

## EVALUATION OF THE MULTILINEAR CAPACITY OF CANINE MESENCHYMAL STEM CELLS

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### **Abstract**

*Interest of the medical world towards regenerative therapy using mesenchymal stem cells has become increasingly prominent, given the many recent successes. Dogs are ideal candidates for testing the methods of isolation, cultivation and differentiation of mesenchymal stem cells into multiple cell lines and their use in regenerative therapy. The aim of this paper was to test the multipotency and multilinearity of mesenchymal stem cells derived from canine bone marrow and umbilical cord blood. Mesenchymal stem cells were obtained from a total of 12 dogs following medular aspiration or by collecting cord blood during caesarean section. Samples were processed using Histopaque 1077 and then cultured in  $\alpha$ -MEM supplemented medium. In order to assess the stemness and multipotency of mesenchymal cells isolated from canine bone marrow and umbilical cord blood, their phenotype was characterized by assessing the Oct4 gene expression followed by the evaluation of their differentiation potential towards bone, cartilage, fat and nerve cells. Canine bone marrow and umbilical mesenchymal stem cells expressed the Oct4 gene. This gene expression was not identified after differentiation, however was shown in cells grown in propagation medium. Osteogenic, chondrogenic, adipogenic and nervous differentiation was demonstrated by identifying specific morphology, specific stainings and by assessing the gene expression of genes of interest. Canine mesenchymal stem cells have a high multilineage capacity, being able to differentiate towards osteogenic, chondrogenic, adipogenic and nervous lines. These properties can be exploited in order to use this type of cell therapy in homologous, heterologous and even xenogenic regenerative therapies.*

**Key words:** canine, mesenchymal, stem cells, differentiation.

### **INTRODUCTION**

The study of multipotent mesenchymal stem cells and their differentiation potential towards various cell lines, like bone, cartilage adipose tissue or neural tissue, represent an extremely important research topic. (Zucconi et al., 2010; Pall et al., 2009; Malgieri et al., 2010). Interest of the medical world towards regenerative therapy using mesenchymal stem cells has

become increasingly prominent, given the many recent successes (Martin et al., 2002; Livingstone et al., 2003; Krampera et al., 2007). Dogs are ideal candidates for testing the methods of isolation, cultivation and differentiation of mesenchymal stem cells into multiple cell lines and their use in regenerative therapy (Bianco et al., 2001; Cancedda, 2003; Baertschiger, 2005). The aim of the present study was to test the multipotence and multilinearity of mesenchymal stem cells derived from canine bone marrow and umbilical cord blood.

## **MATERIALS AND METHODS**

Mesenchymal stem cells were collected from 12 dogs by medular aspiration as well as collection of cord blood during caesarean section. Samples were processed using Histopaque 1077 and then cultured in  $\alpha$ -MEM medium. In order to assess the stemness and multipotency of mesenchymal cells the following protocols were used: assessment of Oct4 gene expression and evaluation of differentiation potential towards bone, cartilage, fat and nerve cells by evaluating the specific morphology of cells, performing specific staining and assessing the gene expression of genes of interest. Cellular differentiation was performed by culturing cells in specific culture media, as follows:

- For osteogenic differentiation
  - DMEM –LG (Sigma-Aldrich™);
  - 10 mM  $\beta$  glycerophosphate (Sigma-Aldrich™);
  - 300  $\mu$ M ascorbic acid (Sigma-Aldrich™);
  - 10% FCS (Gibco®);
  - 1% antibiotic-antimycotic mix (Gibco®).
- For chondrogenic differentiation:
  - DMEM –LG (Sigma-Aldrich™);
  - 10<sup>-7</sup> mol/l Hydrocortison (Sigma-Aldrich™);
  - 100  $\mu$ M Ascorbic acid (Sigma-Aldrich™);
  - 1% ITS (Sigma-Aldrich™);
  - 2% HS (horse serum) (Gibco®);
  - 1% antibiotic-antimycotic mix (Gibco®).
- For adipogenic differentiation

- RPMI medium (Gibco®);
  - 5% FCS(Gibco®);
  - 100 µM ascorbic acid (Sigma-Aldrich™);
  - 0.5 mM Isobutyl-xanthine (Sigma-Aldrich™);
  - 1% antibiotic-antimycotic mix (Gibco®).
- For nervous differentiation:
- NEUROBASAL medium (Invitrogen™).
  - 2 mM glutamax (Sigma-Aldrich™);
  - 10<sup>-6</sup> M retinoic acid (RA) (Sigma-Aldrich™)

The specific differentiation markers identified by PCR are shown in table 1:

Table 1. The specific differentiation markers identified by PCR

Markers	Gene	Primer sequence (5'-3')
Stemness	OCT4	Forward GAGTGAGAGGCAACCTGGAG
		Reverse GTGAAGTGAGGGCTCCCATA
Bone	<i>OSTEOPONTIN</i>	Forward CATATGATGGCCGAGGTGATAG
		Reverse CAAGTGATGTGAAGTCCTCCTC
	OSTEOCALCIN	Forward GAGGGCAGCGAGGTGGTGAG
		Reverse TCAGCCAGCTCGTCACAGTTGG
Cartilage	COL2A	Forward GAAACTCTGCCACCCTGAATG
		Reverse GCTCCACCAGTTCTTCTTGG
Nervous	BIII TUBULIN	Forward GCACACTGCTCATCAACAAG
		Reverse TCTTGCTCTCCTTCATGGA
	GFAP	Forward CGAGTTACCAGGAGGCACTA
		Reverse TCCACGGTCTTTACCACAAT
	NESTIN	Forward GAGAACCAGGAGCAAGTGAA
		Reverse TTTCCAGAGGCTTCAGTGTC

## RESULTS AND DISCUSSIONS

Canine medullar and umbilical mesenchymal stem cells have shown OCT4 gene expression. This gene expression was no longer identified after differentiation, but was shown in cells cultivated on propagation medium (Figure 1).

For osteogenic differentiation, at the end of cultivation the mineralized cellular matrix was noticed as well as the osteogenic nodules. The apparition

of calcium deposits was proven by Alizarin-Red coloration (Figure. 2). The cells expressed the osteopontin and osteocalcin markers (Figure 3). Regarding the chondrogenic differentiation, the cells presented a flattened morphology with aggregation tendency (Figure 4). The chondrogenic phenotype was proven by showing the expression of COL2A marker (Figure 5).

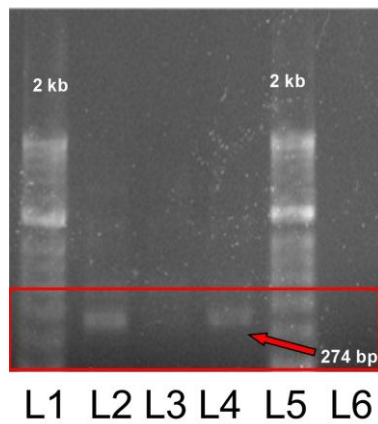


Figure 1 Oct4 expression before and after differentiation (L1, L5 – ladder, L2 – BM MSCs, L4 – UCB MSCs, L3, L6 – osteogenic differentiation)

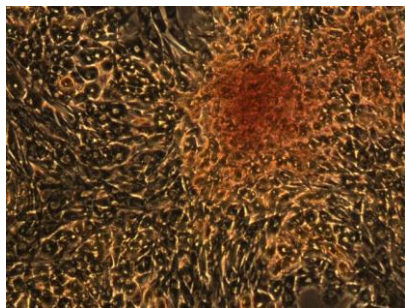


Figure 2 Alizarin red staining -extracellular calcium deposits

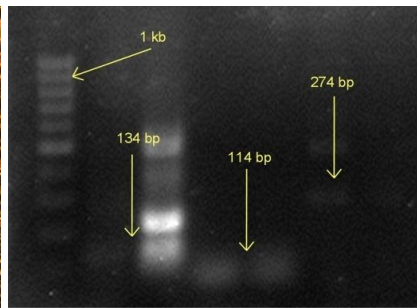


Figure 3 Osteocalcin 134 bp and osteopontin 114 bp expression

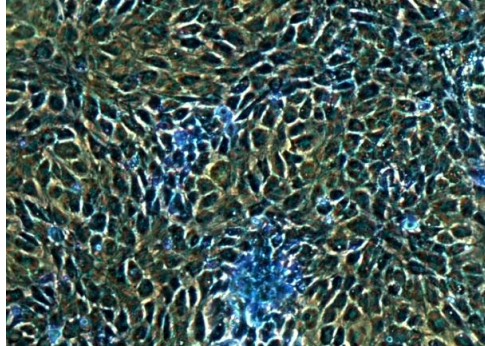


Figure 4 Alcian blue staining  
- glycosaminoglycans deposits

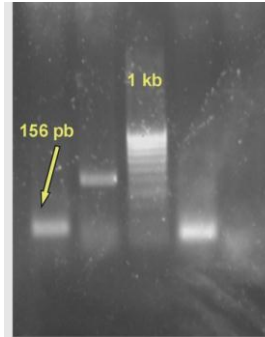


Figure 5 Col2A 156 bp  
expression

For adipose differentiation, lipid vacuoles were noticed shown by Oil Red coloration (Figure 6, 7).

Nervous differentiation showed a remarkable rounding of cells accompanied by increase of cell size (Figure 8). We also showed Nestin 328 bp and GFAP 277 bp expression for early differentiation as well as B3 tubulin for late differentiation (Figure 9).

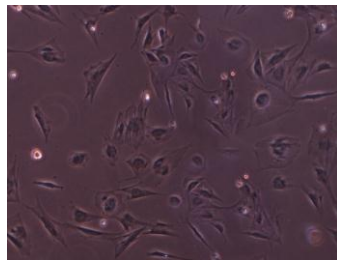


Figure 6 Lipid droplets

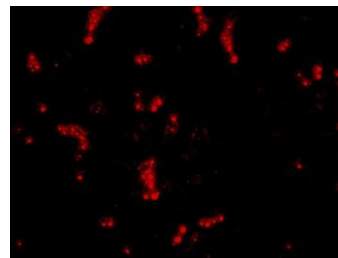


Figure 7 Oil Red staining

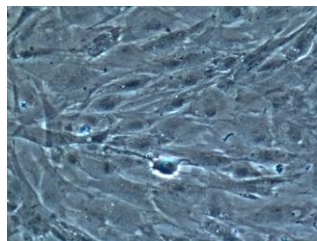


Figure 8 Intercellular filaments

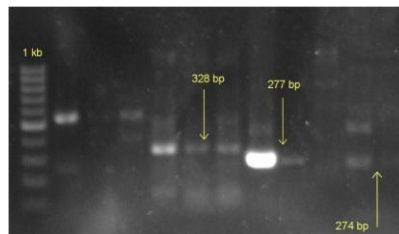


Figure 9 Nestin, GFAP and B3 tubulin

## CONCLUSIONS

Canine mesenchymal stem cells have a high multilineage capacity, being able to differentiate towards osteogenic, chondrogenic, adipogenic and nervous lines.

These properties can be exploited in order to use this type of cell therapy in homologous, heterologous and even xenogenic regenerative therapies.

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