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Cryopreservation does not alter karyotype, multipotency, or NANOG/SOX2 gene expression of amniotic fluid mesenchymal stem cells

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ABSTRACT. Cryopreservation of mesenchymal stem cells from amniotic fluid is of clinical importance, as these cells can be harvested during the prenatal period and stored for use in treatments. We examined the behavior of mesenchymal stem cells from human amniotic fluid in culture that had been subjected to cryopreservation. We assessed chromosomal stability through karyotype analysis, determined whether multipotent capacity (differentiation into adipogenic, chondrogenic, and osteogenic cells) is maintained, and analyzed SOX2 and NANOG expression after thawing. Five amniotic fluid samples were cryopreserved for 150 days. No chromosomal aberrations were observed. The expression levels of NANOG and SOX2 also were quite similar before and after cryopreservation. Capacity for differentiation into adipogenic, chondrogenic, and osteogenic tissues also remained the same. We conclude that cryopreservation of amniotic fluid does

not alter karyotype, NANOG/SOX2 gene expression, or multipotent capacity of stem cells that have been collected from amniotic fluid during pregnancy.

Key words: Amniotic fluid; Cryopreservation; Differentiation; SOX2; NANOG; Stem cell

INTRODUCTION

Amniotic fluid has been a source of research for decades, especially regarding the acquisition of cells (amniocytes) for fetus cultivation and genetic study.

Amniotic fluid contains a heterogeneous population of differentiated and non-differentiated cells from the three germinative layers (Bossolasco et al., 2006). Most of the cells are differentiated in epithelial tissue and tend to present very limited proliferative capacity and differentiation. Within the first trimester, and up to the middle of the second semester, the amniotic fluid consists of cells initially gathered from the scaling off of the vitellinic vesicle, and later of the embryo as well as the placenta and the amnion. From the keratinization of the fetal skin between the 17th and 20th week of gestation, the deglutition and the fetal diuresis become key factors in the composition of the amniotic fluid, considering that, in the second half of the gestation period, the amniotic fluid consists mainly of fetal urine. Secretions within the respiratory and gastrointestinal tracts also contribute to the "pool" of cells within the amniotic fluid (Hoehn and Salk, 1982; Gosden, 1983; Torricelli et al., 1993; Brace, 1997; Trounson, 2007; Marcus and Woodbury, 2008).

The cells obtained from fetal tissues, such as placenta and amniotic fluid, have proven to be a good option for treatments, since they appear to have multipotent cell characteristics with no ethical, legal, or technical obstacles concerning the manipulation of embryonic stem cells (Bossolasco et al., 2006; De Coppi et al., 2007).

Recent observations in cell cultures from amniotic fluid provide evidence that these may well represent new sources for the isolation of mesenchymal stem cells (MSCs), which is further supported by the fact that samples can be easily accessed for cultures and the natural restoration of the aspirated volume (Tsai et al., 2004). In addition, several authors have reported that the amniotic fluid contains cells that express the antigen Oct-4, a specific marker of multipotent stem cells (Prusa and Hengstschlager, 2002; Prusa et al., 2003; Karlmark et al., 2005; Miki et al., 2005; Tsai et al., 2004, 2006; Kim et al., 2007).

The multipotent capacity of MSCs, their easy isolation and culture, in addition to their great potential for differentiation into different cell types and expansion *in vitro*, make these cells an attractive therapeutic tool for a wide variety of clinical applications (Minguell et al., 2000; Koç and Lazaru, 2001; Schoeberlein et al., 2005). The aforementioned aspects make the MSCs from amniotic fluid a possible source of potential use in cell therapy, in turn reducing ethical issues as compared to those seen in the use of embryonic stem cells (Prusa et al., 2003; Kim et al., 2007).

The study of the transcriptional control, which is achieved through SOX2 and NANOG transcription factors, offers an initial clarification of part of the multipotent capacity of the MSCs. When these factors are activated, several differentiation suppressor genes, such as Dnmt3b, Foxd3, Id4, Jmjd2c, and Suz12, are also transcriptionally activated. However,

when these transcription factors are suppressed, the genes responsible for the differentiation are activated (Boyer et al., 2005; Chew et al., 2005; Chickarmane et al., 2006; Ivanova et al., 2006; Loh et al., 2006; Greco et al., 2007; Sharov et al., 2008).

It is known that extensive subcultures for isolation can block cell functions through the emergence of clear signs of senescence and apoptosis. Considering that this sample preservation technique has certain disadvantages, such as limited cell survival, high cost, and a risk of contamination, cryopreservation becomes an attractive technique for the preservation of biological samples. Since the beginning of cryobiology, researchers have sought to gain a greater understanding of the events that control cell behavior during freeze-thaw cycles (Karlsson and Toner, 1996; Diller, 1997; Massumoto et al., 1997). From this perspective, the cryopreservation technique has been carefully reviewed in a number of prior studies so as to obtain protocols that cause less damage to the cells of various biological samples (Massumoto et al., 1997).

This study aimed to verify the behavior of MSCs from human amniotic fluid in culture when submitted to cryopreservation; assess their chromosomal stability; determine the maintenance of multipotent capacity through differentiation in other cell types; as well as to analyze SOX2, and NANOG persistence expression after thawing.

MATERIAL AND METHODS

Amniocentesis

Five women, in the second and third trimesters of pregnancy, who presented some form of obstetric, clinical indication for the performance of amniocentesis, were included in this study. After obtaining the patients' verbal and written consent, the following technique was implemented. A maternal abdomen ultrasound scan, using GE Loqic 5 equipment and a convex probe of 3.5 mHZ, was conducted to determine the site of puncture. After, abdomen antisepsis, with povidine iodine-topic solution and anesthesia on the maternal abdomen skin, using 2 to 3 mL 1% lidocaine, with no vasoconstrictor, was introduced into the uterine cavity by means of a BD-20G needle, viewed through ultrasound. After having collected an initial sample for the primary purpose of examination, 20 mL amniotic fluid was collected for this study.

This study was approved by the Committee on Ethics in Research from the Federal University of Minas Gerais (UFMG-COEP).

Cell culture

Approximately 20 mL amniotic liquid was centrifuged at 1000 rpm for 10 min. The supernatant was discarded, and the button of total cells was resuspended in 5 mL supplemented culture medium Amniomax (Invitrogen®). The content of the tubes was transferred to T25 bottles or blades, which were incubated with 5% CO_2 at 37°C for 5 days. After this period, the medium was exchanged each 3 to 4 days, and the cultures were observed daily as regards the number and confluency of the colonies.

After reaching 80% confluency, the culture was submitted to the three cellular passages in which the cells were detached, using 0.25% Trypsin-EDTA (Invitrogen®), resuspended in the amniomax culture medium and incubated with 5% CO_2 at 37°C for two days.

At the end of this process, to reach 80% of confluency, three final bottles (1, 2, 3) and 6 cultures in blades (4, 5, 6, 7, 8, 9) of adherent cells with fibroblastoid aspects were gathered. Culture 1 was prepared to perform a karyotype analysis; culture 2 was prepared to test the genic expression; culture 3 was cryopreserved, while cultures 7, 8, and 9 were induced into the adipogenic, chondrogenic, and osteogenic differentiations, respectively. Cultures 4, 5, and 6 were used as negative controls of differentiation.

Karyotype

The culture cell division was halted using Kario Max Colcemid Solution (Invitrogen®). Subsequently, the cells were loosened with 0.25% Trypsin-EDTA solution, resuspended in hypotonic solution, 0.075 M KCl (Merck®), and set with methanol/acetic acid solution in a 3:1 proportion (Merck®) (Jean and Katheleen, 1997).

The final cell package was submitted to spreading on a slide, G banding, and chromosomal analysis according to ISCN (International System for Human Cytogenetic Nomenclature).

Cell count by hemocytometer and cell viability analysis

In order to establish the number of cells for RNA extraction, and immunophenotyping for cryopreservation, the cell suspensions were always counted. For this, 20 µL cell suspension was added to 20 mL 0.4% Trypan blue solution (Sigma Chemical, St. Louis, MO, USA). Each sample was homogenized, and 10 mL was transferred to the hemocytometer.

Cell viability, assessed in samples before cryopreservation and after cryopreservation and thawing, was determined by counting the number of stained cells (nonviable) in relation to the total number of cells (cells stained + non-stained cells), and expressed in a percentage. It was counted for each sample a minimum of 100 cells.

Flow cytometric analysis of MSCs

To evaluate the lineage and surface marker phenotype of the cultured MSCs, cells (5 x 10^6 cells/mL) were detached and incubated in ISOTON® II (Beckman Coulter®) with the following fluorescent antibodies: CD14 FITC (BD Pharmingen®) as a monocyte marker; CD90 FITC (BD Pharmingen®) and CD105 PE (Beckman Coulter®) as an MSCs marker; CD34 PE (BD Pharmingen®) as a hematopoietic progenitor cell; CD45 FITC (BD Pharmingen®) as a pan leukocyte marker; CD73 PE (BD Pharmingen®) as a marker of activated T lymphocytes and MSCs; HLA-DR PE-Cy5 (BD Pharmingen®) as a marker of activated T lymphocytes or natural killer cells, and 7-amino-actinomycin D (7-AAD) (BD Pharmingen®) as a viability marker. Cells were analyzed on a fluorescence-activated cell sorter FC 500 MCL System (Beckman Coulter®) with 10,000 events stored.

Cryopreservation

For each patient, one flask of culture, containing 1 mL (1 x 10^4 cells) final cell suspension, was cryopreserved.

The cell suspension was transferred to 2 mL cryogenic tubes, and dimethyl sulfoxide (DMSO) (Cryoserv®) was added to a final concentration of 10% (v/v). The required volume of DMSO was added slowly and the tubes slowly homogenized. Next, the tubes were transferred to the programmed freezing equipment, where they remained for 10 min at 4°C, and were then frozen at a freezing rate of -1°C/min until they reached a final temperature of -80°C. The freezing protocol was optimized to produce an initial cooling stage of 1°C/min up to 4°C, followed by a cooling of 10°C/min until -40°C and a heating up of 12°C/min up to -15°C/min. After, a cooling of 1°C/min to -40°C, a further stage of cooling at a rate of 10°C/min up to -80°C was performed. Finally, cryogenic tubes containing the cells were placed in liquid nitrogen (Woods et al., 2003).

Adipogenic differentiation

The medium of culture 7 was changed by means of a mixture of the medium Amniomax together with 14% Supplement (Invitrogen®), 1 µM dexamethasone, 0.5 µM 3-isobutyl-1-methylxanthine, 0.05 mg/L human insulin, and 200 µM indomethacin (all from Sigma®) (Kim et al., 2007). The medium was exchanged every 3 days, and the cultures were observed daily concerning the intracellular presence of lipid drops, which was confirmed by the appearance of a red oil stain after 2 weeks (Laboratory Methods in Histotechnology; Prophet et al., 1994).

Chondrogenic differentiation

The medium of culture 8 was changed by creating a mixture of the medium DMEM high glucose (Invitrogen®) together with 10 mg/mL bovine pancreas insulin, 5.5 mg/mL human transferrin, 5 µg/mL sodium selenite, 0.5 mg/mL bovine serum albumin, 4.7 mg/mL linoleic acid (ITS + 1), 0.1 µM dexamethasone, 50 µg/mL acid ascorbic-2-phosphate, 100 µg/mL sodium pyruvate, 40 µg/mL L-prolin, and 10 ng/mL transforming growth factor-B1 (all from Sigma®) (Kim et al., 2007). The medium was exchanged every 3 days, and the cultures were observed daily. The differentiation was confirmed by the appearance of a periodic acid-Schiff-Alcian blue (PAS-AB) staining pattern after 3 weeks (Prophet et al., 1994).

Osteogenic differentiation

After reaching 80% confluency, the medium of culture 9 was changed by formulating a mixture of the DMEM high glucose medium, together with 10% fetal bovine serum (Invitrogen®), 0.1 µM dexamethasone, 10 mM B glycerol-phosphate, 50 µM L-ascorbic acid, and 10 nM 1,25-dihydroxy-vitamin D3 (Baksh et al., 2007). The medium was exchanged every 3 days, and the cultures were observed daily. The differentiation was confirmed by the appearance of a von Kossa stain after 2 weeks (Prophet et al., 1994).

Gene expression

The total RNA from 1 x 10^6 cells was obtained by extraction using silica gel. The genomic DNA was extracted using the enzyme DNAse free of RNAse (Promega®). The expression verification of NANOG and SOX2 stem cell marker genes was obtained by RT-PCR

using 2.5 U polymerase rTth DNA enzyme (Applied Biosystems®), 1X enzyme buffer, 0.16 mM dNTP, 2.5 mM Mn, and 0.4 pmol of each primer. The β-globin gene was used as internal control and the conditions of cDNA amplification were: 2.5 U polymerase rTth DNA enzyme, 1X enzyme buffer, 0.12 mM dNTP, 2.5 mM Mn, and 0.16 pmol of each primer. The primers used for stem cell markers were: SOX2-F 5'-GCCGAGTGGAAACTTTTGTC-3', SOX2-R 5'-GTTCATGTGCGCGTAACTGT-3', NANOG-F 5'-CATGAGTGTGGATCCAGCTTG-3', and NANOG-R 5'-CCTGAATAAGCAGATCCATTG-3'. The β-globin internal control primers were PC04 5'-CAACTTCATCCACGTTCACC-3' and GH20 5'-GAAGAGCCAAGGACAGGTAC-3'.

RT-PCRs for SOX2 and NANOG markers were performed using the Thermal 2400 (Applied Biosystems®) according to the following program: a 30-min cycle at 60°C, followed by a denaturation cycle at 95°C for 5 min; 45 cycles at 94°C for 30 s, at 57°C for 30 s, and at 72°C for 1 min, and a final extension stage of 72°C for 10 min. For β-globin amplification, the following program was used: one cycle of 30 min at 60°C, followed by a denaturation stage at 94°C for 5 min; 5 cycles at 94°C for 1 min, at 55°C for 1 min, at 72°C for 1 min; 35 cycles at 94°C for 1 min, at 53°C for 1 min, and at 72°C for 1 min, and a final extension stage at 72°C for 5 min. The reaction products were placed on 2% agarose gel, stained with ethidium bromide, and viewed through an FMBIO® fluorometer.

Thawing and cell cultures after cryopreservation

The thawing of the tube was carried out after 150 days of freezing. The tube was thawed to 37°C and prepared according to that previously described for the initial cell cultures. In the same manner, the cells were again submitted to differentiation in adipogenic, chondrogenic, and osteogenic cultures, according that previously described.

For gene expression studies after cryopreservation, the cells grown for 150 days were submitted to three other stages of culture before performing the gene expression evaluation so that the expressed residual messenger RNA present before cryopreservation would not interfere in the results after freezing.

RESULTS

After the final passages, the cells presented adherent and fibroblastoid characteristics, according to that expected for mesenchymal stem cells (Figure 1). To identify the original chromosomal constitution, and whether or not any detectable change had occurred after culture or cryopreservation, a karyotype analysis with G-banding was performed before the culture, after three passages, and after 150 days of cryopreservation. Karyotype 46,XX (in three samples tested) and 46,XY (in two samples tested) could be observed, for all times evaluated, confirming that no chromosomal alterations had occurred (Figure 2).

Cell viability rate was evaluated from cell cultures after three passages and showed a mean of 94.2 ± 1.55%; after 150 days of cryopreservation showed a mean viability of 88.8 ± 1.75%. The average loss of viability occurred in 5.4%.

The MSCs expressed the markers CD90, CD105 and CD73. However, the MSCs did not express CD14, CD45, CD34, and II HLA-DR. These cells were used in subsequent experiments. The cell viability was higher than 90%.

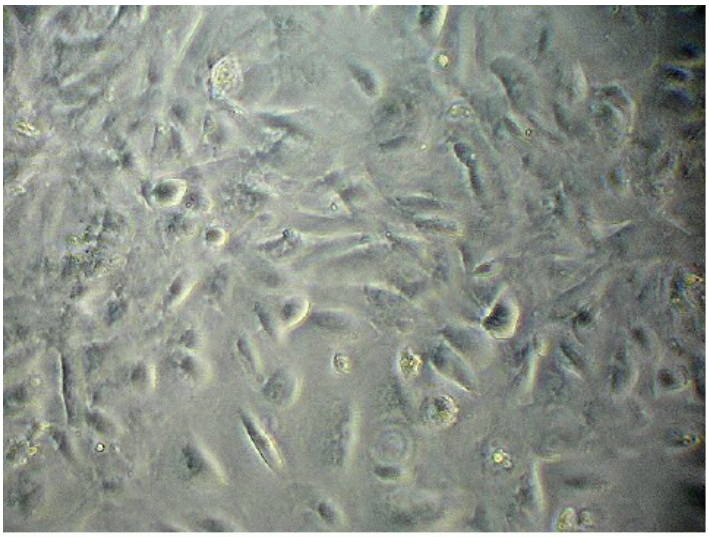

Figure 1. Amniotic fluid stem cells in culture after cryopreservation, showing fibroblastoid characteristics.

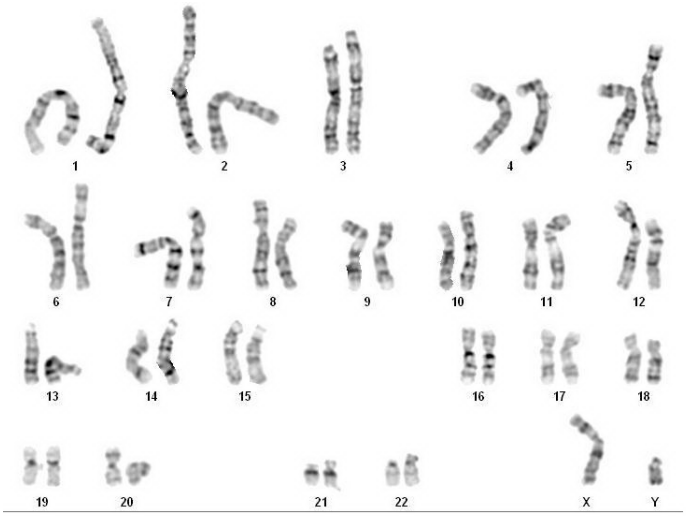

Figure 2. Example of karyotype obtained after cryopreservation.

When submitted to an adipogenic induction for two weeks, the cells presented a large number of lipid droplets stained by oil red (Figure 3A), along with cells with similar aspects to that of adipocytes. When submitted to an osteogenic induction for two weeks, the cells presented an aggregation of micronodules or calcium deposit, stained by von Kossa method (Figure 3B). The chondrogenic differentiation, performed over a 3-week period, could be observed using PAS-AB stain, in which a large number of vacuolized cells, which proved to be positive for the above mentioned staining, could be observed (Figure 3C). The same results

were obtained before and after cryopreservation. Cultures 4, 5, and 6, used as the negative control, were dyed by the same methods cited earlier, but were not induced into the differentiation. None of these control cultures presented similar structures or organelles as previously cited (data not shown).

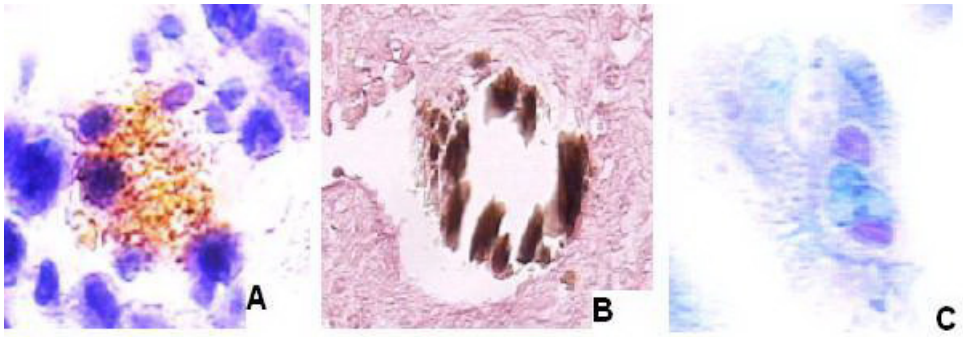

Figure 3. Aspect of the cells after differentiation. **A.** Aspect of the cells after adipogenic induction, showing lipid droplets by oil red stain. **B.** Osteogenic induction showing an aggregation of micronodules or calcium deposit using von Kossa staining. **C.** Chondrogenic differentiation showing vacuolized cells, which proved to be positive for PAS-AB staining.

Amplification products were observed by means of an RT-PCR technique of stem cell transcription factors - SOX2 and NANOG - before and after cell cryopreservation to evaluate the continuity of the expression of these genes under the effect of cryopreservation. Fragment of 264 and 192 bp, corresponding to the amplification of the SOX2 and NANOG genes, respectively, could be observed. This exact pattern remained unchanged before and after the cryopreservation in the five samples studied. Moreover, the control β-globin amplified in all the evaluated samples and times of evaluation (Figure 4).

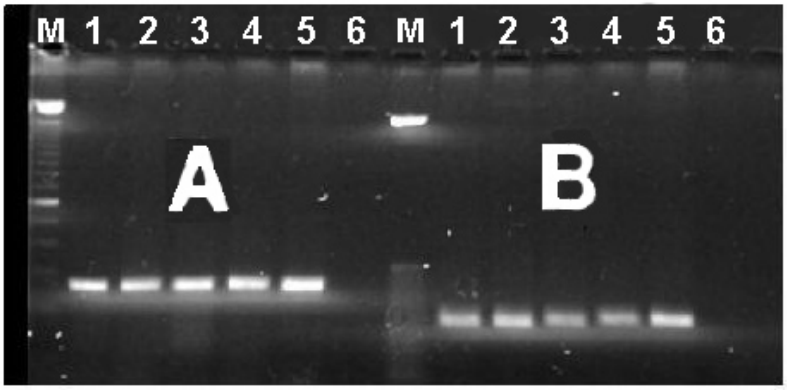

Figure 4. Gene expression of SOX2 and NANOG in stem cells from amniotic fluid. **A.** and **B** are fragments obtained in the RT-PCR of SOX2 and NANOG genes, respectively, before cryopreservation (*lanes 1* and *2*) and after cryopreservation (*lanes 3*, *4*, and *5*). *Lane 6* corresponds to the negative reaction control. *Lane M* = standard molecular weight = 100 bp.

DISCUSSION

There is a vast field of research constantly vigilant for the possible therapeutic applications of MSCs in many metabolic, genetic, immunological, and degenerative diseases in which a loss of structural or functional specific tissue (diabetes, cystic fibrosis, neurological diseases, heart diseases, connective tissue diseases, and hematopoiesis) may occur.

A substantial amount of research has revealed a great interest in the study of the properties of MSCs obtained from amniotic fluid, the possibilities of isolation and culture, the capacities of differentiation, and, most importantly, its potential application in cell therapy (Prusa and Hengstschlager, 2002; Miki et al., 2005; Tsai et al., 2006). These cells have shown a great capacity for differentiation *in vitro* (Karlmark et al., 2005; Kim et al., 2007).

Although all the studies cited herein demonstrate the ability of the cells extracted from amniotic fluid to differentiate themselves into several distinct cell types and indicate a therapeutic potential, it is known that cells kept in culture for relatively long periods of time result in the cell aging process and may lose characteristics, such as plasticity (Bonab et al., 2006).

This study developed a new protocol for isolation, confirmed through flow cytometric analyses, and cryopreservation of MSCs.

The karyotype analysis of these cells indicates that both culture and cryopreservation do not lead to the emergence of chromosomal aberrations, an extremely important fact when considering possible therapeutic use.

Moreover, it could be observed that the capacity of differentiation in adipogenic, chondrogenic, and osteogenic cells of the MSCs was maintained after freezing over a 5-month period, confirming its great potential to generate cells from different tissues. This detail becomes particularly important when one considers that cryopreservation maintains the cell's capacity for proliferation when submitted to a new culture and does not affect its stem cell characteristics, as a capacity of differentiation.

This study also showed that the cells isolated from amniotic fluid express the NANOG and SOX2 genes, which act as markers of MSCs by increasing their proliferate capacity. It could also be observed that even after 5 months of cryopreservation and subsequent thawing, cells maintained the ability to express NANOG and SOX2, corroborating with previous reports that the MSCs after cryopreservation maintained their capacity for proliferation.

MSC cryopreservation from amniotic fluid is of clinical importance, since these cells can be harvested while still in the prenatal period (younger cells with a greater capacity for differentiation) and may be stored to be used in treatments in the immediate post-partum period or later.

Cells derived from amniotic fluid have proven to be of great promise in the field of cell therapy and its probable application in autologous tissue regeneration. This study suggests that the development of the amniotic fluid cell cryopreservation technique can represent an extremely useful tool in the maintenance of these cells collected during pregnancy.

We demonstrated that MSCs from amniotic fluid obtained by amniocentesis can be successfully cryopreserved, which provides an abundant source for research on human MSCs. Moreover, it opens a new way for autologous cellular therapies without inducing tissue rejection and it does not raise any ethical issues that are associated with human embryonic stem cell research. Considering the easy culture and cryopreservation, the amniotic fluid may provide an excellent alternative source for the potential therapeutics of MSCs.

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REFERENCES

Baksh D, Yao R and Tuan RS (2007). Comparison of proliferative and multilineage differentiation potential of human mesenchymal stem cells derived from umbilical cord and bone marrow. *Stem Cells* 25: 1384-1392.

Bonab MM, Alimoghaddam K, Talebian F, Ghaffari SH, et al. (2006). Aging of mesenchymal stem cell *in vitro*. *BMC Cell Biol.* 7: 14.

Bossolasco P, Montemurro T, Cova L, Zangrossi S, et al. (2006). Molecular and phenotypic characterization of human amniotic fluid cells and their differentiation potential. *Cell Res.* 16: 329-336.

Boyer LA, Lee TI, Cole MF, Johnstone SE, et al. (2005). Core transcriptional regulatory circuitry in human embryonic stem cells. *Cell* 122: 947-956.

Brace RA (1997). Physiology of amniotic fluid volume regulation. *Clin. Obstet. Gynecol.* 40: 280-289.

Chew JL, Loh YH, Zhang W, Chen X, et al. (2005). Reciprocal transcriptional regulation of Pou5f1 and Sox2 via the Oct4/Sox2 complex in embryonic stem cells. *Mol. Cell Biol.* 25: 6031-6046.

Chickarmane V, Troein C, Nuber UA, Sauro HM, et al. (2006). Transcriptional dynamics of the embryonic stem cell switch. *PLoS Comput. Biol.* 2: e123.

De Coppi P, Bartsch G Jr, Siddiqui MM, Xu T, et al. (2007). Isolation of amniotic stem cell lines with potential for therapy. *Nat. Biotechnol.* 25: 100-106.

Diller KR (1997). Engineering-based contributions in cryobiology. *Cryobiology* 34: 304-314.

Gosden CM (1983). Amniotic fluid cell types and culture. *Br. Med. Bull.* 39: 348-354.

Greco SJ, Liu K and Rameshwar P (2007). Functional similarities among genes regulated by OCT4 in human mesenchymal and embryonic stem cells. *Stem Cells* 25: 3143-3154.

Hoehn H and Salk D (1982). Morphological and biochemical heterogeneity of amniotic fluid cells in culture. *Methods Cell Biol.* 26: 11-34.

Ivanova N, Dobrin R, Lu R, Kotenko I, et al. (2006). Dissecting self-renewal in stem cells with RNA interference. *Nature* 442: 533-538.

Jean HP and Katheleen WR (1997). Pre Natal Chromosome Diagnosis. In: The AGT Cytogenetics Laboratory Manual (Barch MJ, Knutsen T and Spurbeck JL, eds.). Lippincott-Raven, Philadelphia, 199-258.

Karlmark KR, Freilinger A, Marton E, Rosner M, et al. (2005). Activation of ectopic Oct-4 and Rex-1 promoters in human amniotic fluid cells. *Int. J. Mol. Med.* 16: 987-992.

Karlsson JO and Toner M (1996). Long-term storage of tissues by cryopreservation: critical issues. *Biomaterials* 17: 243-256.

Kim J, Lee Y, Kim H, Hwang KJ, et al. (2007). Human amniotic fluid-derived stem cells have characteristics of multipotent stem cells. *Cell Prolif.* 40: 75-90.

Koç ON and Lazarus HM (2001). Mesenchymal stem cells: heading into the clinic. *Bone Marrow Transplant.* 27: 235-239.

Loh YH, Wu Q, Chew JL, Vega VB, et al. (2006). The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. *Nat. Genet.* 38: 431-440.

Marcus AJ and Woodbury D (2008). Fetal stem cells from extra-embryonic tissues: do not discard. *J. Cell Mol. Med.* 12: 730-742.

Massumoto CM, Mizukami S, Campos MF, Silva LA, et al. (1997). Cryopreservation of bone marrow and peripheral blood stem cells using a controlled rate freezing system: experience with 86 procedures. *Rev. Assoc. Med. Bras.* 43: 93-98.

Miki T, Lehmann T, Cai H, Stolz DB, et al. (2005). Stem cell characteristics of amniotic epithelial cells. *Stem Cells* 23: 1549-1559.

Minguell JJ, Conget P and Erices A (2000). Biology and clinical utilization of mesenchymal progenitor cells. *Braz. J. Med. Biol. Res.* 33: 881-887.

Prophet EB, Mills B, Arrington JB and Sobin LH (1994). Laboratory Methods in Histotechnology - Armed Forces Institute of Pathology. American Registry of Pathology, Washington.

Prusa AR and Hengstschlager M (2002). Amniotic fluid cells and human stem cell research: a new connection. *Med. Sci. Monit.* 8: RA253-RA257.

Prusa AR, Marton E, Rosner M, Bernaschek G, et al. (2003). Oct-4-expressing cells in human amniotic fluid: a new source for stem cell research? *Hum. Reprod.* 18: 1489-1493.

Schoeberlein A, Holzgreve W, Dudler L, Hahn S, et al. (2005). Tissue-specific engraftment after *in utero* transplantation of allogeneic mesenchymal stem cells into sheep fetuses. *Am. J. Obstet. Gynecol.* 192: 1044-1052.

Sharov AA, Masui S, Sharova LV, Piao Y, et al. (2008). Identification of Pou5f1, Sox2, and Nanog downstream target genes with statistical confidence by applying a novel algorithm to time course microarray and genome-wide chromatin immunoprecipitation data. *BMC Genomics* 9: 269.

Torricelli F, Brizzi L, Bernabei PA, Gheri G, et al. (1993). Identification of hematopoietic progenitor cells in human amniotic fluid before the 12th week of gestation. *Ital. J. Anat. Embryol.* 98: 119-126.

Trounson A (2007). A fluid means of stem cell generation. *Nat. Biotechnol.* 25: 62-63.

Tsai MS, Lee JL, Chang YJ and Hwang SM (2004). Isolation of human multipotent mesenchymal stem cells from second-trimester amniotic fluid using a novel two-stage culture protocol. *Hum. Reprod.* 19: 1450-1456.

Tsai MS, Hwang SM, Tsai YL, Cheng FC, et al. (2006). Clonal amniotic fluid-derived stem cells express characteristics of both mesenchymal and neural stem cells. *Biol. Reprod.* 74: 545-551.

Woods EJ, Liu J, Pollok K, Hartwell J, et al. (2003). A theoretically optimized method for cord blood stem cell cryopreservation. *J. Hematother. Stem Cell Res.* 12: 341-350.