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RABBIT AMNIOTIC FLUID AS A POTENTIAL ALTERNATIVE SOURCE OF BROADLY MULTIPOTENT STEM CELLS

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ABSTRACT

A small animal model such as rabbit to serve as a biological model for *in vivo* tissue engineering experiments, would be desirable. The aim of our preliminary study was to describe a method of isolation of rabbit multipotent stem cells from the amniotic fluid and to examine the phenotype of these cells using flow cytometry. New Zealand White rabbit amniotic fluid was mixed with the culture medium and transferred into a T25 tissue culture flask. Analysis of rabbit stem cells was realized using specific antibodies and flow cytometry (BD FACSCanto II flow cytometry analyzer).
Our preliminary resutls show rapid proliferation of amniotic fluid-derived adherent cells. We hypothesize that these cells have a broadly multipotent mesenchymal stem cell phenotype basing on their morphology and the expression of CD44. Furthermore, these cells can potentially serve as an excellent and amenable cell source for reprogramming experiments into naive induced pluripotent stem cells.

Key words: rabbit; stem cell; amniotic fluid; proliferation

INTRODUCTION

Amniotic fluid is known to contain multiple cell types derived from the developing fetus (Priest *et al.*, 1978; Polgar *et al*., 1989). Cells within this heterogeneous population can give rise to diverse differentiated cells including those of adipose, muscle, bone and neuronal lineages (DeCoppi *et al*., 2001; In't Anker *et al.*, 2003; Tsai *et al*., 2004; Prusa *et al*., 2004).

De Coppi *et al.* (2007) described lines of broadly multipotent AFS cells, and used retroviral marking to verify that clonal human AFS cells can give rise to adipogenic, osteogenic, myogenic, endothelial, neurogenic and hepatic lineages, inclusive of all embryonic germ layers. Therefore, the authors postulated that they meet a commonly accepted criterion for pluripotent stem cells, without implying that they can generate every adult tissue.

Amniotic fluid stem cells clearly display a unique phenotype that is mostly multipotent but borders on pluripotency. Due to this fact, it does not come as a surprise that they have been shown to be more amenable to reprogramming (Li *et al.*, 2012) into the induced pluripotent stem cells that are virtually identical to embryonic stem cells (Takahashi and Yamanaka, 2006).

Remarkably enough, Moschidou *et al.* (2012) showed that c-KIT+ human first-trimester amniotic fluid stem cells (AFSCs) can be fully reprogrammed to pluripotency without any ectopic factors at all, by culture on Matrigel in human embryonic stem cell (hESC) medium supplemented with only the small-molecule histone deacetylase inhibitor (HDACi) - valproic acid (VPA). The cells share 82 % transcriptome identity with hESCs and are capable of forming embryoid bodies (EBs) *in vitro* and teratomas *in vivo*. After long-term expansion, they maintain genetic stability, protein level expression of key pluripotency factors, high cell-division kinetics, telomerase activity, repression of X-inactivation, and

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capacity to differentiate into lineages of the three germ layers, such as definitive endoderm, hepatocytes, bone, fat, cartilage, neurons, and oligodendrocytes. The authors conclude that AFSC can be utilized for cell banking of patient-specific pluripotent cells for potential applications in allogeneic cellular replacement therapies, pharmaceutical screening, and disease modeling.

Amniotic fluid stem cells have also been demonstrated to represent a feasible cell source for advanced applications such as tissue engineering of living, autologous heart valves. The feasibility of using these cells for tissue engineering has been demonstrated *in vitro* as well as *in vivo* in sheep model (Schmidt *et al*., 2007; Schmidt *et al*., 2008; Weber *et al*., 2012).

However, using sheep as a model for tissue engineering expreiments is logistically and financially cumbersome. Therefore, a small animal model such as rabbit to serve as a biological model for *in vivo* tissue engineering experiments, would be desirable (Slamečka and Chrenek, 2010). Here, we describe a method of isolation of rabbit multipotent stem cells from the amniotic fluid and we examine the phenotype of these cells using flow cytometry.

MATERIAL AND METHODS

Cell Culture

New Zealand White rabbit amniotic fluid was recovered from the uterus at about 23 day of gravidity using sterile syringe. The composition of the culture medium for the amniotic fluid stem cells was as follows: EBM-2 basal medium (CC-3156, Lonza) supplemented with 20 % fetal calf serum (FCS); recombinant growth factors: bFGF, EGF, R3-IGF-1; vitamin C (all are part of the medium kit); penicillin and streptomycin (15140122, Life Technologies). Rabbit amniotic fluid was mixed with the culture medium in proportion of 5:6, respectively. 5 ml of this mixture was then transferred into a T25 tissue culture flask. Approximately 5 days following plating, colonies of adherent cells start to be visible. At this point, a medium change is performed and from this point onwards, the medium is changed every day. On the day 10, the adherent outgrowths are after washing with PBS harvested using 2ml of Accutase (A1110501, Invitrogen) for 5 min at 37°C and 5 % CO_2. The cells were counted and replated for the purpose of increasing the cell numbers. We found the optimum density of the cells to be 6-8×10^4/cm^2 of cell culture surface.

Cryopreservation

Freezing of the rabbit amniotic fluid stem cells was performed using a medium consisted of 1:1 mixture of culture medium and freezing medium, respectively. The freezing medium consists of serum supplemented with 20 % DMSO (D2650, Sigma-Aldrich). Each cryovial contains about one million cells.

Flow Cytometry

For the purpose of flow cytometry analyses, the rabbit amniotic fluid stem cells are harvested using Accutase and washed with PBS. Then, the cells are fixed in 2 % paraformaldehyde (P6148, Sigma-Aldrich) for 20 minutes and washed in PBS. The cells were subsequently permeabilized in BD Perm Buffer III (558050, BD Biosciences) on ice for 30 minutes and then washed in PBS supplemented with 2 % FCS. The cells were then incubated with primary antibodies for 45 minutes on ice, washed twice and incubated with the secondary antibody for another 45 minutes on ice. Following washing, the cells were resuspended in PBS + 2 % FCS and analyzed using BD FACSCanto II flow cytometry analyzer. The antibodies used were the following: CD44 (sc-59758; SantaCruz Biotechnology), CD45 (304002; BioLegend), CD73 (344002; BioLegend), CD90 (328102; BioLegend), CD105 (sc-18838; SantaCruz Biotechnology), IgG1 kappa isotype control (400102; BioLegend), IgG2a isotype control (400202; BioLegend) and Cy2 secondary antibody (115-225-003, Jackson ImmunoResearch).

RESULTS AND DISCUSSION

The derivation of adherent rabbit amniotic fluid stem cells from cells suspended in the amniotic fluid was surprisingly rapid. While in case of humans, relatively small colonies of amniotic fluid stem cells just start to appear usually on the day 5 following the initial plating, the colonies of rabbit amniotic fluid stem cells, we observed on the day 5, were much bigger and comprised of considerably higher number of cells.

We found two predominant types of morphology in the cultures of rabbit amniotic fluid stem cells – "spindle-shaped" and "tile-shaped". Spindle-shaped rAFSC grow in form of colonies comprised of the individual cells being more spread out with bigger gaps (Figure 1A). This morphology of the cells suggests a mesenchymal phenotype. The other morphology observed might indicate an epithelial phenotype (Figure 1B) but further experiments, preferably immunocytochemistry using labeled antibodies specific to epithelial cell markers, would have to be performed to confirm this hypothesis. Following passaging, the tile-shaped cells still appear (Figure 2B) but the proliferation rate of the spindle-shaped (Figure 2A) cells is higher and they will ultimately take over. Following 13 days of culture, a total of 6.85 million cells were derived and cryopreserved.

We analyzed the expression of several markers typically used to profile mesenchymal stem cells – CD44, CD90, CD73, CD45 and CD105 (Figure 3). CD45

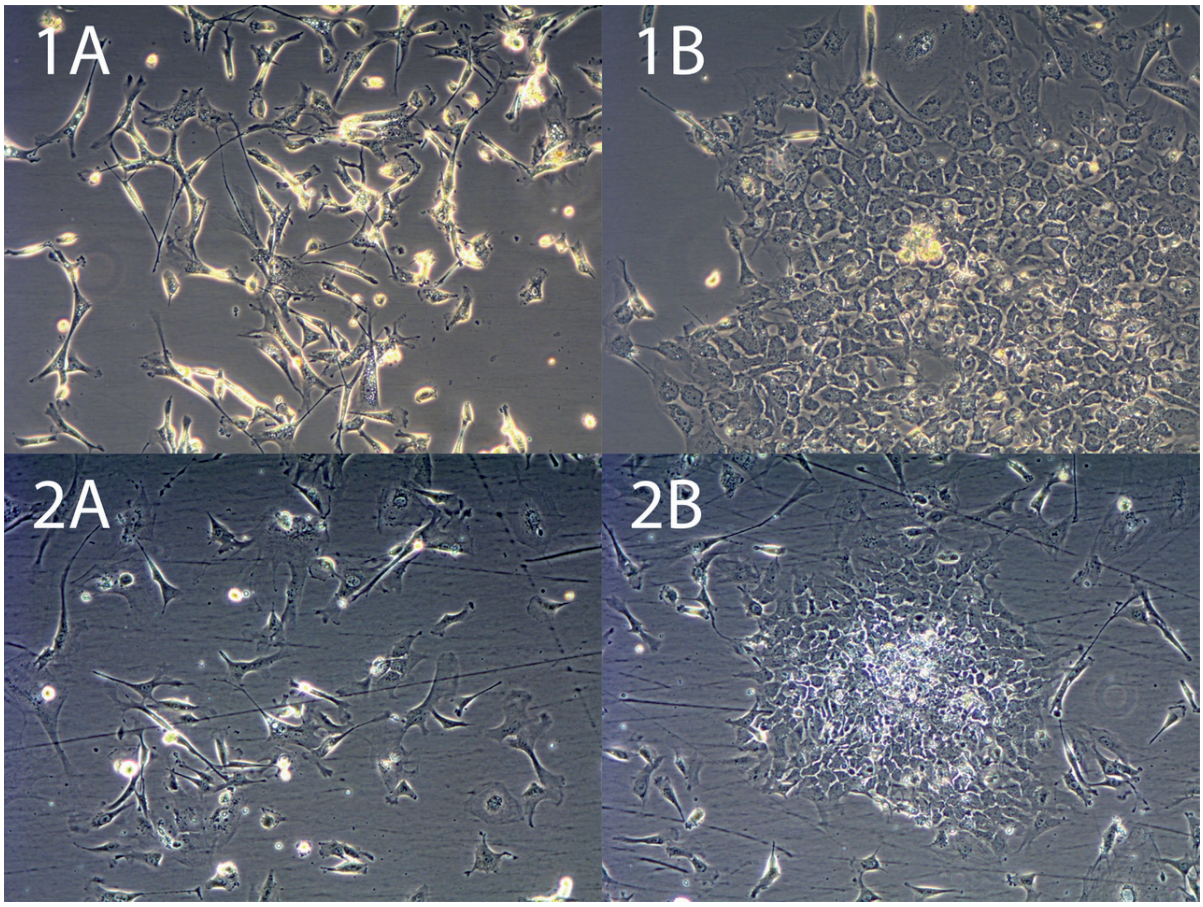

Fig. 1-2: Morphology of the cells recovered from rabbit amniotic fluid. 1A - Spindle-shaped rAFSC grow in form of colonies comprised of the individual cells being more spread out with bigger gaps. 1B - The morphology observed might indicate an epithelial phenotype. The tile-shaped cells still appear (Figure 2B) but the proliferation rate of the spindle-shaped cells is higher and they ultimately take over

expression is typically not observed in mesenchymal stem cells as opposed to the cells of the hematopoietic lineage and therefore, it serves as a negative marker. We detected the expression of CD44 only out of all mesenchymal stem cell-reactive markers, the expression was 47.8 % (Figure 3B). We hypothesize that the other mesenchymal stem cell markers can still be expressed but the anti-human antibodies we used have not cross-reacted with the proteins. This assumption is based on the fact that the cells have been derived under conditions identical to those used to derive human amniotic stem cells and the fact that the morphology of the rabbit amniotic fluid-derived adherent cells is "spindle-shaped" typically associated with mesenchymal stem cells.

Our preliminary results show rapid proliferation of amniotic fluid-derived adherent cells. We hypothesize that these cells have a broadly multipotent mesenchymal stem cell phenotype basing on their morphology, the expression of CD44 (Figure 3B) and the fact that they have been derived under conditions that have been shown to suport the mesenchymal stem cell phenotype of the human amniotic fluid stem cells (Schmidt *et al.*, 2007). However, this is not enough to confirm our hypothesis and therefore, we will seek for a more profound scrutiny of the phenotype of these cells. For that, a set of specific antibodies reactive against mesenchymal stem cell markers, will have to be designed. As rabbit-specific antibodies are scarcely available, an in-depth scrutiny of the cross-reactivity of anti-human or anti-mouse antibodies is going to have to be carried out which might prove to be a daunting challenge. Once such a set of antibodies is desinged, detailed multiplex flow cytometry analysis and immunocytochemistry confocal examination will be caried out. Rapidly proliferating broadly multipotent rabbit mesenchymal stem cells would prove to be valuable for tissue engineering and cell therapy

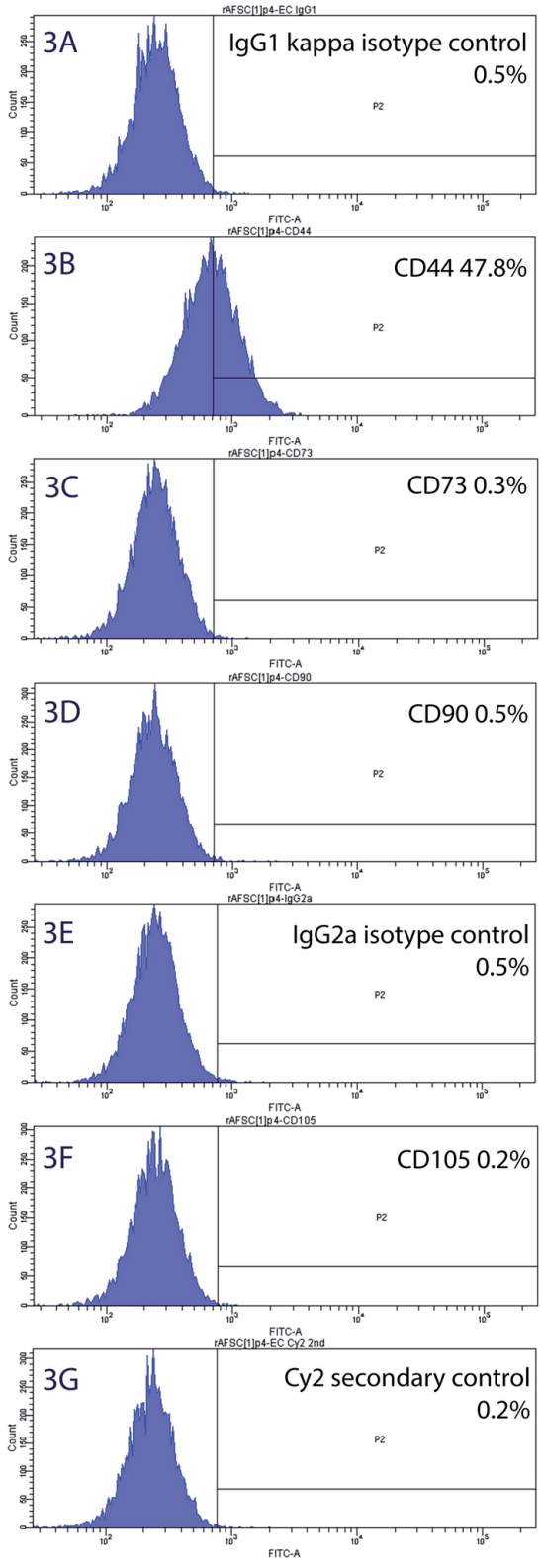

Fig. 3: Expression of several markers typically used to profile mesenchymal stem cells – CD44, CD90, CD73, CD45 and CD105 on rabbit amniotic fluid-derived stem cells

experiments enabling rabbit to become a sought-after small-animal biological model for these applications. Furthermore, these cells can potentially serve as an excellent and amenable cell source for reprogramming experiments into naive induced pluripotent stem cells.

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