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ISOLAMENTO E CARACTERIZAÇÃO DE CÉLULAS-TRONCO DO SACO VITELINO DE SUÍNOS DOMÉSTICOS (*Sus scrofa*)

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RESUMO: Anexos fetais como cordão umbilical, membrana amniótica e líquido amniótico foram recentemente sugeridos como fontes ideais de diferentes linhagens de células-tronco. As células-tronco embrionárias dão origem a células fetais, onde estas são responsáveis pelo crescimento e desenvolvimento fetal. Portanto, este estudo teve como objetivo analisar as células do saco vitelino para caracterizar esta como células e seu potencial como possível fonte de células-tronco pluripotentes para uso futuro em terapia regenerativa. O tecido foi coletado do saco vitelino de embriões com 30 dias de gestação, o tecido foi colocado com meio de cultura contendo 15% de soro fetal bovino e incubado a 37 ° C. Foram feitos ensaios para concentração e viabilidade celular, curva de crescimento, caracterização por citometria de fluxo (CD105, NANOG, CD45 e Out-3/4), imunocitoquímica (CD90, CD105, CD117, vimentina, Stro-1, Oct-4, VEGF, beta tubulina, citoqueratina e PCNA) e ensaios de diferenciação condrogênica, adipogênica e osteogênica. As células do saco vitelino mostraram aderência ao plástico e uma morfologia fusiforme, atingiram confluência de 70% em cerca de 20 dias, e essas células foram mantidas até passagem 4, onde ocorreu posterior morte celular. Essas células possuíam imunofenotipagem positiva para marcadores como CD105, CD90, CD117, vimentina, Stro-1, Oct-4, VEGF, Beta tubulina, citoqueratina, Nanog e PCNA. Logo, estas células podem ser classificadas como células mesenquimais e pluripotentes, ou seja, possuem auto renovação e diferenciação em várias linhagens, por exemplo, condrócitos, adipócitos e osteócitos. Sendo assim, as células do saco vitelino proporcionam uma fonte de células ideal para medicina regenerativa e engenharia de tecidos.

Palavras-chave: cultura de células; células-tronco mesenquimais; membranas extraembrionárias; saco vitelino suíno; *Sus scrofa*.

ISOLATION AND CHARACTERIZATION OF STEM CELLS FROM YOLK SAC DOMESTIC PORCINE (*Sus scrofa*)

ABSTRACT: Fetal attachments as umbilical cord, amnion and amniotic fluid have recently been suggested as ideal sources of different strains of stem cells. The embryonic stem cells give rise to fetal cells, where are responsible for fetal growth and development. Therefore, this study aimed to analyze the cells from the yolk sac to characterize this cells as to potential as a possible source of pluripotent stem cells for future use in regenerative therapy. The yolk sac tissue was collected from the embryos in the early stage of gestation (30d) and explants from YS were plated with medium culture 15% fetal bovine serum and incubated at 37ºC in a humidified atmosphere of 5% CO2. Trials were made for concentration and cell viability, cell growth evaluation, and the characterization by flow cytometry using specific antibodies (CD105, NANOG, CD45 and Oct-3/4), immunocytochemistry using the following antibody: CD90, CD105, CD117, vimentin, Stro-1, Oct-4, VEGF, beta tubulin,

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Cytokeratin and PCNA and Osteogenic, adipogenic, and chondrogenic differentiation assays. The yolk sac cells showed adherence to plastic and a spindle-like morphology, they reached confluence of 70% in about 20 days, and these cells were maintained until passage 4, where later cell death occurred. These cells had similar imunofenoipagem the mesenchymal stem cells and hematopoietic cells expressing markers such as CD105, CD90, CD117, vimentin, Stro-1, Oct-4, VEGF, Beta Tubulin, Cytokeratin, Nanog and PCNA. These cells can be classified as pluripotent cells, in other words, possessed self-renewal and multi-lineage differentiation potency, which could differentiate into chondrocytes, adipocytes and osteocytes. The pYS cells would provide an ideal cell resource for regenerative medicine and tissue engineering.

Key Words: cell culture; extraembryonics membranes; mesenchymal stem cell; swine yolk sac; *Sus scrofa*

INTRODUCTION

Embryonic stem (ES) cells are pluripotent cells that are able to generate a whole organism. The ES cells give rise to rapidly dividing fetal stem (FS) cells, which are responsible for fetal growth and development. After birth, FS cells are replaced by tissue-specific adult stem (TSS) cells. According to this classification, FS cells are operationally located between ES and TSS cells. In vitro cultured FS cells were shown to be isolated from extra-embryonic membrane and fetal tissues, including hematopoietic tissues (Evans and Kaufman, 1981; O'Donoghue and Fisk, 2004).

Embryonic membranes are of great importance to mammals, especially the yolk sac, it holds the primary development of the fetus produces a spectrum of serum proteins (Tiedemann and Minuth, 1980; Janzen *et al.*, 1982; Young and Klein, 1983; Shi *et al.*, 1985) and functions as a site of primaries blood cell formation (Liwska and Grabinski-Baranowski, 1994; Niimi *et al.*, 2002; Bertassoli *et al.*, 2012). Such functions are of particular importance for the swine because, in this species, the formation of the placenta is beginning only after the18th of the gestation (Talbot *et al.*, 2007).

Mesenchymal stem cells (MSCs) are defined as a group of multipotent stem cells that can functionally differentiate into at least bone, fat, and cartilage in vitro and in vivo. Under appropriate culture conditions, MSCs are readily amplified in vitro for several passages without signs of senescence and differentiation. These properties render them as an intriguing source in cell therapy and tissue engineering. These stem cells (MSCs) can be isolated from various organs of the body, such as for example, bone marrow, adipose tissue, synovial membrane, muscle, dermis, deciduous tooth, umbilical cord, placenta, liver, spleen and thymus (Meirelles *et al.*, 2006; Bertassoli *et al.*, 2013).

Pig is a main domestic animal and has many biophysical and biochemical similarity to human beings. The use of pig models for pre-clinical testing is well established and the availability of embryonic stem cells may open the way to pre-clinical experimentation for any kind of cell therapy. However, thus far, the stable embryonic stem cell lines have not yet been generated from pig embryos, but rather obtained by normal fertilization or by parthenogenetic activation (Keefer *et al.*, 2007; Chen *et al.*, 2011).

Another important characteristic about research with embryos of mammals, showing interest on stem cells from pig embryos, is that they can be exploited for the production of transgenic animals for use in xenotransplantation donors, and thus decrease the bounce rate (Kim *et al.*, 2010).

In this paper, we isolated, characterized and demonstrated the multipotency and plasticity of pYS cells. Our study revealed that these cells were similar to mesenchymal and hematopoietic stem cells, and could differentiate into cell derivatives of the three tissues: bone, cartilage and adipose tissue.

MATERIALS AND METHODS

Animals

This research study was approved by the Ethical Committee of the School of Veterinary Medicine and Animal Science of São Paulo University (n. 2659/2012). Swine fetuses (n=20) were obtained from female swine, race Landrace, previously inseminated, with 30 days of gestation, from the group Cabo Verde located in Monte Santo, Minas Gerais, Brazil and group Umburama located in Atibaia. The pregnant uterus was obtained through the slaughter of females in the municipal abattoir of the city.

Isolation and growth of pYS cells

The yolk sac tissue was collected from the embryos in the early stage of gestation (30d) and wash with PBS. Tissue explants from YS were plated in 6-wells culture plate with medium culture - Dulbeco's Modified Eagle Medium "D-MEM" (LGCBio), 15% fetal bovine serum (LGCBio) and incubated at 37ºC in a humidified atmosphere of 5% CO2. After 48 h, no-adherent cells were removed and the medium was replaced. When reached 80% confluence, cells were treated with 0.05% Trypsin- EDTA and replaced in 25 cm2 and 75 cm2 flasks (1 x 107 cells). The average cell numbers for each six wells were calculated and the growth curve was plotted with the average number of cells at different times.

Flow cytometry analysis

The surface antigens of pYS cells (passage 3) were examined by cytometry analysis. The cells were harvested by the treatment of trypsin and centrifuged 269 g for 10 minutes. The cells was washed three times with PBS and incubated with primary antibodies prepared in phosphate-buffered saline 0,9% (PBS) for 1 hour. To determine the surface antigens of pYS cells were stained with fluorescein isothiocyanate (FITC)-conjugated antibodies against CD105 (Abcam, UK), CD45 (Abcam, UK), Nanog (Santa Cruz, USA) and OCT-3/4 (Santa Cruz, USA). The flow cytometry analysis of the cells was completed by using FACS Calibur Flow cytometry Win Mdi 2.8 system. Ten thousand events were counted for each sample.

Immunophenotyping

The cells were grown at 37°C in a humidified CO2 incubator until they were 60–70% confluent, were washed twice in tris-buffered saline (20mM Tris-HCl [pH 7.4] 0.15M NaCl, and 0.05% Tween-20) and fixed for 24h in 4% paraformaldehyde solution, permeabilized with 0.1% Triton X-100 (Sigma) and blocked with 5% bovine serum albumin (BSA) for 1 h. The blocked cells were incubated for 1 hour with primary antibody at a dilution of 1:100 at room temperature. Primary antibodies used for immunophenotypic profile were as follows: antimouse vimentin (Santa Cruz, USA), anti-mouse oct-3/4 (Santa Cruz, USA), antimouse vascular endothelial growth factor (VEGF) (Santa Cruz, USA), anti-cytokeratin peptide 18 (Abcam, UK), antimouse Stro-1 (Abcam, UK), polyclonal anti -B – tubulin (Abcam, UK), antimouse PCNA-3 (Abcam, UK), anti-mouse CD117, (Abcam, UK) antimouse CD105 (Chemicon International, USA) and antimouse CD90 (Chemicon International, USA). After washing the antibodies thrice in tris-buffered saline, fluorescein isothiocyanate conjugated antibody was added and the antibodies were incubated for 40 min at room temperature. The cells were incubated with anti-rabbit secondary antibody conjugated to Texas-Red, anti-goat conjugated FITC (F0479, Dako Cytomation, CA, USA) or anti-mouse IgG conjugated FITC (AP308F, Chemicon International, Temecula, CA, USA) at a dilution of 1:100 for one hour in a dark chamber. Microscope slides were mounted in Vectashield mounting medium with or without 40.6-diamidino-2-phenylindol (DAPI; Vector Laboratories). Digital images were acquired with a cooled CCD camera (PCO, VC44) and processed using the ISIS software (MetaSystem).

Osteogenic, adipogenic, and chondrogenic differentiation assays

All differentiation assays were performed in triplicate. To promote osteogenic differentiation, 1x104 cells were plated in six well plates in culture basal medium. After 24 h, the medium was changed, and osteogenic medium

composed of Dulbecco's modified Eagle's medium (DMEM)-low glucose (LGCBio), 2% fetal bovine serum (FBS), 50mM ascorbate-2- phosphate (Invitrogen), and 0.1mM dexamethasone (Sigma) was added and changed every 3 days. After 10 days, osteogenic medium was supplemented with 10mM b-glycerolphosphate (Sigma). At day 21, cells were washed twice in PBS, fixed for 24 h in 4% paraformaldehyde, and stained with von Kossa reagent (Invitrogen).

Adipogenic differentiation was performed according to the protocol for canine MSCs. A total of 1x104 MP cells were plated in six-well plates. After 24 h, adipogenic differentiation was induced by addition of DMEM-high glucose (HG) supplemented with 2% FBS, 1mM dexamethasone, 100mM indomethacin (Sigma), 0.5mM 1-methyl-3-isobutyxanthine (Sigma), 10 mg/mL insulin, and 1% antibiotic. The medium was changed every 3 days. At day 10, differentiated cells were washed twice in PBS and fixed as just described. Oil Red O (Sigma) staining was used to detect intracellular lipid accumulation.

Chondrogenic differentiation was carried out using pellet culture technique. For the preparation of each pellet, YS cells were trypsinized, counted, and 2x106 cells were resuspended in culture medium containing DMEM-HG supplemented with 6.25 mg/mL insulin, 6.2 mg/mL transferin, 6.25 mg/mL selenous acid, 5.33 mg/mL linoleic acid (ITS; Premix, BD Biosciences), 0.1 Mm dexamethasone, 1mM sodium pyruvate, 50mg/mL ascorbate-2-phosphate, and without transforming growth factor b1 (R&D System, LGC Biotechnology). YS cells were maintained at 37ºC in a humidified atmosphere with 5% CO2, for 21 days, by changing chondrogenic culture medium every day. After that, cell aggregates were embedded in paraffin (Media-Paraplast Plus, Oxford Lab.). Sections were obtained at 4–5 mm using a Leica microtome and stained with Masson Trichrome following the routine protocol (Sigma).

RESULTS

The pYS cells isolated from yolk sac tissue

The porcine yolk sac was collected in the early stage gestation (30 days, Fig. 1A). After 7 days of culture, both fibroblast-like cells and epithelioid cells appeared. For 2 to 3 passages, most cells exhibited fibroblast-like phenotype (Fig. 1B e C). In early culture passages due to the existing mixed cell populations and cells not adherent was removed in the medium culture change. After 2 passages, the cell populations exhibited the uniform fibroblast-like morphology. The pYS cells were maintained until passage 6, after this they began to undergo cell death with low rates of survival and proliferation (Figure 1D)

For routine culture, cells were grown in the 35 mm culture plate, and when reached 80% confluence (0.86102 cells/plate), cells in one plate were replaced in 25 cm2 flasks. The cells were frozen at passage 4.

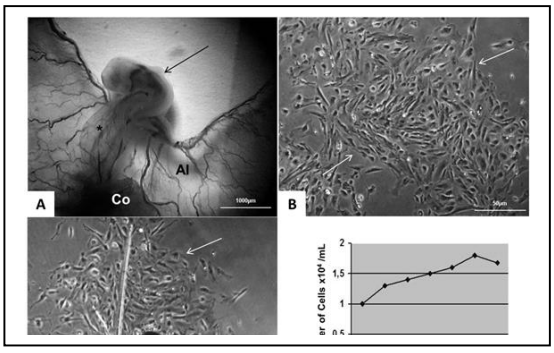

Figure 1 - Isolation of pYS cells from the porcine fetus. (A) The porcine fetus with the extraembryonic membranes: allantois (Al), chorion (Co), Yolk sac (asterisk), amnion (thin arrow). The yolk sac was collected under sterile condition. (B e C) The morphology of pYS cells after

culturing for 3 passages in vitro. Most cells exhibited fibroblast-like shape (arrow). (D) The growth curve of the pYS cells (at 2th passage).

Characterization of pYS cells

Flow cytometry was used to determine whether the morphologically homogenous FACs possessed uniform surface markers. As demonstrated in Figure 3 and 4, the pYS cells were found to be positive for CD105, Nanog and Oct3/4, in contrast, small quantity of cells were labeled for CD45 (almost nil), indicating these cells as pluripotent and mesenchymal.

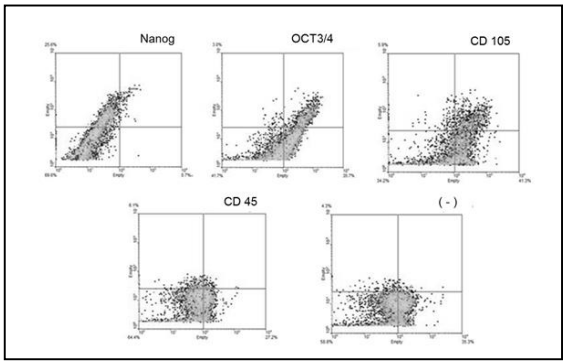

Figure2 – Surface markers of FACs from porcine YS cells. Flow cytometric analysis shows that the immunophenotypes of FACs from pYS cells. They are positive for CD105 (SH2), Nanog, Oct3/4 and negative for CD45.

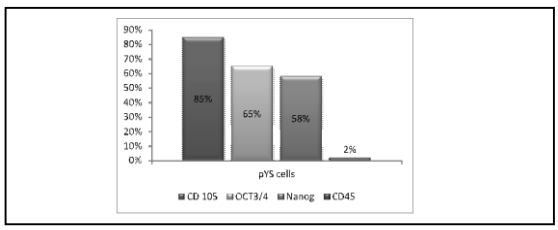

Figure 3 – Figure supplementary of flow cytometry marker CD105, Oct3/4, Nanog and CD45 in percentage.

The immunofluorescence analysis of specific markers and showed that pYS cells expressed some marker of mesenchymal stem cells (CD105, CD90, vimentin and cytokeratin) (Figure 6), hematopoetic stem cells (CD117), pluripotent stem cells (Stro-1 and Oct-4) and cellular proliferation (PCNA) (Figure 7). These results revealed that the isolated pYS might be at the pluripotent cell transition from the embryonic stem cell phase to the mesenchymal and hematopoietic cells

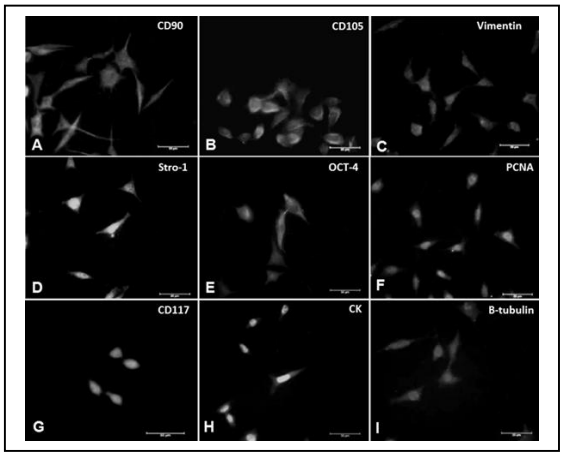

Figure 4 - Immunofluorescence in pYS cells. (A, B, C) Mesenchymal surface marker (CD90, CD105 and Vimentin), (D, E) pluripotent surfece marker (Stro-1 and Oct-4), (F) cellular proliferation (PCNA), hematopoietic surface marker (CD117) and cytoskeletal marker for control (Citokeratin-18 and B-tubulin). Nuclei stained with DAPI (blue). Scale bar: 50µm.

Diferentiation of pYS cells

Adipogenic differentiation was induced under conditions that are routinely used for MSCs (FERNANDES *et al.*, 2012), and adipose cell formation was observed. Oil Red staining demonstrated the presence of lipid droplets within the cytoplasm (Figure 5A). Control pYS cell culture maintained in basal culture medium did not show lipid droplets (Figure 5D).

Osteogenic differentiation of the pYS cells occurred within 21 days. Formation of a robust extracellular matrix

and mineralization was revealed by Von-Kossa staining in the cells from pYS maintained in culture medium, which induces osteogenic differentiation (Figure 5B). Control pYS cell culture maintained in basal culture medium did not show mineralization of extracellular matrix (Figure 5E).

The chondrogenic differentiation capability of p YS cells was tested with a cell pellet formation protocol. Cells were able to undergo chondrogenic differentiation. Masson Trichrome staining demonstrated the presence of large collagen areas, which were more robust in the cell pellet culture from pYS cells (Figure 5C). Control pYS cell culture maintained in basal culture medium did not show collagens areas (Figure 5F).

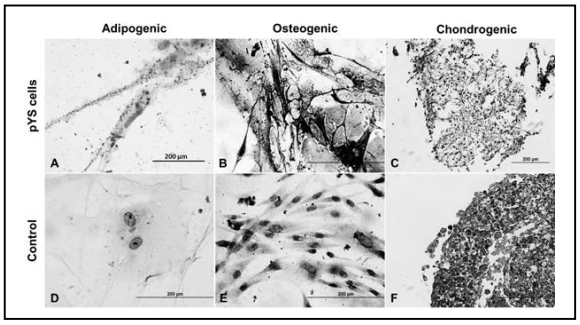

Figure 5 – Differentiation of pYS stem cells. (A) Adipogenic differentiation, (B) Osteogenic differentiation, (C) Chondrogenic differentiation, (D) control adipogenic differentiation, (E) control osteogenic differentiation, (F) control chondrogenic differentiation. Scale bar: 200µm.

DISCUSSION

The cells derived from the porcine yolk sac possessed morphology spindle-shaped, justifying his classic description of fibroblastoid, large nucleus and projections at both ends, findings that come into agreement with Ringe *et al.* (2002), Tagami *et al.* (2003), Bosch *et al.* (2006) where the authors reported in their study that MSCs have the format described above.

Deans and Moseley (2000) reported in their study that the MSCs when cultured at low density the adhesion and formation of colonies is rapid, results that corroborate our results with MSCs yolk sac (80% confluence in about 10 days).

Stem cell suffer from the passages and tripsinization, occurring to a slow proliferation and cell death (Fehrer and Lepperdinger, 2005), corroborating this study is ours, where we can see the decrease of proliferation and increased cell death from the already passage 6 (Figure 1D).

The yolk sac cells showed positivity for markers such as CD105, CD90, Stro-1, OCT-4 and PCNA, markers of mesenchymal stem cells, proliferation and pluripotency, plastic adherence, fibroblastoid morphology and when grown in low concentration, showed a high proliferation, thus, according Kolf *et al.* (2007) and Lacono *et al.* (2010) these characteristics are proposed by the International Society for Cellular Therapy for classification of mesenchymal cells.

According to Sabatini *et al.* (2005) and Souza *et al.* (2010) there are several markers of mesenchymal stem cells, however, the markers that have been used to better selection of mesenchymal stem cells are Stro-1, CD90 and CD 105, soon, when subjected to immunocytochemistry, the yolk sac cells expressed these markers, which makes us believe in the potential of these cells like mesenchymal cells.

CD105 (endoglin) is express in proliferating vessels during angiogenesis (Vasconcelos *et al.*, 2011), i.e., is expressed in cells that give rise to other cells (stem cells). In cells in the yolk sac was shown that expression immunocytochemistry on, so these cells are derived from the yolk sac of the embryo initial vasculogenesis process.

Vimentin, and cytokeratin-18 are cytoskeletal intermediate filaments and are commonly used to define stem cells (Wenceslau *et al.*, 2011). Vimentin is expressed in various non-epithelial cells, especially in MSCs of fetal and adult origin (Bittencourt *et al.*, 2006). In this study the pYS cells reacted positively to vimentin. Human fetal MSC from blood, LV, BM, and kidney also showed to be vimentin-positive (O'Donoghue and Fiks, 2004). Cytokeratin-18, known to represent the epithelial-mesenchymal cells transition, was expressed in. Accordingly, its expression has also been reported in cells derived of extraembryonics membranes in dogs (Wenceslau *et al.*, 2011; Fernandes *et al.*, 2012), fetal liver in mice and progenitors form umbilical cord (Rosada *et al.*, 2003). These results lead us to think again that these cells studied here have great features of mesenchymal stem cells, as also expressed these markers when used the immunocytochemistry technique.

The flow cytometry analysis revealed positive expression of Oct3/4 and Nanog of the cells from YS. These transcription factors are usually expressed in early stages of development (morula, blastocyst) reflecting the stem cell pluripotency (Evans and Kaufman, 1981)

In contrast to the above mentioned, regarding the classification of these cells as mesenchymal results, these cells were positive for CD117, being this marker expressed during vasculogenesis. Its expression among cell populations isolated from YS may suggest the presence of hemangioblasts, which are common precursors of hematopoietic and endothelial cells (Auerbach *et al.*, 1996; Cumano *et al.*, 2001; Palis and Yoder, 2001; Meirelles *et al.*, 2006). This result indicates that the yolk sac might be responsible for production of blood cells and endothelial, which initiates the the construction of the embryonic vascular plexus (Baron, 2003; ChoiI, 2002; Galdos *et al.*, 2010; MCgrath and Palis, 2005; Santos *et al.*, 2012).

Finally, the pYS cells were able to undergo both osteogenic, chondrogenic and adipogenic differentiation. In early studies, human (Campagnoli *et al.*, 2001), canine (Wenceslau *et al.*, 2011; Fernandes *et al.*, 2012) and porcine fetals cells (Chen *et al.*, 2011) were also able to undergo the three tissues differentiation.

CONCLUSION

In conclusion, we successfully isolated and characterized pYS cells. These cells can be classified as pluripotent cells, in other words, possessed self-renewal and multi-lineage differentiation potency, which could differentiate into chondrocytes, adipocytes and osteocytes. The pYS cells would provide an ideal cell resource for regenerative medicine and tissue engineering. Moreover, the molecular mechanisms involved in the self-renewal and multipotency of pYS-MSCs remains unclear and need to be further investigated.

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