# Phenotype and ultrastructure of stem cells derived from amniotic fluid of Nitra rabbit

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## Abstract

The isolation of amniotic fluid-derived mesenchymal stem cells (AF-MSCs) has been already shown in human and several other species including rabbit. However, prior to the preclinical research on various animal models it is desirable to define AF-MSCs by a panel of surface protein markers. Therefore, the aim of this preliminary study was to detect the expression of several protein markers on the surface of AF-MSCs isolated from local breed of Nitra rabbit. Amniotic fluid was collected from humanely sacrificed rabbits (n = 3) and AF-MSCs were cultured to a third passage. Flow cytometry was used to detect surface protein marker expression and for viability testing. Rabbit AF-MSCs (rAF-MSCs) were also analyzed by transmission electron microscopy to define the ultrastructure. rAF-MSCs showed both sufficient viability (more than 80%) and low apoptosis rates at third passage and highly expressed CD29 (88.17 ± 7.17%) and CD44 (80.00 ± 2.28%). However, a dim expression of CD90 (17.24 ± 1.31%) and negative expression of CD73 (1.21 ± 0.56% and 4.41 ± 1.46%), CD105 (1.67 ± 0.37%) and CD166 (0.96 ± 2.26%) was observed. Additionally, ultrastructure analysis revealed eccentrically located nucleoli, an abundance of thin pseudopodia on cells' surfaces and proved the presence of typical mesenchymal stem cell features. In conclusion, this set of data contributes to more detailed information on rAF-MSCs, which were previously proposed feasible for preclinical stem cell research and as a suitable source for the cryopreservation of animal genetic resources in gene bank.

**Keywords:** amniotic fluid, mesenchymal stem cells, rabbit, surface markers, ultrastructure

## Introduction

Recently, researchers have identified the amniotic fluid as an untapped source of stem cells that are multipotent, possess immunomodulatory properties and do not have the ethical and legal limitations of embryonic stem cells (Park et al., 2011; Loukogeorgakis and De Coppi, 2016). Due to their ease of isolation, high proliferation rate, pluripotency features, low immunogenicity and inability to form tumors, AF-MSCs are a promising type of stem cell for allogeneic cell transplantations in diseases of mesenchymal origin (Phermathai et al., 2010; Colosimo et al., 2013).

To date, AF-MSCs have been isolated from various animal species for preclinical research (Steigman et al., 2009; Klein et al., 2010; Chen et al., 2011; Filioli Uranio et al., 2011; Choi et al., 2013; Colosimo et al., 2013). Regarding rAF-MSCs, there are reports demonstrating their isolation and characterization by a few molecular markers (Fei et al., 2013; Slamecka and Chrenek, 2013; Kováč et al., 2016) and showing their potential in preclinical research (Steigman et al., 2009; Klein et al., 2010).

Rabbit as a biological model for AF-MSC research was proposed to be very feasible due to cost-effectiveness, logistical needs and common occurrence of large litters (Klein et al., 2010). Furthermore, rabbit mesenchymal stem cells have been shown to possess both cellular and tissue physiology closely resembling that of human MSCs (Fox, 1984; Warden, 2007). However, rAF-MSCs have not yet been characterized by multiple surface markers commonly used to define mesenchymal stem cell populations or described by ultrastructure analysis.

## Materials and Methods

Rabbit does (n = 3) of Nitra rabbit breed were used in the experiments. Pregnant mare serum gonadotropin (PMSG) at 25 I.U. (Sergon, Bioveta, Czech Republic) was administered to each doe 48 h before artificial insemination. Rabbit does were artificially inseminated with fresh doses of semen, followed by intramuscular injection of synthetic gonadotropin-releasing hormone (2.5 µg per doe; Supergestran, Nordic Pharma, Czech Republic).

### Isolation and culture of rAF-MSCs

Uteri of rabbits were obtained from humanely sacrificied gravid does at gestation day 23. Amniotic fluid was recovered from each fetus under sterile conditions using a lancet and pipette. Amniotic fluid was diluted (1:1) with culture medium; EBM-2 basal medium (Lonza, USA) supplemented with 20% fetal bovine serum (Sigma-Aldrich, UK), EGM-2 SingleQuots™ Kit (Lonza, USA) and 1% penicillin/streptomycin (Life Technologies, Slovak Republic). Primary cells were transferred to 25 $cm^2$ tissue culture flasks (Sigma-Aldrich, UK). Cultures were maintained at 37˚C in a humidified atmosphere with 5% $CO_2$. Culture medium was completely replaced on day 5 in order to remove non-adherent cells. Thereafter, the culture medium was completely replaced every other day until the adherent cell population reached ~ 80-90% confluence. Subsequently, cells were dissociated using Accutase (Invitrogen, Carlsbad, CA, USA) for 5 min at 37˚C in a humidified atmosphere with 5% $CO_2$ and counted by EVE™ Automated cell counter (NanoEntek, USA). Cells were then

reseeded as first passage (P1) into new culture flasks at density of 1.2 - 1.5 x $10^4$ cells/$cm^2$. Second and third passage (P2 and P3) were created using the same procedure.

### Flow cytometry analyses

Cultured rAF-MSCs at P3 were harvested using Accutase dissociation reagent, washed with PBS (without Ca and Mg; Biowest, USA) and pelleted by centrifugation for 5 min at 600 × g and 4 ˚C (same conditions for each centrifugation). For each individual doe, the cells were divided into 12 aliquots, each containing at least $10^6$ cells. One aliquot was used per one surface marker (9 aliquots and 1 control) and 2 aliquots were used for viability and apoptosis evaluation. Flow cytometry analyses were carried out using FACS Calibur™ (BD Biosciences, USA) and Cell Quest Pro™ (BD Biosciences, USA) software.

For surface proteins analysis, pellets were resuspended in 50 µl rabbit serum to block Fc receptors. Cell aliquots were then incubated with primary antibodies: anti-rabbit CD29 (Merck, Slovak Republic), anti-human CD34 (Thermo Fisher Scientific, USA), anti-rabbit CD44 (Bio-Rad, UK), anti-rabbit CD45 (Bio-Rad, UK), anti-mouse CD73 (eBioscience, Austria), anti-human CD73 (eBioscience, Austria), anti-rat CD90 (BD Biosciences, USA), anti-rabbit CD105 (GeneTex, USA), anti-rabbit CD166 (Bioss, USA). Double staining by fluorochrome conjugated secondary antibodies was performed using anti-mouse IgG1-PE (Miltenyi Biotec, Germany) and anti-rabbit IgG-FITC (Bio-Rad AbD Serotec, UK). Incubation with primary and secondary antibodies and proper isotype controls were performed for 15 min at 4˚C.

Rabbit AF-MSC viability and apoptosis rates were evaluated at P3. Cells were harvested and counted (as above). Cell aliquots were stained by Annexin-V-FLUOS Staining Kit (Roche Slovakia, Slovak Republic) to detect early apoptotic rates. Cell aliquots were washed with Annexin-V binding buffer (part of Staining Kit) and centrifuged. Pellets were resuspended in 50 µl staining solution and incubated at room temperature for 20 min. After incubation, cells were washed and centrifuged. Supernatant was discarded and cells were resuspended in 250 µl of Annexin-V binding buffer. Propidium iodide (Molecular Probes, USA) was added to each tube to evaluate overall viability. Cell populations were gated using Cell Quest Pro™ software as follows: $AnV^+/PI^-$ (early apopototic cells); $AnV^-/PI^-$ (non-apoptotic/live cells); $AnV^+/PI^+$ (late apoptotic and dead cells) $AnV^-/PI^+$ (non-apoptotic/dead cells).

Data were processed using SigmaPlot software (Systat Software Inc., Germany) and expressed as mean values ± SEM.

### Transmission Electron Microscopy

For TEM, rAF-MSCs at P3 were washed in 0.15 M cacodylate buffer (pH 7.1-7.3) and embedded in agar to form pellets. Agar pellets of rAF-MSCs were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.15 M cacodylate buffer (pH 7.1-7.3) for 1 h and then washed in cacodylate buffer. Samples were then post-fixed in 1% osmium tetroxide in cacodylate buffer, washed in distilled water, dehydrated in acetone and embedded in Durcupan ACM (Fluka Analytical – Sigma-Aldrich,

Switzerland). Blocks of AF-MSCs were cut into semi-thin sections (1-2 μm) using a UC 6 Leica ultramicrotome (Leica Microsystems, MIKRO Ltd., Slovak Republic). Sections were collected on copper grids and examined through a transmission electron microscope (JEM 2100, Jeol, Japan) operating at 80kV.

## Results and discussion

In this preliminary study, rAF-MSCs were successfully isolated, expanded and defined by multiple surface markers. Cell viability and apoptosis rates at P3 were examined by flow cytometry to determine the proportion of dead and/or early apoptotic cells. Overall viability of cultured rAF-MSCs was more than 80%. In addition, apoptotic cells were present, however rates were favorably low (Table 1). These results demonstrate that rAF-MSCs can be easily cultured and retain a high viability level at P3.

Table 1. Viability and apoptosis rates of rAF-MSCs in %

| $AnV^-/PI^-$ | $AnV^+/PI^-$ | $AnV^+/PI^+$ | $AnV^-/PI^+$ |
|---|---|---|---|
| 85.82 ± 1.78 | 1.04 ± 0.32 | 5.65 ± 0.86 | 7.49 ± 2.03 |

Surface markers commonly used to define MSCs were detected to verify mesenchymal nature of isolated cells. The expression of particular markers is shown in Table 2. Stem cells are typically characterized by the presence of surface markers associated with self-renewal without differentiation. Flow cytometry is a fundamental technique in the identification of stem cells through detection of these markers (Dziadosz et al., 2016). In this preliminary study, rAF-MSCs highly expressed CD29 and CD44. These two markers were not proposed by International Society for Cellular Therapy (ISCT; Dominici et al., 2006), however they are often used to detect MSC populations. There are many other studies of AF-MSCs, which found theses markers to be expressed in human (In't Anker et al., 2003; Tsai et al., 2004; De Coppi et al., 2007), rabbit (Steigman et al., 2009; Klein et al., 2010; DeKoninck et al., 2013; Fei et al., 2013; Kováč et al., 2016) and other species (Park et al., 2011; Chen et al., 2011; Nadri and Soleimani, 2007; Iacono et al., 2012; Choi et al., 2013). Investigators therefore use CD29 and CD44 together with some of molecular markers proposed for human MSCs by ISCT. Among these markers, a dim expression of CD90 and a negative expression of CD73, CD105 and CD166 were observed. Also, hematopoietic lineage markers CD34 and CD45, used as a negative control, were not expressed.

Although rAF-MSCs were previously defined by a few studies (Steigman et al., 2009; Klein et al., 2010; DeKoninck et al., 2013; Fei et al., 2013), some of the MSC markers, like CD73, CD105 and 166 were not previously screened in rAF-MSCs. A negative expression of surface markers detected by flow cytometry could also be possibly attributed to antibodies specificity. However, for CD105 and CD166 anti-rabbit antibodies were used. Hence it confirms the negative expression of CD105

and CD166. For CD73, both anti-mouse and anti-human antibodies were used, as no rabbit-specific antibody was currently commercially available. However, a negative expression of CD73 was detected using both antibodies. Additionaly, a dim expression of CD90 was observed using anti-mouse antibody. Hence, CD73 and CD90 results could be attributed to low binding affinity of used antibodies and therefore it would be desirable to use other analyses to detect the expression of these markers. For instance, an mRNA analysis could elucidate whether these markers are expressed at mRNA level to avoid dependence of antibodies specificity and cross-reactivity. mRNA expression studies were carried out on other animal models, like dog (Filioli Uranio et al., 2011), goat (Pratheesh et al., 2013), horse (Lovati et al., 2011) and pig (Chen et al., 2011). Moreover, an extensive study of typicall MSCs markers expressed by AF-MSCs in different species would be also beneficial for understanding the species diversity in expression of these markers, as there are reports suggesting that markers proposed by ISCT may not be expressed in particular animal species (Nadri and Soleimani, 2007; Eslaminejad et al., 2011).

MSC markers have also numerous distinct functions. For example CD105 plays a role during MSC chondrogenic differentiation and is also involved in cytoskeletal organization (Dominici et al., 2006). CD73 is an ecto-5-nucleotidase purine catabolic enzyme with broad substrate specificity that catalyzes the dephosphorylation of purine and pyrimidine ribo- and deoxyribonucleoside monophosphates (Barry et al., 2001). CD90 was originally discovered as a thymocyte antigen. Its exact function has not yet been fully elucidated but it has been proposed to mediate cell-cell and cell-matrix interactions (Williams and Gagnon, 1982). Therefore, in addition to mRNA expression analysis, another possibility of detecting the presence of these surface markers could be screening of their proposed functions.

It has been reported, that the isolation and characterization of stem cells derived from amniotic fluid and various sources are important issues in cell therapy, regenerative medical research, and tissue engineering (Miki et al., 2005; De Coppi et al., 2007; Hipp and Atala, 2008; Iacono et al., 2012). This preliminary study extends the definition of rAF-MSCs, which could be potentially used in preclinical studies as previously shown by (Steigman et al., 2009; Klein et al., 2010) or for the preservation of animal genetic resources.

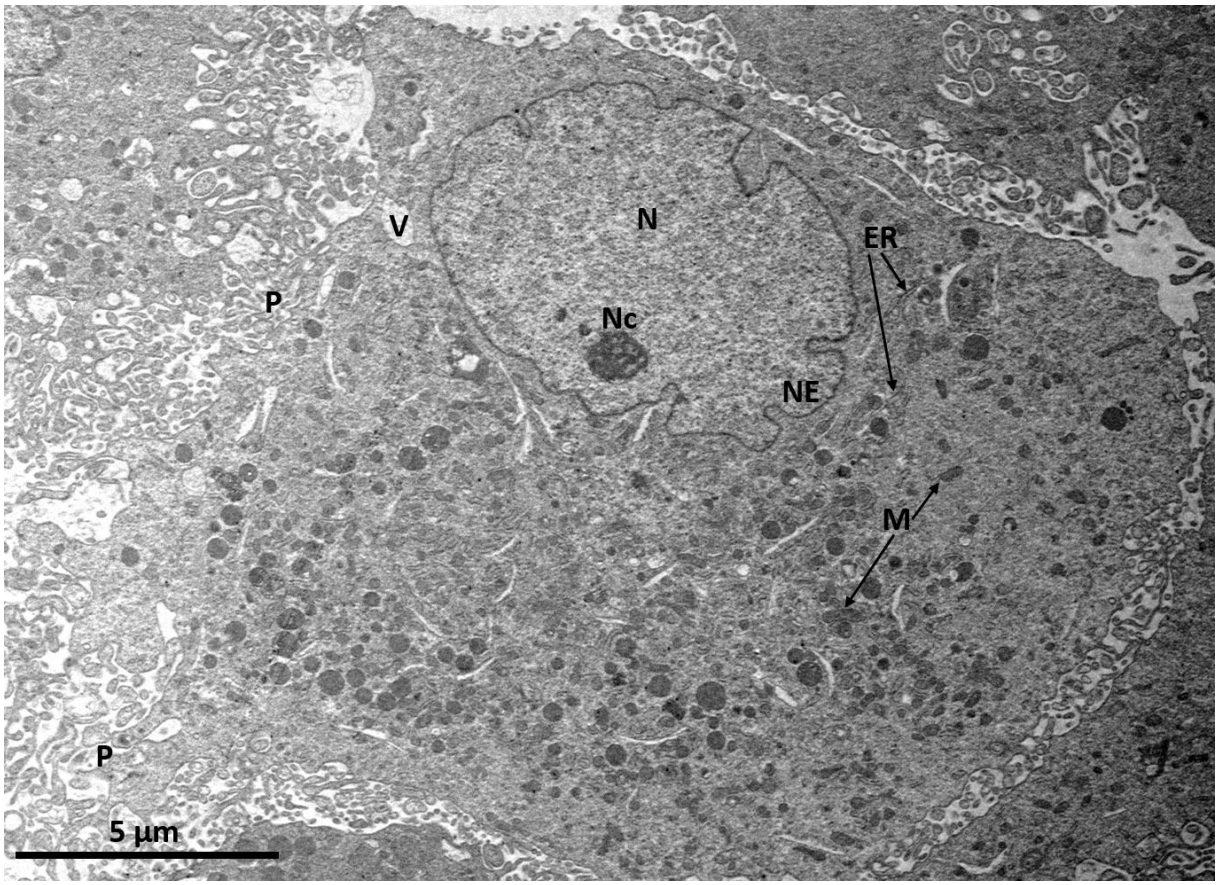
Table 2. Expression of surface protein markers in %

| CD29 | CD44 | hCD73 | mCD73 | CD90 | CD105 | CD166 | CD45 | CD34 |
|---|---|---|---|---|---|---|---|---|
| 88.17 ± 7.17 | 80.00 ± 2.83 | 1.21 ± 0.56 | 4.41 ± 1.46 | 17.24 ± 1.31 | 1.67 ± 0.37 | 0.96 ± 0.26 | 3.95 ± 1.92 | 1.18 ± 0.50 |

h – anti-human antibody; m – anti-mouse antibody

Ultrastructure of rAF-MSCs was investigated using transmission electron microscopy to further characterize rAF-MSCs. An abundance of thin pseudopodia on the surface of cells was observed (Figure 1). In addition, rAF-MSCs displayed eccentrically located nuclei and a cytoplasm containing numerous endoplasmic

reticula. Mitochondria and vacuoles were present in moderate quantities. These findings correlate with description of rabbit bone marrow mesenchymal stem cells (Tan et al., 2013). These observations suggest that mesenchymal nature of isolated AF-MSCs can be approved also by conducting ultrastructure analysis.




N – oval-shaped indented nucleus that is eccentrically located, Nc – nucleolus, NE – nuclear envelope, V – vacuoles, P – thin pseudopodia, ER – endoplasmic reticulum, M – mitochondria

Figure 1. Ultrastructure analysis of cultured rAF-MSCs

## Conclusions

AF-MSCs have a remarkable potential in curing various diseases and application in agriculture or veterinary medicine due to their ideal stem cell properties. rAF-MSCs in this preliminary study expressed CD29 and CD44. However a dim expression of CD90 and a negative expression of CD73, CD105 and CD166 were observed. In conclusion, this preliminary study extends the surface marker expression profile of rAF-MSCs which is demanded for preclinical trials to exclusively define particular stem cell populations. However, additional analysis of mRNA surface marker expression would be desirable for markers detected by non-rabbit-specific antibodies. Ultrastructure was also defined in this study to further characterize rAF-MSCs. Furthermore, this cell population is also a suitable source for the cryopreservation of animal genetic resources in gene bank.

## Acknowledgements

This work was supported by the grant APVV-14-0043 and APVV-14-0348 coordinated by the Slovak Research and Development Agency and VEGA 1/0611/15. This work was also supported by Research Center AgroBioTech built in accordance with the project Building Research Centre „AgroBioTech" ITMS 26220220180.

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