Ilea Ioana Cristina, Pall Emoke, Ciupe Simona, Cenariu M., I.S.Groza

[1]University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Faculty of Veterinary Medicine, Cluj-Napoca, Romania, criss_vet@yahoo.com

Abstract

Mesenchymal stem cells (MSCs) are defined as bone marrow derived cells which have the capacity to differentiate into the three classical mesodermal lineages (adypocites, osteoblasts and chondrocytes). Different studies revealed the ability of bone marrow derived MSCs to differentiate into ectodermic lineages including neurons. The aim of our study was to evaluate the multipotency of mouse bone marrow derived MSCs by phenotypic characterization during neuronal induction.
Mouse MSCs were isolated from bone marrow by flushing the femurs with α MEM (Gibco) medium supplemented with 1% penicillin-streptomycin (Gibco). Isolated cells were cultured in a propagation medium containing DMEM-F12 medium supplemented with 20% FCS (Gibco), 1% penicillin-streptomycin (Gibco), 5% horse serum (Sigma) and 10µg/5ml MycoZap (Mycoplasma Elimination Reagent, Lonza).
For neural induction, cells were cultured in Neurobasal medium supplemented with 0.1mM β-mercaptoethanol and 1% glutamax for 2 weeks. For phenotypic characterization, were evaluated the expression of S-100 protein and neuron specific enolase (NSE) during differentiation. Our results confirmed the multipotency of isolated cells by neuronal differentiation. At 3 days after neurogenic induction, cells morphology changed, appearing star-shaped cells and at day 4 were present specific neuritic networks. At 2 weeks after induction, the immunostaining showed the presence of S-100+ cells, confirming the glial differentiation, as well as NSE+ cells, an indicator of neuronal differentiation.

Key words: *mouse MSCs, multipotency, neuronal differentiation.*

INTRODUCTION

Mesenchymal stem cells (MSCs) are defined as bone marrow derived cells which have the capacity to differentiate into the three classical mesodermal lineages (adypocites, osteoblasts and chondrocytes) (Pall. et al., 2010; Ippokratis et al., 2005; Tondreau et al., 2004). Based on these properties, MSCs arise interest of many researchers, by generating important promises as a potential source for cellular therapies and tissue engineering (Witte et al., 1997; Baksh et al., 2004,). Currently, hematopoietic stem cells transplantation is used for the treatment of different types of leukemia (Tabbara et al., 2002). The potential use of bone marrow derived MSCs in different types of degenerative disease is related with the ability of MSCs to differentiate into ectodermic lineages including endothelial cells (Groza et

al., 2011), neurons, astrocytes and oligodendrocytes (Shihabuddin et al., 2000; Sekiya et al., 2002). In 2002, Kim et al., indicated the capacity of embryonic stem cells to differentiate in dopamine neurons after transplantation into a rat model of Parkinson's Disease. Also, in a rat model with spinal cord injury the transplantation of differentiated stem cells leeded to a long term functional improvement (Kim et al., 2002). The results of this studies advocate the use of MSCs in neurodegenerative cellular therapy, but the extrapolation to human medicine requires the development of rapid and less complicated protocols for isolation and culture. Also human medicine involve rigorouse protocols for transplantationin in order to prevent the rejection and graft-versus-host-disease.
To minimize this challenges, the aim of our study was to evaluate the multipotency of mouse bone marrow derived MSCs by phenotypic characterization during neuronal differentiation with a simple induction medium.

MATERIALS AND METHODS

Mouse MSCs were isolated from 12-14 weeks old CD1 mice in accordance with the international ethical standards. The bone marrow was harvested after femoral dissection, by removing the epiphyses and flushing the shaft with a 27G needle and 1 ml washing medium (figure 1).

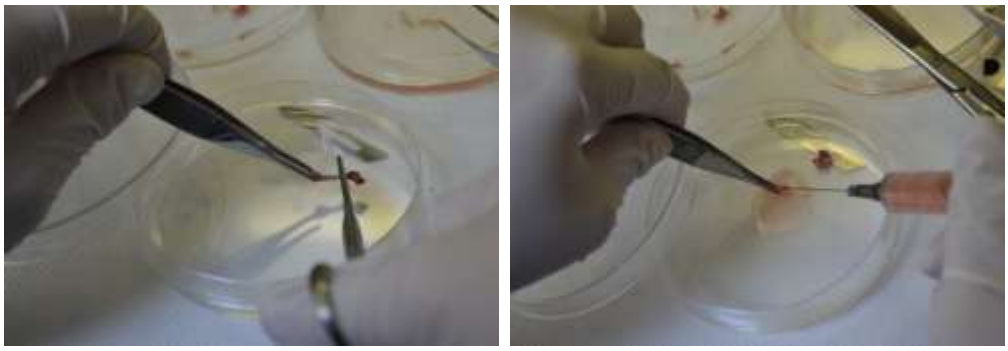

Figure 1. – Bone marrow isolation

Isolated cells were cultured in DMEM-F12 medium. For neural induction, MSCs 4x were cultured in Neurobasal medium for 2 weeks. The phenotypic characterization was done by evaluation of the expression of S-100 protein and neuron specific enolase (NSE) during differentiation. The

immunostained cultures were examined using a fluorescence microscope. In figure 2 the detailed protocol of work is shown.

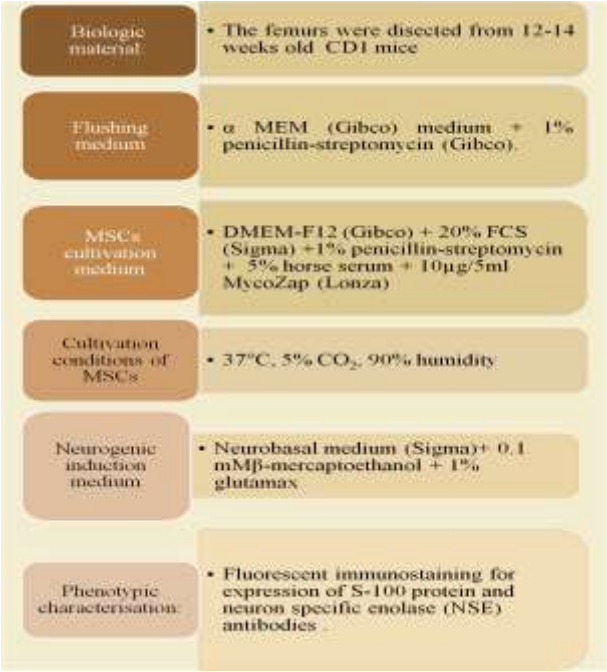

Figure 2. – Detailed protocol of MSCs isolation and characterization

RESULTS AND DISCUSSIONS

The study of MSCs biology and phenotypic characterization can be realized through experimental research on mouse, because it is a very suitable animal model. These cells are promising for regenerative medicine due to their capacity to regenerate injured tissue, to prevent pathologic fibrotic remodeling and to stimulate endogenous progenitors (Meirelles et al., 2009; Rodrigues et al., 2010). MSCs could be use as treatment of coronary artery disease due to their capacity to generate the novo myocardium after local transplantation (Orlic et al., 2001), and as a treatment of myocardial infarct (Stamm et al., 2003). Also, it was demonstrated their potential use in the therapy of muscular dystrophy (Gussoni et al., 1999), and their protection properties in lung injuries, including inflammation and collagen deposition (Ortiz et al., 2003). In orthopedic medicine, MSCs are used for segmental

bone repair (Quarto et al., 2001), craniotomy defects (Krebsbach et al., 1998) and in regeneration of meniscus tissue (Murphy et al., 2003).
In order to evaluate the multipotent capacity of mouse MSCs, the cells after recovery and culture were characterized both morphologically and imunophenotipically.
The primary culture (P1) (figure 3) was maintained for 7 days. In the d 4 of propagation, in culture was present a heterogenous cell population, composed by adherent fibroblast-like cells, rounded and hexagonal shaped cells. Also in suspension were present macrophages, monocytes and erytrocites. Martin et al demostrated in 1997 that the primary cultures are usually maintained for 12–16 days, during which time the nonadherent haematopoietic cell fraction is depleted. Due to the highly incresead doubling number of MSCs, we maintened the culture only until d7, in order to avoid the contact inhibition. First passage was done at a 70% confluence.

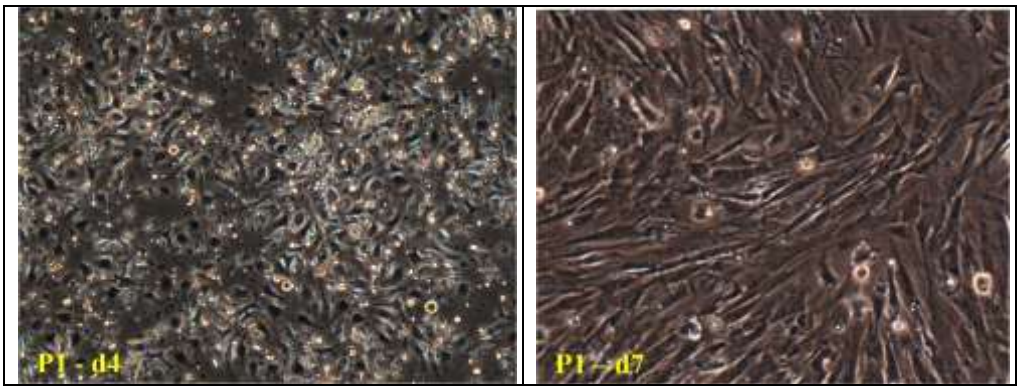

Figure 3. – The morphological aspect of primary culture

At P2, the heterogeneity of the cultured cell population was maintained, but the number of fibroblast-like cells arrived at 60%, a diverse morphology being observed at 40% of the cells. At P3, in culture were present just a percentage of 10% hexagonal shaped cells, the rest of 90% being adherent fibroblast-like cells, special characteristic of MSCs. A homogenous fibroblast-like cells culture was obtained at P4, reason why we started the neuronal induction.
After neurogenic induction with Neurobasal medium, at d3, the cells morphology started to change, appearing neuron-like cells, characterized by rounded somas and long spindly processes, indicating an early stage of neuronal differentiation. At day 4, the number of neuron-like cells increased

significantly (60%) and the neuritic networks started to be apparent (figure 4).

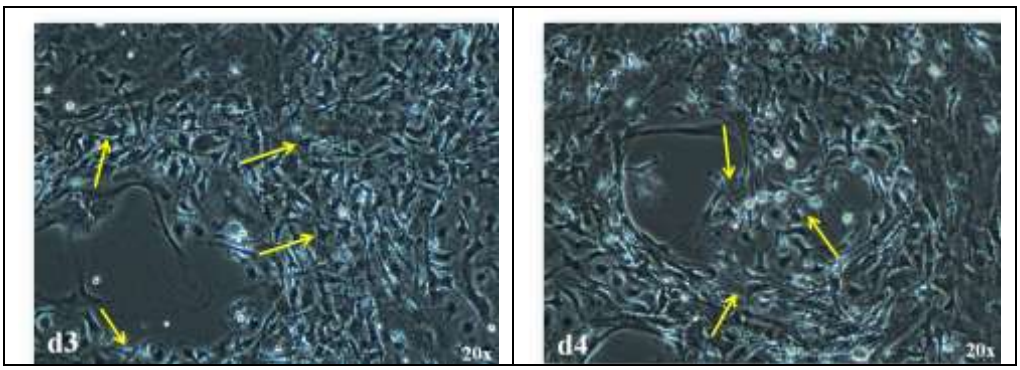

Figure 4. – The morphological aspect during neuronal differentiation

The immunostaining was done at d14 of differentiation. In the differentiated culture, positive cells for S-100+ protein were found, which are specific for astrocytes and glial cells, and also NSE+ cells, an indicator of neuronal differentiation (figure 5).

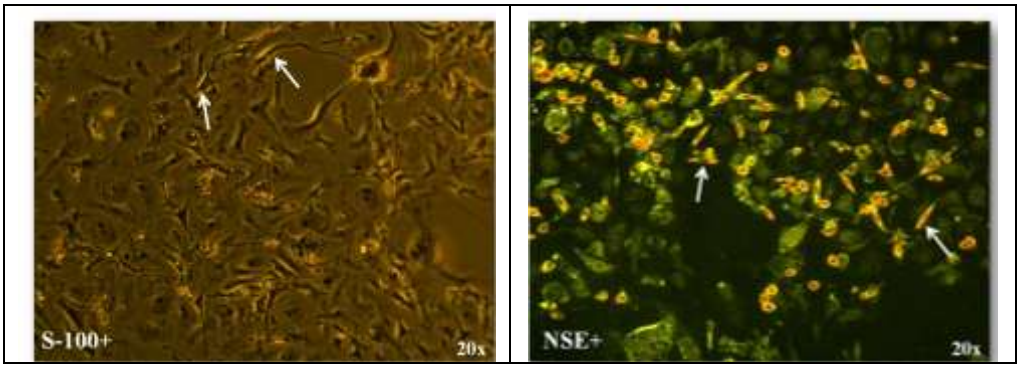

Figure 5. – Immunofluorecent staining

S-100 is a protein found in vertebrates and expressed on the cells derived from the neural crest, including glial, astocytes, and dentritic cells (Krampera et al., 2007). Previous reports showed that bone marrow derived MSCs cannot differentiate in astrocytes, neither *in vivo* and *in vitro* (Wehner et al., 2003). Contrary to these results, in our study we showed the possibility to obtain S-100+ cells after induction with Neurobasal medium.

NSE is a protein that usually is used for the identification of neuronal cells and other cells with neuroendocrine differentiation (Völlner et al., 2009). The expression of NSE on culture-differentiated cells suggest the presence of immature and differentiating neurons (Muńoz-Elías et al., 2003,), accompanied by changes in cell morphology and mitotic activity (neuronal). According to these findings, the morphological and phenotypical changes suggest that during differentiation, the neuron-like cells express diverse specific neuronal proteins, which means that the culture was composed by cells in different stages of differentiation.

CONCLUSIONS

Neuronal differentiation of mouse bone marrow derived MSCs is recommended at P4, when there is a homogenous culture of fibroblast-like cells.

The supplemented Neurobasal medium was optimal to induce neurogenic differentiation after 14 days.

The multipotency of mouse bone marrow derived MSCs was confirmed by the presence of S-100+ cells and NSE+cells, indicating differentiation capacity.

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RESEARCH ON THE THORACIC LIMB JOINT AT THE DOMESTIC PIG (*SUS SCROFA DOMESTICA*)