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Gene expression of stem cells at different stages of ontological human development

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ABSTRACT

Objectives: To compare multipotent mesenchymal stem cells (MSCs) obtained from chorionic villi (CV), amniotic fluid (AF) and placenta, with regard to their phenotype and gene expression, in order to understand if MSCs derived from different extra-embryonic tissues, at different stages of human ontological development, present distinct stemness characteristics.
Study design: MSCs obtained from 30 samples of CV, 30 of AF and 10 placentas (obtained from elective caesarean sections) were compared. MSCs at second confluence cultures were characterized by immunophenotypic analysis with flow cytometry using FACS CANTO II. The expression of the genes Oct-4 (Octamer-binding transcription factor 4, also known as POU5F1), Sox-2 (SRY box-containing factor 2), Nanog, Rex-1 (Zfp-42) and Pax-6 (Paired Box Protein-6), was analyzed. Real-time quantitative PCR was performed by ABI Prism 7700, after RNA isolation and retro-transcription in cDNA. Statistical analysis was performed using non-parametric test Kruskal–Wallis (XLSTAT 2011) and confirmed by REST software, to estimate fold changes between samples. Each gene was defined differentially expressed if p-value was <0.05.
Results: Cells from all samples were negative for haematopoietic antigens CD45, CD34, CD117 and CD33 and positive for the typical MSCs antigens CD13, CD73 and CD90. Nevertheless, MSCs from AF and placentas showed different fluorescence intensity, reflecting the heterogeneity of these tissues. The gene expression of OCT-4, SOX-2, NANOG was not significantly different among the three groups. In AF, REX-1 and PAX-6 showed a higher expression in comparison to CV.
Conclusions: MSCs of different extra-embryonic tissues showed no differences in immunophenotype when collected from second confluence cultures. The expression of OCT-4, NANOG and SOX-2 was not significantly different, demonstrating that all fetal sources are suitable for obtaining MSCs. These results open new possibilities for the clinical use of MSCs derived from easily accessible sources, in order to develop new protocols for clinical and experimental research.

1. Introduction

Stem cells are primitive non-specialized cells, able to regenerate themselves and to differentiate into specific cell types [1]. Stem cells are classified as follows: totipotent, able to give rise to all cell types of the organism, including extra-embryonic tissues [2,3]; pluripotent, able to differentiate into cell types derived from the three germ layers but not into extra-embryonic tissues [4]; multipotent, capable of generating a limited number of cell types, restricted to a single germ layer [3]; unipotent, able to generate a single cell type. They are found in various adult tissues [5].

Stem cells can also be classified into embryonic (ESCs) and adult stem cells (ASCs) [6]. Both of these two cell types have some limitations: ESCs are pluripotent and have a high grade of self-renewal [7] but may form tumours and develop host immune rejection [5]. Moreover, their use opens many ethical concerns. ASCs are multipotent and have a lower differentiation and proliferative potential than ESCs but do not present ethical problems [3,5].

To overcome these limitations, many efforts have been made to isolate stem cells from other sources [8]. On the one hand, patient-specific pluripotent stem cells from specialized adult cells have

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been obtained by nuclear reprogramming (induced pluripotent stem cells) [7]; on the other hand, multipotent stem cells have been isolated from the fetus but with high risk of morbidity, and from extra-embryonic tissues (fetal membranes, placenta and umbilical cord blood) [9]. Whereas umbilical cord blood is a source of haematopoietic stem cells, extra-embryonic tissues are utilized as an important source of stem cells with a high potential for differentiation. Among the different populations, multipotent mesenchymal stem cells (MSCs) represent a very promising tool in clinical applications for their differentiating potential towards mesoderm-derived lineage.

More than 120 clinical trials are in progress utilizing MSCs for different therapeutic applications [10,11]. In clinical practice, the main source of MSCs is adult bone marrow. The percentage of MSCs in the bone marrow, however, is very low (0.001–0.01%), it decreases with age [12] and the retrieval techniques are dangerous. For these reasons it is important to find alternative sources of MSCs, such as extra-embryonic tissues [9] and adipose tissue, which offers a greater number of cells and is easily accessible [13,14].

Moreover, MSCs from extra-embryonic sources have intermediate characteristics between ESCs and ASCs [9,15]. In a recent study, MSCs obtained from 10 samples of chorionic villi showed multipotent properties in common with ESCs [16]. Although MSCs have well-known characteristics [17], their differences among different sources in terms of gene expression have not been determined yet [18]. It should be really interesting to understand if stem cell potential is reduced during human ontological development.

This topic has been previously explored [19] but the originality of our study derives from the contemporaneous analysis of the three different samples at different stages of human development. The rationale of this experimental study was to understand if MSCs derived from different extra-embryonic tissues, at different stages of human ontological development, presented different stemness potential.

We analyzed the expression of the stem cell master genes *OCT*-4 (Octamer-binding transcription factor 4, also known as POU5F1), *SOX-2* (SRY box-containing factor 2) and *NANOG* in all samples. Moreover, we evaluated the expression of two other genes, *REX*-1 (Zfp-42) and *PAX*-6 (Paired Box Protein-6), expressed by stem cells under the control of Oct-4, Sox-2 and Nanog.

2. Materials and methods

In this experimental study, a comparison, in terms of phenotype and gene expression, among three different extraembryonic tissues – chorionic villi (CV), amniotic fluid (AF) and placenta – was carried out.

CV and AF samples were obtained from 30 pregnant women for each group, aged 23–45 years, from the 11th to 14th and the 15th to 21st weeks respectively. The patients underwent chorionic villus sampling (CVS) and amniocentesis to look for chromosomal abnormalities. The great majority of the samples analyzed came from healthy pregnancies. Only 3 samples out of 60 (5%) had a trisomy (trisomy 21). No difference in terms of immunophenotypic and gene expression analysis was found between the healthy pregnancies and those with chromosomal abnormalities. The placental samples were obtained from 10 pregnant women, aged 25–39 years and derived from elective caesarean sections, carried out around 38 weeks of gestation.

The study was approved by the local Ethics Committee (“Ethics Committee of ANDROS Day Surgery, Palermo, Approval date: November 15, 2010, Reference number 03/MR/10”).

CV was first mechanically fragmented and then enzymatically digested using pronase (Merck) and collagenase (Sigma) for 15 min for both phases. Enzyme activity was arrested with Hank’s Balanced Salts solution (Sigma). Cells were plated in non-coated 25 cm^2 polystyrene culture flasks in Chang Medium (Irvine Scientific), a complete culture medium supplemented with penicillin, streptomycin, amphotericin, 10% fetal bovine serum (FBS) and glutamine (EuroClone).

AF samples, obtained from amniocentesis, were centrifuged at 1400 RPM for 10 min. Cells were then plated in non-coated 25 cm^2 polystyrene culture flasks in Chang Medium. All samples were collected in order to carry out prenatal diagnosis and were then used for the present study at the second passage of culture with the consent of each woman.

Placenta samples were first mechanically fragmented and then enzymatically digested using pronase (Merck) and collagenase (Sigma) for 30 min for each phase. Enzyme activity was arrested with culture media containing FBS, cells were counted and 5×10^5 cells were plated in non-coated 25 cm^2 polystyrene culture flasks in Chang Medium (Irvine Scientific). These samples were also analyzed at the second passage of culture.

In order to confirm the fetal origin of the placenta samples, a 16 autosomal STR DNA genotyping (D3S1358, D19S433, D2S1338, D16S539, D18S51, TH01, D21S11, vWA, D8S1179, FGA, SE33, D22S1045, D10S1248, D1S1656, D12S391, D2S441), carried out using Powerplex 17 ESI System (Promega), was performed.

Flasks from all samples were stored at 37 °C in a CO_2 incubator. Regarding placenta samples, non-adherent cells were removed and fresh medium was added after 48 h. Culture medium was replaced twice a week in all samples and cells were replaced after reaching 80% (about every week) confluence using trypsine/EDTA (Sigma). MSCs were initially investigated for the spindle-shape morphology, absence of contamination by pathogens and for their proliferative potential in culture.

Cells from all samples were evaluated for cell surface antigens expression by flow cytometry, performed by FACS CANTO II (BD Bioscience). Four lasers evaluated simultaneously the physical parameters, Forward Scatter (FSC) and Side Scatter (SSC), and the expression of six different surface antigens on a single tube. Cells from all samples were collected at confluence, pelleted, suspended in 100 μl of PBS at the concentration of 1×10^4 cells/μl and stained with the antibodies. Cells were incubated in the dark for 15 min at room temperature. After incubation, cells were washed with PBS and suspended in 100 μl of PBS to be analyzed with Facs Canto II.

Cells were incubated with the following antibodies: CD13-FITC (Dako), CD90-PE (Beckman Coulter), CD73-PE, CD34- PerCP-CY5.5, CD117- PE-CY7, CD33-APC and CD45-APC-CY7 (BD Biosciences).

Flow cytometer settings were established using unstained cells. Cells were gated by FSC to eliminate debris. A minimum of 10,000 events was counted for each analysis. Some samples were assayed by flow cytometry at different culture passages to determine any changes in surface molecule expression.

Cells in culture were treated with PBS and 2× RNA Nucleic Acid Purification Solution (Applied Biosystem) to lyse cells and preserve RNA. Total RNA was extracted by the platform ABI PRISM 6100 Nucleic Acid PrepStation (Applied Biosystem). The RNA concentration and purity were measured with a spectrophotometer by determining the absorbance ratio of 260 nm to 280 nm (>1.8). To assess the integrity, RNA fragments were resolved on a 1.2% agarose gel looking for subunits18S and 28S of rRNA. 1 μg of total RNA was then reverse-transcribed with High-Capacity cDNA Archive kit (Applied Biosystem). The reaction was performed in a thermocycler in a final volume of 12 μl (10 min at 25 °C, 60 min at 37 °C and 7 min at 4 °C).

Real-time quantitative PCR was conducted on a ABI Prism 7700 (Applied Biosystem). 2.5 μl of cDNA were added with the PCR master mix (Applied Biosystem) and the specific primers and probes (TaqMan Gene Expression Assay, Applied Biosystem). The