osteogenic differentiation marker (10X). **(E)** Immunocytochemical staining for Olig-4 neural differentiation marker (10X). **(F)** Immunocytochemical staining for the SOX2 neural differentiation marker (10X).

Dıscussıon

Surface antigen expression, which allows for a rapid identification of a cell population, has been used extensively in immunology and haematology. To identify MSCs, cells should express specific cell surface markers and lack the HSC markers. MSCs, unlike HSCs, do not have a single definitive marker. Instead, MSCs are characterised by the presence or absence of a combination of surface phenotypic markers (Bieback et al., 2007; Tyndall et al., 2007; Hussain et al., 2012). Confirmation of the identity of the cultured cells was accomplished using immunofluorescence and immunophenotyping. Analysis by flow cytometry showed that these cells were negative for HSC markers (CD34 and CD45) and found to express CD29, CD44, CD73 and CD105, which strongly demonstrates that the cells cultured in our laboratory were indeed HUCB-derived MSCs/progenitor cells.

The aim of this study was to evaluate HUCB as a source of MSCs with embryonic characteristics. The expression of the selected ESC markers defines the stemness of those cells. The presence of these markers on the surface of MSCs suggests that these cells are more primitive or earlier progenitors than the cells not expressing these markers. Qualitative and quantitative methods were used to identify the ESC markers. Our study showed that ESC markers are present on MSCs from HUCB-cultured cell populations. The expression of CD9, SSEA-4, SSEA-1, E-cadherin PODXL, SOX2, NANOG and OCT-3/4 was detected, and these proteins are known markers for ESCs, as CD9 is a member of the transmembrane-4 superfamily and is an important protein in sperm-oocyte fusion. Most of these proteins play a role in cellular development (Kaji et al., 2002). It has been shown in our study that MSCs derived from HUCB express this marker (90%), and these results were confirmed using immunocytochemistry.

McGuckin *et al.* 2005 reported that cord blood stem cells were able to express SSEA-3 and 4, which are sialoproteins that characterise human ESCs, but these cells did not express SSEA-1(McGuckin et al., 2005). However, in this study, the flow cytometry and immunocytochemistry analyses confirmed the expression of SSEA-4 and SSEA-1 from HUCB MSCs. The presence of these markers in the MSC population suggests that the cells are in an undifferentiated state because their expression is down regulated following stem cell differentiation (Jonathan et al., 2004). E-cadherin markers are transmembrane-1 proteins that are required for cell adhesion and cell signalling. They are involved in directing the cells to their proper tissue locations during development, and they are dependent on calcium for optimal function; furthermore, E-cadherin proteins are responsible for the aggregation of the inner cell mass of the blastocyst (Bryant & Stwo, 2004).

Mariottim *et al.* (2010) reported that the MSCs derived from placenta and BM lack E-cadherin expression (Mariottim et al., 2010). However, in this

study, 84% of the MSCs derived from HUCB expressed E-cadherin, as determined by immunocytochemical staining (qualitative technique). The transmembrane expression of PODXL decreases during maturation of haematopoietic cells. Therefore, PODXL is used as an embryonic stem cell marker. It has been demonstrated that PODXL mRNA is expressed in human CD34+ cells and in lineage-committed erythroid, megakaryocyte and myeloid progenitors (Kerosuo et al., 2004). In our study, the MSCs showed low levels of PODXL expression (40%) compared with other markers. Immunocytochemistry confirmed this low level expression. However, PODXL expression is still poorly defined.

Masahiro and colleagues (2008) showed that the MSCs from BM can express the SOX2 marker as a transcription factor for developing the central nervous system (Baer et al., 2007; Masahiro et al., 2008). The SOX2 marker is necessary for the pluripotency of stem cells and might play a role in the regulation of OCT-3/4 expression in the maintenance of multipotent differentiation capability through upstream transcription factors (Ellisd et al., 2004). In this study, we observed SOX2 expression in MSCs using immunocytochemical staining. The isolated MSCs also expressed other ESC markers, such as the transcription factor NANOG, which is expressed in the ICM of the blastocyst, the morula, and ESCs. NANOG is important in maintaining pluripotency and works in concert with other factors, such as OCT-3/4 and SOX2, to maintain embryonic stem cell identity (Mitsui et al., 2003). In this study, we demonstrated that the expression was intracellular and within the nucleus.

The expression of OCT-3/4 is crucial to sustain the self-renewal and pluripotency of ESCs through an octamer motif containing a cis-element. OCT-3/4 is responsible for gene transcription and is expressed in totipotent embryonic stem and germ cells in the pre-implantation embryo (Babaie et al., 2007). It controls pluripotency and plays a critical role in regulating the path for cell differentiation (Buitrago et al., 2007). In our study, it was demonstrated that the expression of OCT 3/4 was intracellular using immunocytochemical staining. This finding is not consistent with the results from previous studies (McGuckin et al., 2005). However, our results are consistent with the data of Masahiro *et al.* (2008).

The biological property that most uniquely identifies MSCs is their capacity for multilineage mesenchymal differentiation. Thus, cells have been shown to differentiate into osteoblasts, chondroblasts, neurocytes and adipocytes using standard *in vitro* tissue culture-differentiating conditions. In our study, the induction of adipogenic differentiation was apparent by the accumulation of lipid vacuoles and expression of the adipogenic marker FABP-4. In osteogenic medium, which contains specific supplements, the MSCs were transformed from fibroblastic to an osteoblast-like morphology

and formed a mineralised bone matrix with calcium deposition, which was confirmed by the presence of positive Alizarin red staining after 21 days of culture. The osteogenic lineage differentiation was confirmed by labelling of the osteocalcin protein after 21 days of culture. Our findings are consistent with previously reported data (Bieback et al., 2004; Lee et al., 2004; Hussain et al., 2012).

MSCs are being used to treat articular cartilage defects. However, the potential of HUCB-derived cells to differentiate into chondrocytes that produce type II collagen has been shown by a previous study (Lee et al., 2004). In our study, we assayed MSCs for chondrogenic differentiation and showed the ability to differentiate after 21 days in a serum-free chondrogenic medium. This ability was confirmed using a special cryosectioning method to analyse three-dimensional cell pellets for cartilage cell protein aggrecan by immunocytochemistry. Neurogenic differentiation was demonstrated by the expression of OLIG-4 and SOX2 neural markers.

Conclusion

Flow cytometry and imunoctochemistry were used to demonstrate the positive expression of MSCs surface markers CD29, CD44, CD73 and CD105 and the presence of the embryonic stem cell markers CD9, PODXL, E-cadherin, OCT3/4, SSEA-1, SSEA-4, NANOG and SOX2 as well as multilineage differentiation. It can be concluded that HUCB is a rich source of MSCs with embryonic characteristics and that they may be utilised for clinical purposes.

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