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Pluripotent stem cells isolated from umbilical cord form embryonic like bodies in a mesenchymal layer culture

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Summary. Recently the matrix of umbilical cord began to use as an alternative source of stem cells additionally to the blood of umbilical cord. Umbilical cord has been used mainly for mesenchymal stem cell banking. The immunological characteristics of mesenchymal stem cells in combination with their ability to avoid rejection make them an attractive biological material for transplantations. In this study the isolation of small in size pluripotent stem cells from umbilical cord expressing early transcription factors with characteristics that resemble to embryonic stem cells is investigated.

Pluripotent stem cells were isolated from human umbilical cords, by a new strategy method based on unique characteristics such as the small size and the positivity on early transcription factors OCT and Nanog.

An enriched population of $CXCR4^+$ OCT^+ $Nanog^+$ $CD45^-$ small stem cells from the cord was isolated. This fraction was able to create alkaline phosphatase positive like spheres forms in a mesenchymal layer with multilineage differentiation capacity. Our results were assessed by RT PCR and electophoresis for the pluripotent genes.

These data suggest that umbilical cord provides an attractive source not only of mesenchymal stem cells but moreover of pluripotent stem cells. The method described herein should be applied in the field of stem cell banking in addition to the classical umbilical cord harvesting method.

Isolation of a population of cells with pluripotent characteristics from umbilical cord. Adoption of a second centrifugation step for the pluripotent stem isolation. Increasing the value of the cord and explaining the pluripotency. This work will enhance the value of umbilical cord harvesting.

Key words: Umbilical cord, Pluripotent stem cells, Differentiation, Transplantation, Very small embryonic like stem cells, Regenerative medicine, Plasticity

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Introduction

Stem cell based therapy, referring to both adult and embryonic stem cells, is widely used in medicine, mainly in regenerative medicine. Stem cell based therapy aims to treat tissue damage which has been the result of injuries, diseases, genetics and/or birth defects. Stem cells are distinguished by their ability to renew themselves and differentiate into various cell types.

Adult stem cells can be isolated from various sources, including bone marrow, cord blood, placental tissues, amniotic fluid, adipose tissue and skin. The drawback with adult stem cells is that they are multipotent, meaning that they only have the potential to differentiate to a limited number of cell types.

Embryonic stem cells are pluripotent, that is, they possess the potential to differentiate into derivatives of all three embryonic germ layers: endoderm, mesoderm and ectoderm. Although embryonic stem cells have great potential, there are many ethical concerns involved with their use, since they are derived from the inner cell mass

of blastocyst-stage embryos.

The plasticity of stem cells isolated from cord blood it is already known. In 2004 Kogler et al. found a non-haematopoietic stem cell population of umbilical cord blood called unrestricted somatic stem cells, capable of differentiating into all three germ layers (Kogler et al., 2004). Recently, researchers worldwide found stem cells isolated from umbilical cord blood that expressed early transcription factors found typically in the embryonic stem cells (Kucia et al., 2007). These cells are first described by Kucia et al., 2006, in a fraction of murine bone marrow stem cells (Kucia et al., 2006), and named Very Small Embryonic Like Stem cells (VSELS). VSELS are very small (2-4 mμ) CD34 and CD45 negative stem cells that strongly express CXCR4 Sca-1$^+$ antibody and embryonic transcription factors such as OCT and Nanog. These transcription factors are considered markers of mouse and human embryonic stem cells playing a basic role in stem cell pluripotency (Rosner et al., 1990; Chambers et al., 2003; Mitsui et al., 2003). VSELS were also described in several murine organs such as brain, liver, kidney, heart, and skeletal muscles (Zuba-Surma et al., 2008). Recently, some investigators found these cells in mobilized peripheral blood in patients after stroke (Paczkowska et al., 2009) and after acute myocardial infarction (Wojakowski et al., 2009). The investigators thought that these cells represent a primitive population of stem cells that maybe play a major role in regenerative medicine and in stem cell therapies in general.

Umbilical cord is already known as a source of multipotent stem cells. The mesenchymal stem cells (MSCs) isolated from umbilical cord harvesting can differentiate into nerve cells (Karahuseyinoglu et al., 2007), chondrocyte (Wang et al., 2004; Karahuseyinoglu et al., 2007), osteoblasts (Wang et al., 2004; Conconi et al., 2006), cardiac muscle cells (Karahuseyinoglu et al., 2007), skeletal muscle cells (Wang et al., 2004), adipose cells (Conconi et al., 2006; Lu et al., 2006), dopaminergic neurons (Fu et al., 2006; Weiss et al., 2006) and germ-like cells *in vitro* (Huang et al., 2010). Other studies have referred to the isolation of pluripotent stem cells from the porcine umbilical cord that express Nanog and Oct antibodies and are alkaline phosphatase positive, without referring to the size of isolated stem cells and without characterizing these stem cells as VSELS (Carlin et al., 2006).

Here we describe for the first time a new strategy method for pluripotent stem cell isolation from the total length of the umbilical cord that have many similar characteristics to the VSELS first described by Kucia et al. (2006, 2007, 2008) with the ability to create forms that look like embryoid bodies in a mesenchymal layer culture.

Materials and methods

Umbilical cords were obtained from term-gestation newborns after birth from consenting parents. Umbilical cords were transferred to the laboratory in a carrying solution (70% normal saline, 30 % CPD containing antibiotics (Penicillin G, Sigma Oakville, ON, Canada no P-3032), gentamicin (50 μg/ml; Sigma no.G-1397), and amphotericin B (5 μg/ml; Sigma no. A9528).

Isolation of mesenchymal stem cells

Once in the laboratory the cords were washed until the removal of all blood. The procedure of enzymatic digestion was based on recently published protocol where the total length of the umbilical cord was used (Tsagias et al., 2011). The protocol method leads to the isolation of a large number of mesenchymal stem cells in total, due to the fact that more tissue is used. Also, the method leads to a greater number of stem cells per cm of tissue. An enzymatic cocktail comprising 2.7 mg/ml collagenase type I (Gibco) and 0.7 mg/ml hyalouronidase (Aplichem), followed by the addition of 2.5% trypsin (Biochrom AG) was used. Cells were centrifuged at 300g for 20 min. The pellet was resuspended in a complete medium. Separated cells from the pellet served as layer for the culture of the very small embryonic like stem cells.

Isolation of pluripotent stem cells from umbilical cord

Based on the knowledge that very small embryonic like stem cells are a very small in size population, a second high centrifugation was applied to the supernatant of our samples in order to isolate the small pluripotent population. Cells were centrifuged at 1100g for 60 min. The new pellet was resuspended in a complete alpha Modification of Eagle's Medium (Biochrom AG, Berlin, Germany). Flow cytometry was used for the count of the small pluripotent stem cell population (Beckman-Coulter, Miami, FL, USA).

Flow cytometry protocol for the small pluripotent stem cell gating

For the very small pluripotent stem cell count we use a new gating protocol of flow cytometry based on the unique characteristics of the VSEL, such as the very small size the positivity of early transcription factors Oct and Nanog, CXCR184 positivity and the CD45-depletion. Cells were stained with CD45 antibody conjugated with APC (Allophycocyanin) and CD184 antibody conjugated with PC5 (Phucoerythrin-cyanine 5). Cells were incubated for 20 min in the dark. Cells were then washed and fixed with 4% paraformaldeyde for 20 min and then were permeabilized with 0.1% Triton for 10 min. The permeabilization buffer was removed and the cells were stained with Oct3/4 antibody conjugated with Alexa Fluor^R and Nanog antibody conjugated with Phycoerythrin-PE (BD, Pharmigen, USA). The analysis was done in a Beckman-Coulter FC

500 flow cytometer (Beckman Coulter). The second centrifugation step consisted only of small size cells since the larger cells were removed from the first centrifugation step.

Mesenchymal feeder layer process

The phenotype of isolated MSCs from the total length of the umbilical cord was tested by flow cytometry in a Beckman-Coulter FC500 flow cytometer (Beckman-Coulter, Miami, FL, USA). Phycoerythrin (PE)-conjugated antibody against CD105, antibodies and fluorescein isothiocyanate (FITC)-conjugated antibody against CD29, CD90, CD44, antibodies were used for mesenchymal stem cell characterisation. Mesenchymal stem cells were seeded in 6-well plates (Costar, Cambridge, MA, USA) with alpha Modification of Eagle's Medium (Biochrom AG, Berlin, Germany), supplemented with 10% FCS (Biochrom AG, Berlin, Germany), 100 μM L-ascorbic acid 2-phosphate (Applichem), 100mg/ml Pen/Strep (Biochrom AG, Berlin, Germany). When the mesenchymal stem cells reached more than 80 % confluence served as a feeder layer for the very small embryonic like stem cell culture. Mesenchymal stem cells used as feeder layer after at least three passages.

Co-culture of the small pluripotent stem cells with the feeder mesenchymal layer-Formation of embryoid bodies

The selected population of the second centrifugation was cultured with the mesenchymal feeder stem cells with alpha Modification of Eagle's Medium (Biochrom AG, Berlin, Germany), supplemented with 10% FCS (Biochrom AG, Berlin, Germany), 100 μM L-ascorbic acid 2-phosphate (Applichem), 100 mg/ml Pen/Strep (Biochrom AG, Berlin, Germany), for a sufficient period of time in order to induce formation of the EB-like bodies. The ability was maintained even after the passages.

Alkaline phosphatase detection

Embryoid like bodies were tested for alkaline phosphatase activity to determine their pluripotency, using an alkaline phosphatase kit (Millipore's Alkaline Phosphatase Detection Kit, Catalog number SCR004) in accordance with the manufacturer's instructions. Millipore's Alkaline Phosphatase Detection Kit is a specific and sensitive tool for the phenotypic assessment of undifferentiated pluripotent embryonic stem cells (Pease et al., 1990).

Plasticity of the embryoid like bodies

To determine the plasticity of the isolated stem cells from the second centrifugation stage we cultured the embryoid bodies in neurogenic and osteogenic differentiation medium.

Neurogenic differentiation

To identify that the embryoid bodies had neurogenic potential, cultured clusters were directed towards neurogenic differentiation medium as described by Safford et al. (2002). After disassociation of the clusters the medium was changed with neuronal induction medium composed of DMEM with butylated hydroxyanisole (BHA, 200lM final concentration), KCl (5 mM), valproic acid (2μM) forskolin (10 μM), hydrocortisone (1 μM), and insulin (5g/ml). Changes in morphology were detected by inverted microscope.

Osteogenic differentiation

Differentiation of embryoid-like bodies was performed by culturing them after disassociation in osteogenic differentiation medium as described previously (Kern et al., 2006). The growth medium was changed to the osteogenic differentiation medium consisting of DMEM-LG supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin, 100 nM dexamethasone, 0.2 mM L-ascorbate, and 10 mM b-glycerophosphate (Sigma-Aldrich, St Louis, MO, USA). Cells were incubated for 28 days and the medium was changed every 3 days. Osteogenic differentiation was examined by staining for alizarin red- S following the manufacturer's instructions. Cells were fixed with 10% formalin (Sigma) for 15 minutes and stained with alizarin red solution (Sigma) for 15 minutes. Changes in morphology of cells were detected by inverted microscope.

RNA Isolation

Total RNA was isolated using RNeasy Mini Kit (Nucleospin FFPE RNA, Macherey-Nagel, Duren Germany). The mRNA (10ng) was reverse-transcribed with One Step RT-PCR according to the manufacturer's instructions.

Quantitative real time PCR

Total RNA was isolated using the RNeasy Mini Kit (Nucleospin FFPE RNA, Macherey-Nagel, Duren Germany). 10ng of total RNA was used for each qRT-PCR reaction. mRNA levels of OCT4 and Nanog genes were estimated in samples isolated from the first or the second centrifugation stage. qRT-PCR reactions were performed using a KAPA SYBR Fast 1-step qRT-PCR Universal kit (KAPA BIOSYSTEMS) in a Rotor-Gene 6000 cycler (Qiagen), according to the manufacturer's instructions. Relative gene expression was calculated by subtracting the ΔCt values of target genes from the ΔCt values of ACTB reference gene. Primer sequences for target genes were: OCT4 forward primer: 5'-AGCCCTCATTTCACCAGGCC-3' OCT4 reverse primer: 5'- TGGGACTCCTCCGGGTTTTG-3' Nanog forward primer: 5'-AGTCCCAAAGGCAAACAACC

CACTTC-3' Nanog reverse primer: 5'-TGCTGGAG GCTGAGGTATTTCTGTCTC-3' Amplified qRT-PCR products were finally analyzed in a 2% Agarose gel electrophoresis.

Results

A specific flow cytometry protocol was designed for small size stem cells gating expressing the phenotype CD45$^-$ CXCR4$^+$ Nanog$^+$ and OCT4$^+$. Based on the knowledge that this population is a very rare population, a second centrifugation step was added, for the isolation of a population of very small stem cells that is highly enriched in cells with the CD45$^-$ CXCR4$^+$ Nanog$^+$ and OCT4 phenotype (Fig. 1).

Stem cells from the second centrifugation stage after plating over a mesenchymal umbilical cord feeder layer begin to form small clusters in the supernatant medium. As the culture continues forms like embryoid bodies are detected in the co-cultures. These forms varied in size and did not present the ability to adhere to the plastic. Furthermore, the cells maintained their ability to form these bodies even after their dissociation with the pasteur pipette or after the passages. The clusters increased in

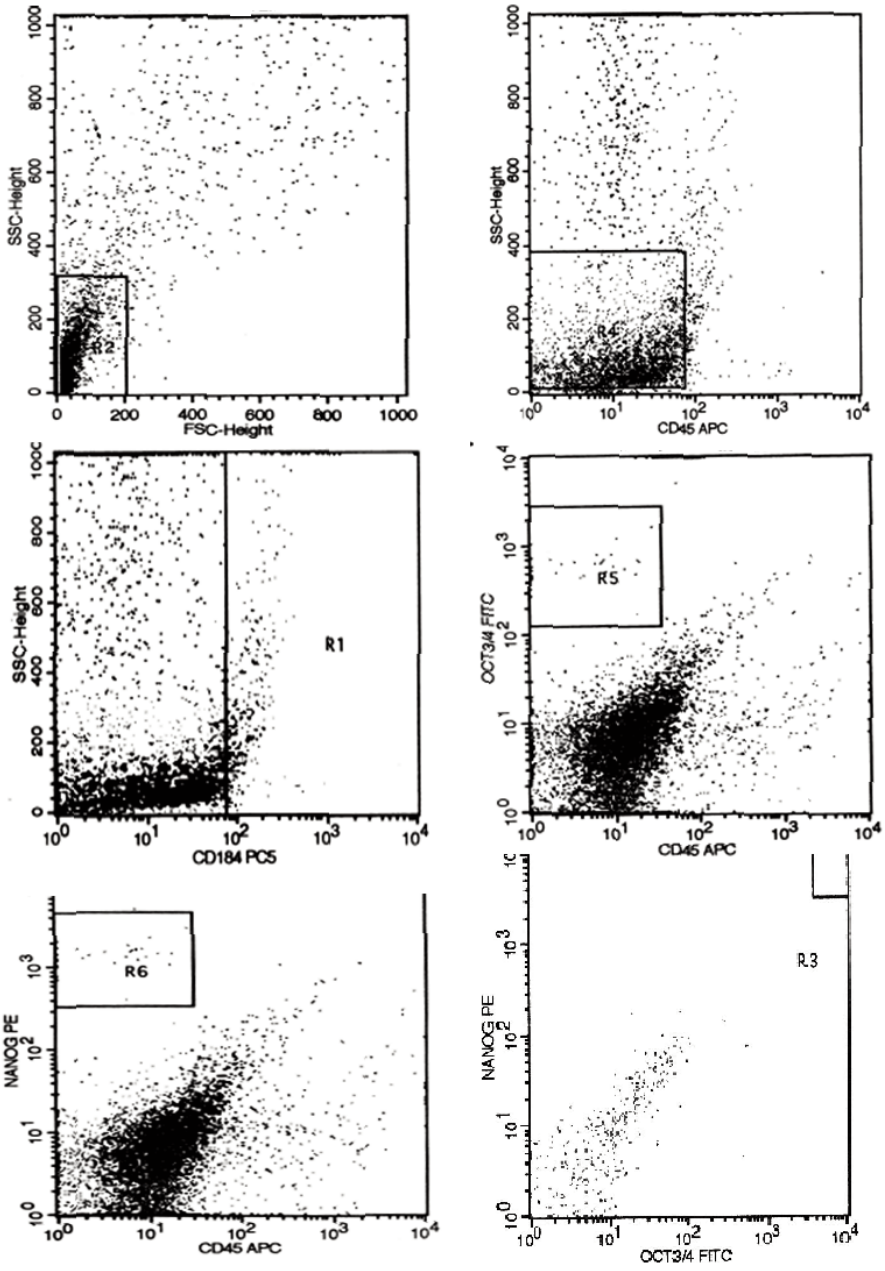

Fig. 1. Flow cytometry protocol for pluripotent very small stem cells gating. R1 region includes stem cells that express the CXCR4 (CD 184) receptor. R2 region consisted of stem cells with low FSS and SSC (small size stem cells) while the region R3 includes the CD45- negative population. The regions R5 and R6 characterize the cells that are additionally Nanog and Oct positive and also express all the above characteristics while R3 region includes all the Nanog and Oct positive stem cells.

size during the culture (Fig. 2).

Embryoid like bodies stained positive for the fetal isoform of alkaline phosphatase (Fig. 3).

The mRNA levels of OCT4 and Nanog embryonic stem cell markers were estimated in cells isolated during the first or second centrifugation samples (Wharton A and Wharton B). qRT-PCR showed that both the fractions contained OCT4 and Nanog mRNA indicating that the cord is a source of pluripotent stem cells. The main difference is that the Wharton B population mainly consisted from a purified population enriched in Oct and Nanog positive stem cells, while the Wharton A population contained a variable population consisting mainly of mesenchymal stem cells (Fig. 4).

The identity of amplified fragments was validated by Agarose gel electrophoresis. OCT4 and Nanog primers

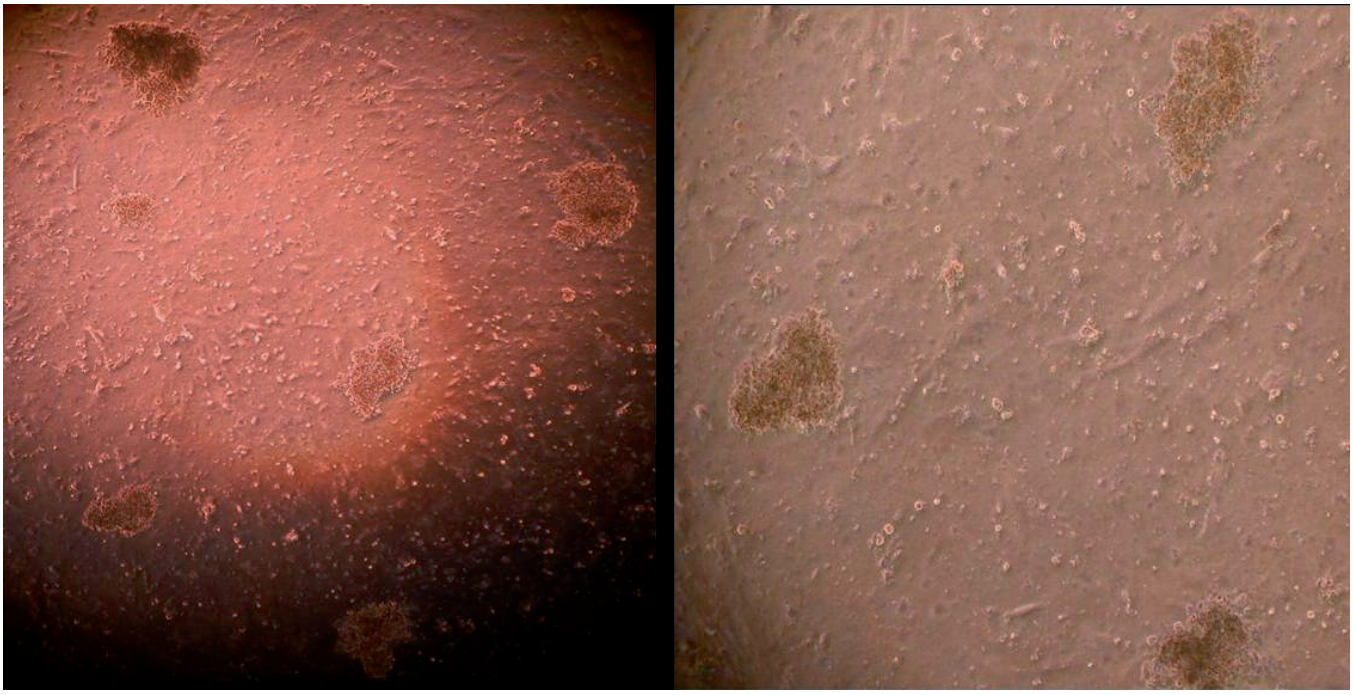

Fig. 2. a. Pluripotent stem cells from umbilical cord form clusters that resemble embryoid bodies when cultured in umbilical cord mesenchymal layer. **b.** The forms do not have the ability to attach to the plastic surface and continue to increase in size in the suspension.

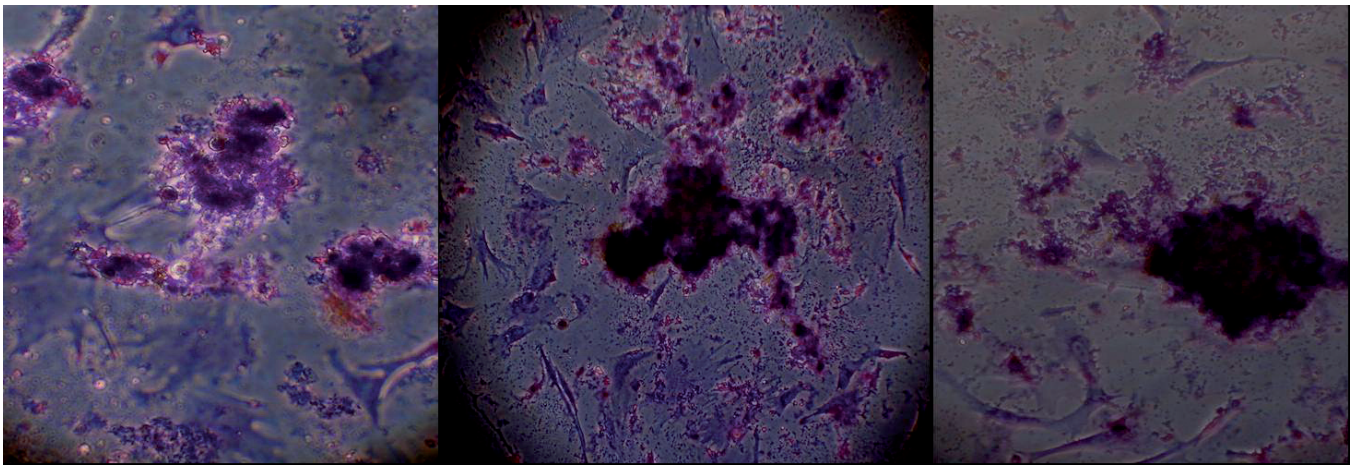

Fig. 3. Positive staining of the clusters like spheres with alkaline phosphatase during the culture. Only the clusters on the suspension medium stained positive. No alkaline phosphatase positive staining was observed in the mesenchymal feeder layer.

amplified specific cDNA fragments with the expected molecular weight of 168bp for OCT4 and 161bp for Nanog (Fig. 5).

All the samples were negative for bacterial and fungal contamination. To determine the pluripotency of the supernatant forms the embryoid bodies were incubated in neurogenic and osteogenic differentiation medium. After 3-5 days the embryoid like bodies began to change, exhibiting a neuronal appearance. After 7 days on the neurogenic differentiation medium neuronal like stem cells began to create formations that assembled to synapses (Fig. 6).

Osteogenic differentiation of the second stage of centrifugation was performed by culturing cells in osteogenic differentiation medium. Osteogenic differentiation was examined by staining for alizarin red-S (Fig. 7).

Discussion

The presently disclosed subject matter provides for the first time a method of isolation of an enriched population of small embryonic-like stem cells from the umbilical cord. We provide an enriched population of small embryonic-like stem cells from such heterogeneous populations isolated by a new isolation strategy method based on two steps of centrifugation. With the first stage of centrifugation a population of larger cells is collected. This population consists of the mesenchymal population of umbilical cord stem cells.

However, prior to the collection of the sediment, the supernatant is also collected in a second sterile container and is centrifuged at high rotation speed, for a long period of time. The population collected after the second centrifugation is an enriched population of small embryonic-like umbilical cord derived stem cells. This population is CD45$^-$ and Oct-4$^+$, Nanog$^+$, CXCR4$^+$. A new flow cytometry protocol was created for the small stem cells gating based in the unique characteristics of these cells. From our flow cytometry data the population derived from the second centrifugation is an enriched pluripotent stem cell population consisting of OCT4 and Nanog positive stem cells in 75%.

The cells described herein are capable of forming embryoid-like bodies when placed in a mesenchymal feeder layer. After 5-7 days the increased clusters began to appear in the suspension. These forms were totally detached from the plastic and continued to increase and to proliferate. Moreover the ability to create these forms like spheres was maintained even after dissociation with a pasteur pipette and after three or four passages in new cultures. The cells maintained their ability to create undifferentiated clusters until a confluence of the flasks achieved. The clusters increased in size during the culture. Embryoid like bodies stained positive for the fetal isoform of alkaline phosphatase as has been shown that consist an ideal marker of undifferentiated pluripotent stem cells (Pease et al., 1990).

Our results were confirmed by qRT-PCR of the mRNA levels of the pluripotent transcription factors Oct-4, Nanog, and with electrophoresis of the cDNAs of OCT and Nanog genes.The mRNA levels of OCT4 and Nanog embryonic stem cell markers were estimated in cells isolated during the first or second centrifugation samples (Wharton A and Wharton B). qRT-PCR showed that both two fractions contained OCT4 and Nanog mRNA, indicating that the cord is a source of pluripotent stem cells. The main difference is that the Wharton B population mainly consisted of a purified population enriched in Oct and Nanog positive stem cells while the Wharton A population contained a variable population consisting mainly of mesenchymal stem cells.

The identity of amplified fragments was validated by Agarose gel electrophoresis. OCT4 and Nanog primers amplified specific cDNA fragments with the expected molecular weight of 168bp for OCT4 and 161bp for Nanog .

They also possess a multi-lineage differentiation potential and self-renewing capability. Moreover, the spheres differentiated into cells with neural-like phenotype and also to bone like alizarin red positive stem cells. After 3-5 days the embryoid-like bodies began to change, exhibiting a neuronal appearance. After 7 days on the neurogenic differentiation medium

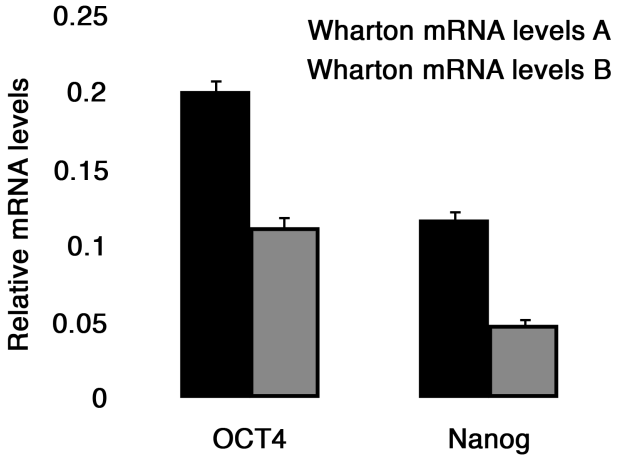

Fig. 4. Estimation of OCT4 and Nanog mRNA levels in cells isolated during the first (Wharton A) and the second centrifugation steps (Wharton B). Relative mRNA levels of OCT4 and Nanog compared to ACTB reference gene.

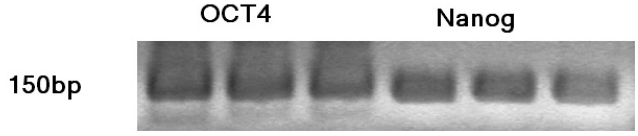

Fig. 5. Agarose gel electrophoresis of amplified cDNA fragments. Gel shows OCT4 and Nanog cDNA fragments in triplicate.

neuronal-like stem cells began to create formations that assembled into synapses. Osteogenic differentiation of the second stage of centrifugation was performed by culturing cells in osteogenic differentiation medium Osteogenic differentiation was examined by staining for alizarin red-S. The generation of cells of at least two of the three germ layers in combination with the morphological criteria and the expression of the pluripotent Nanog and OCT4 genes in our cultures strongly demonstrate their pluripotency.

It is remarkable that the differentiation occurred in the upper buffy coat while no differentiation occurred in the feeder layer. Furthermore, the morphological changes of the differentiation were observed faster in

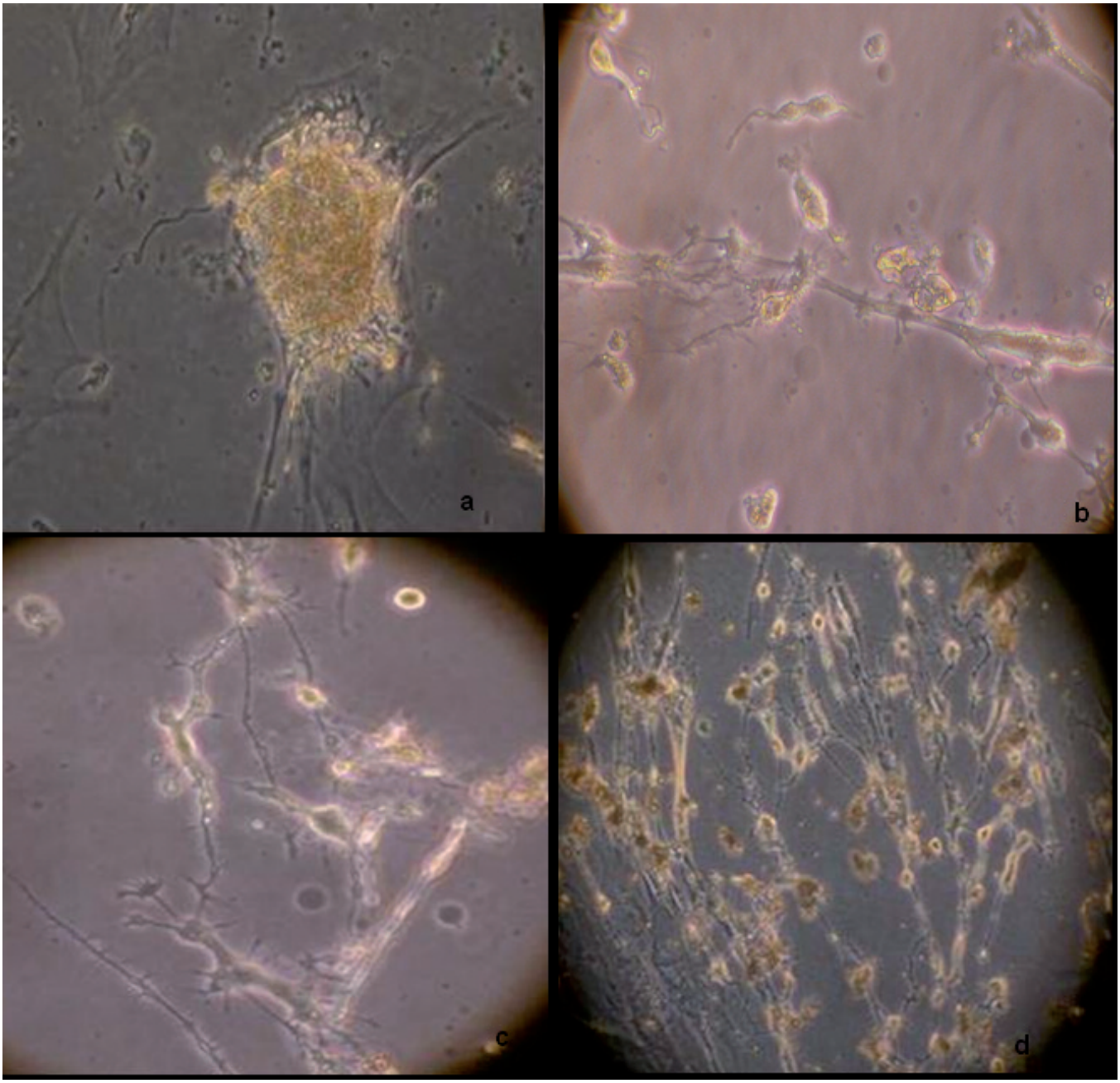

Fig. 6. a. Morphological changes of the supernatant embryoid like bodies of the small umbilical cord stem cells under neuronal differentiation medium during the culture. **b.** Cells appeared phase bright nuclei and extend long thin neurite-like morphology. **c, d.** After 7 days on the neurogenic differentiation medium neuronal like stem cells began to create formations that assembled into synapses.

comparison with the differentiation of mesenchymal stem cells. This fact was especially observed during the neurogenic differentiation (data non shown). Not only were morphological changes observed in cells that began to show neural-like phenotype, but moreover connections between the cells were formed which assembled into synapses. More experiments are needed to determine the neuro-like phenotype of these stem cells and the possible differences from the mesenchymal stem cells that can reach neurogenic differentiation.

Embryoid bodies is a unique characteristic of pluripotent stem cells and has recently been described for the unrestricted somatic stem cells that have been isolated from human umbilical cord blood (Zaibak et al., 2009). These stem did not require a feeder layer while in fetal calf serum cultures formed an adherent monolayer. These spheres were pluripotent and many characteristics, such as heterogenity in shape and loss of the adherence ability, reformation after dissociation and pluripotent potential are common with our forms. Unrestricted somatic stem cells are already well known as pluripotent stem cells. The common characteristics support our findings that our enriched population consisted of very primitive stem cells. Similar spheres are also described by VSEL cells that have been isolated from bone marrow, murine fetal liver thymus and spleen (Kucia et al., 2008).

The stem cells described herein differ totally from those that have been described from a porcine umbilical cord (Carlin et al., 2006). The basic process of cord harvesting is totally different, the cells are cultured with growth factors without a feeder layer and no reference concerning the size of the isolated stem cells exists.

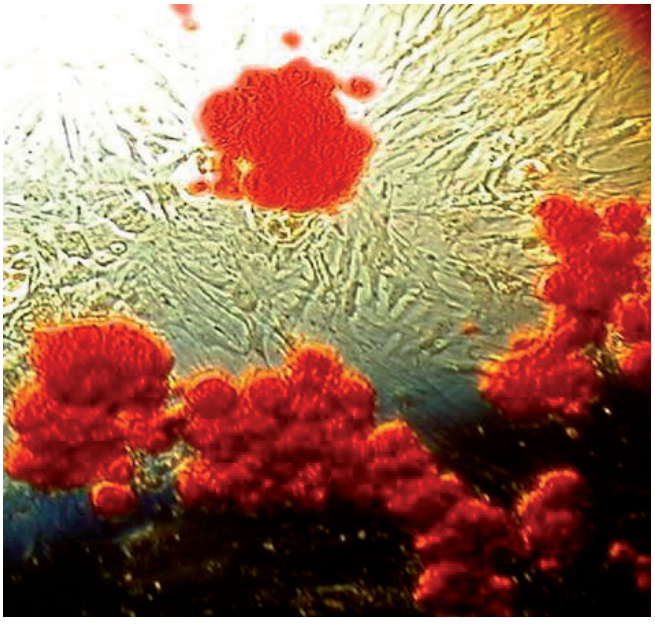

Fig. 7. Osteogenic differentiation of the small stem cells from the umbilical cord cultured in a mesenchymal layer. Formation of mineralized matrix by Alizarin Red evidenced osteogenic differentiation.

We noticed that these small population could be purified by a second stage of centrifugation based on the small size of this stem cells. Indeed, these observations were supported by RT-PCR and electrophoresis.

This population has been identified only in umbilical cord blood and not from the matrix of the umbilical cord. Other controversies about these stem cells already exist. Recently, researchers considered that this population is not equivalent to the murine VSELS and that umbilical cord blood stem cells lack their stem cell characteristics (Danova-Alt et al., 2012). Moreover, a new study supports that in the majority of umbilical cord blood processing the VSELS are lost because of their small size. This, according the researchers, occurs during the centrifugation and the Ficoll-Hypaque processing for red blood cell sedimentation (Bhartiya et al., 2012). For this reason many researchers suggest the RBC lysis before the processing (Halasa et al., 2008). An advantage of umbilical cord harvesting is that the amount of red blood cells is very low and neither a ficoll paque gradient centrifugation nor a lysis of RBC is necessary. We claim that because of the lower mass of the VSELs only a higher centrifugation speed is required for the upper buffy coat. With this method no hypotonic mediums are used which could be dangerous for VSEL viability.

From the second centrifugation an enriched population of small pluripotent stem cells is obtained, while from the first stage a heterogeneous population consisting of mesenchymal and pluripotent stem cells is isolated. Because of the small number of these stem cells -in comparison with the mesenchymal amount these stem cells probably could not form these spheres in a simple mesenchymal culture and they were discarded quickly as supernatant during the culture's process. Moreover, in a classic mesenchymal culture a small number of mesenchymal stem cells is placed in which the corresponding number of pluripotent small stem cells is much smaller. It is very likely that these stem cells are responsible for the neural-like phenotype that was observed from the matrix cord stem cells. All these thoughts and questions should be under investigation.

Many researchers tried to explain the pluripotency of the VSELS analyzing their possible developmental origin. It is supported that VSELs are epiblast-derived stem cells that are deposited early during gastrulation/organogenesis in developing tissue (Kucia et al., 2008; Ratajczak et al., 2008) From the other side umbilical cord formed early during organogenesis and the vessels and the matrix of the cord are derived from extra embryonic mesoderm and/or embryonic mesoderm mainly form the epiblast (Cetrulo and Cetrulo, 2006; Slader, 2004; Weiss and Troyer, 2006).

Taken together these data could easily explain the presence of pluripotent stem cells in the umbilical cord.

The potential relationship of our isolated small stem cells by a second centrifugation with the VSELs found

first in BM and in umbilical cord blood is under investigation. We strongly support that it is the same population and that our enriched population has the same characteristics as VSELs.

This the first time that a population of small size pluripotent stem cells has been purified from umbilical cord harvesting. With our novel strategy it is strongly supported that the umbilical cord is a very useful source of pluripotent stem cells for new therapeutic applications. Taking into consideration the population of small stem cells found by our team we suggest that a new step of centrifugation must be added to existing harvesting methods to isolate stem cells with higher regenerative potential.

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Conflict of interest. Dr Koliakos is a Professor of the Department of Biological Chemistry, Medical School, Aristotle University Thessaloniki and also CEO and President of the Biohellenika SA company

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