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Characterization of Mandible and Femur Canine Mesenchymal Stem Cells: A Pilot Study

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Abstract
Mesenchymal stem cells (MSCs) are emerging donor grafts for bone regeneration in dentistry. MSCs are phenotypically and functionally skeletal site- specific based on extensive studies using human and rodent MSCs but there is paucity of information on canine MSCs (cMSCs) and their regenerative applications in veterinary dentistry. We hypothesized that cMSCs are functionally skeletal-site specific and that mandible cMSCs (M-cMSCs) are highly osteogenic relative to femur cMSCs (F-cMSCs). Trabecular bone samples were obtained from mandible and femur of 2 healthy beagle dogs (ages: 3 weeks, females). Primary M-cMSCs and F-cMSCs were established in culture. Using early passage cells, colony-forming units (CFU), cell proliferation and population doubling capacity were assessed. Using established induction culture conditions, in vitro osteogenesis, chondrogenesis, adipogenesis, and neurogenesis were also assessed. Western blotting and real time PCR were used to assess the following osteogenic markers: alkaline phosphatase (ALP), bone sialoprotein (BSP), osteocalcin (OCN) and osteopontin (OPN). Chondrogenesis was assessed using pellet culture method and histologic sections were stained with Alcian blue; adipogenically induced-cultures were stained with Oil Red O. Neural differentiation was evaluated using morphological analysis and immunostaining to nestin and βIII-tubulin antibodies. Furthermore, in vivo osteogenesis was assessed using the mouse model of in vivo bone regeneration. Transplants were harvested at 6, 8 and 12 weeks for histological analysis.The M-cMSCs demonstrated 1.5 to 2 fold increases in cell proliferation ($p = 0.006$) and life span (five more passages of survival) relative to F-cMSCs. Similar pattern was displayed by M-cMSCs based on expression levels of BSP (14 days $p=0.05$), ALP (14 days $p= 0.004$) and OCN (14 days $p= 0.03$) but OPN levels were not significantly different. Adipogenesis based on number of stained lipid droplets per unit area in M-cMSCs was significant higher than F-cMSCs ($p=0.007$) and chondrogenic response was also significant higher in M-cMSCs compared with F-cMSCs (4 weeks $p= 0.009$). Canine MSCs induced substantial in vivo bone formation. The canine MSCs phenotypic and functional properties are site-dependent as the M-cMSCs were apparently more responsive to multi-lineage differentiation relative to F-cMSCs. While the sample size in this study is limited, our findings are still consistent with previous studies using human, mouse and rat MSCs for site-to-site comparative characterizations (Akintoye et al, 2006; Yoshimura et al, 2007; Aghaloo et al, 2010; Lee et al, 2011). Additionally, it is imperative to further confirm these in a larger sample size and in other dog breeds since dogs exhibit an extremely wide range of body physique. New information will advance our understanding of pre-clinical applications of orofacial MSCs as donor graft materials for oral bone regeneration.

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CHARACTERIZATION OF MANDIBLE AND FEMUR CANINE MESENCHYMAL STEM CELLS: A PILOT STUDY

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A thesis submitted in partial fulfillment of the requirements for the

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DEDICATION

EVERY CHALLENGING WORK NEEDS SELF-EFFORTS AS WELL AS SUPPORT OF THOSE WHO ARE VERY CLOSE TO OUR HEART.

THIS WORK IS DEDICATED TO THE MEMORY OF MY LOVING PARENTS WHOSE AFFECTION AND ENCOURAGEMENT MADE ME THE PERSON I AM TODAY

TO MY WIFE, MERCEDES, AND SON, NICOLAS, WHOSE UNCONDITIONAL LOVE, SUPPORT AND PATIENCE ARE THE JOY AND STRENGTH THAT GOD GIVES ME EVERY DAY...

WORDS CANNOT EXPRESS MY FEELINGS, NOR MY THANKS FOR ALL YOUR HELP

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ABSTRACT

Mesenchymal stem cells (MSCs) are emerging donor grafts for bone regeneration in dentistry. MSCs are phenotypically and functionally skeletal site-specific based on extensive studies using human and rodent MSCs but there is paucity of information on canine MSCs (cMSCs) and their regenerative applications in veterinary dentistry. We hypothesized that cMSCs are functionally skeletal-site specific and that mandible cMSCs (M-cMSCs) are highly osteogenic relative to femur cMSCs (F-cMSCs).

Trabecular bone samples were obtained from mandible and femur of 2 healthy beagle dogs (ages: 3 weeks, females). Primary M-cMSCs and F-cMSCs were established in culture. Using early passage cells, colony-forming units (CFU), cell proliferation and population doubling capacity were assessed. Using established induction culture conditions, *in vitro* osteogenesis, chondrogenesis, adipogenesis, and neurogenesis were also assessed. Western blotting and real time PCR were used to assess the following osteogenic markers: alkaline phosphatase (ALP), bone sialoprotein (BSP), osteocalcin (OCN) and osteopontin (OPN). Chondrogenesis was assessed using pellet culture method and histologic sections were stained with Alcian blue; adipogenically induced-cultures were stained with Oil Red O. Neural differentiation was evaluated using morphological analysis and immunostaining to nestin and βIII-tubulin antibodies. Furthermore, *in vivo* osteogenesis was assessed using the mouse model of *in vivo* bone regeneration. Transplants were harvested at 6, 8 and 12 weeks for histological analysis.

The M-cMSCs demonstrated 1.5 to 2 fold increases in cell proliferation ($p = 0.006$) and life span (five more passages of survival) relative to F-cMSCs. Similar pattern was displayed by M-cMSCs based on expression levels of BSP (14 days $p= 0.05$), ALP (14 days $p= 0.004$) and OCN (14 days $p= 0.03$) but OPN levels were not significantly different. Adipogenesis based on number of stained lipid droplets per unit area in M-cMSCs was significant higher than F-cMSCs (p=

0.007) and chondrogenic response was also significant higher in M-cMSCs compared with F-cMSCs (4 weeks p= 0.009). Canine MSCs induced substantial *in vivo* bone formation.

The canine MSCs phenotypic and functional properties are site-dependent as the M-cMSCs were apparently more responsive to multi-lineage differentiation relative to F-cMSCs. While the sample size in this study is limited, our findings are still consistent with previous studies using human, mouse and rat MSCs for site-to-site comparative characterizations (Akintoye *et al*, 2006; Yoshimura *et al*, 2007; Aghaloo *et al*, 2010; Lee *et al*, 2011). Additionally, it is imperative to further confirm these in a larger sample size and in other dog breeds since dogs exhibit an extremely wide range of body physique. New information will advance our understanding of pre-clinical applications of orofacial MSCs as donor graft materials for oral bone regeneration.

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ABREVIATIONS USED

1. ALP: Alkaline phosphatase
2. b-FGF: Basic fibroblast factor
3. BM: Bone marrow
4. BMSCs: Bone mesenchymal stem cells
5. CAP: Carbonated hydroxyapatite
6. cMSCS: Canine mesenchymal stem cells
7. CSPG: Cartilage specific proteoglycans
8. DMP1: Dentin matrix protein 1
9. DSPP: Dentin sialophosphoprotein
10. EGF: Epidermal growth factor
11. EGFP: Enhanced green fluorescent protein
12. FABP4: Fatty acid binding-protein 4
13. FBS: Fetal bovine serum
14. GAG: Glycosaminoglycans
15. GFAP: Glial fibrillary acidic protein
16. GM-CFU: Granulocyte-Macrophage Colony-Forming Unit
17. GVHD: Graft-versus-host disease
18. HLA-DR: Human leucocyte antigen-DR
19. IGFs: Insulin-like growth factors
20. LP: Lipoprotein lipase
21. MAP-2: Microtubule-associated protein 2
22. M-CSF: Macrophage Colony-stimulating Factor
23. MEPE: matrix extracellular phosphoglycoprotein

24. MHC-I: Major histocompatibility complex-I
25. MHC-II: Major histocompatibility complex-II
26. MSCs: Mesenchymal stem cells
27. NCCs: Neural crest cells
28. OA: Osteoarthritis
29. OCN: Osteocalcin
30. OI: Osteogenesis imperfect
31. OPG: Osteoprotegerin
32. OPN; Osteopontin
33. PA: Pharyngeal arches
34. PAX1: Paired box 1
35. PDGF: Platelet-derived growth factor
36. PPARϒ: Peroxisome proliferative-activated receptor ϒ
37. RA: Rheumatoid arthritis
38. RANK: Receptor Activator of Nuclear Factor κ B
39. RANKL: Receptor activator of nuclear factor-kB ligand
40. rmhGCSF: recombinant methionyl human granulocyte colony-stimulating factor
41. SDF-1: Stromal cell-derived factor
42. SIBLINGS: Small Integrin-Binding Ligand N-linked Glycoprotein
43. TNF: Tumor necrosis factor
44. UC: Umbilical cord
45. UCB: Umbilical cord blood
46. VEGF: Vascular endothelial growth factor
47. α-MEM: Alpha- Minimum Essential Medium

1 INTRODUCTION

1.1 Development of craniofacial, axial, and appendicular skeleton

One of the hallmarks that distinguish vertebrates from invertebrates is the formation of bones, their associated cartilages, and joints. The first sign of skeletal development is formation of mesenchymal condensations, in which mesenchymal progenitor cells aggregate at future skeletal locations. These mesenchymal cells arise from different cell lineages. The mesenchyme that gives rise to the axial skeletal elements (i.e., vertebral column, ribs, and sternum) originates from the sclerotomal portion of the mesodermal somites, whereas the appendicular skeleton (pectoral girdles, upper and lower limbs, pelvis) is derived from the mesenchyme of the lateral plate mesoderm. The developmental origin of the craniofacial skeleton is more complex. Some cranial bones (e.g., the bones making up the roof and much of the base of the skull) are mesodermal in origin, but the facial bones and some other cranial bones arise from mesenchyme derived from the ectodermal neural crest. Skeletal formation progresses through two major mechanisms: intramembranous and endochondral ossification. The type of ossification and anatomic properties of the bones are determined by the location of each skeletal element. Consequently, the deep skeletal parts of the body typically first appear as cartilaginous models of the bones that will ultimately be formed. At specific periods during embryogenesis, the cartilage is replaced by true bone through the process of endochondral ossification. By contrast, during intramembranous ossification, the superficial

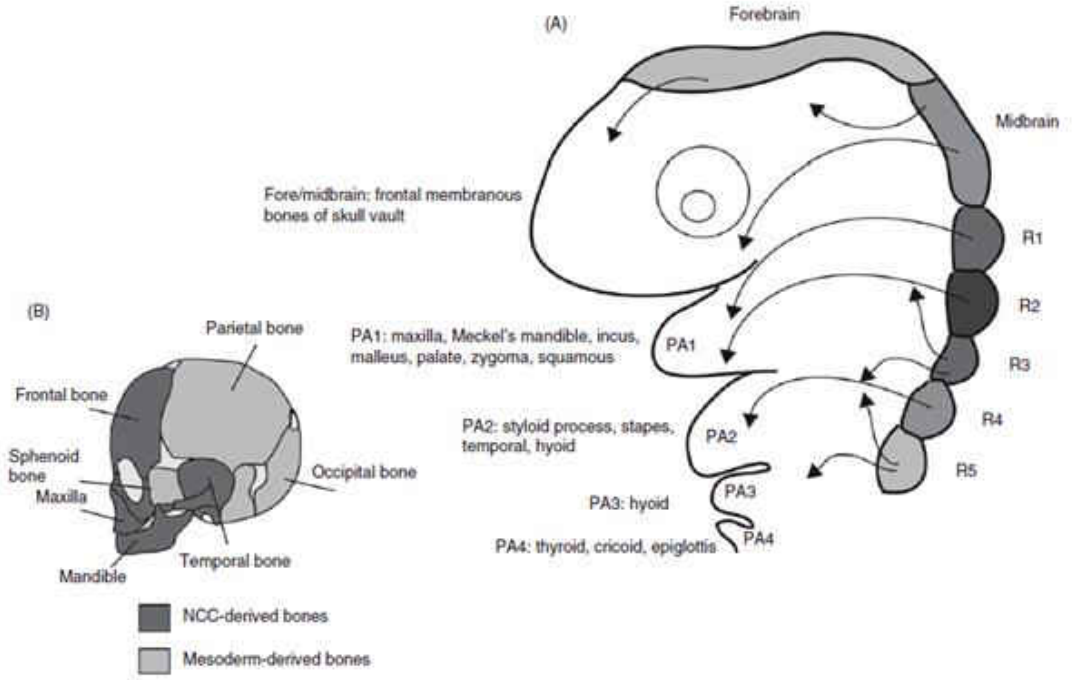
bones of the face and skull are formed by direct ossification of mesenchymal cells without an intermediate cartilaginous stage (Helms and Schneider, 2003; *Carlson*, 2014; Clifford *et al*, 2013).

Mammalian craniofacial skeleton is a complex structure of bones and cartilages that is generally divided in two main components: the neurocranium and the viscerocranium. The neurocranium encloses and supports the brain and cranial sense organs. It comprises the skull vault and base. The viscerocranium provides the structural scaffolding for sight, olfaction and, together with the mandible, mastication (Lawson, 2008). Frontal, parietal, and a part of the occipital bones form the skull vault. The frontal bones are developed from cranial neural crest cells (NCCs), while others are mostly derived from mesoderm cells. These bones are interconnected by cranial sutures which are the primary sites of osteogenesis during skull development. The skull vault is formed through intramembranous ossification. Ethmoid, sphenoid, basioccipital bones, and parts of the temporal bones build the cranial base. The anterior-most skull base is derived from cranial NCCs, while the posterior region comes from paraxial mesoderm. Contrasting with other craniofacial skeletal components, bones from the skull base develop through endochondral ossification (Clifford *et al*, 2013; McBratney-Owen *et al*, 2008).

Early craniofacial development is characterized by several massive migrations and displacements of cells and tissues. Bones that come into being the viscerocranium are derived from cranial NCCs. These cells develop in dorsal midline ectoderm of the midbrain and the rhombencephalon (or hindbrain), in a

number of transversal swellings called rhombomeres, undergo an epithelial to mesenchymal transition, delaminate, and then migrate ventrolaterally between the ectoderm and endoderm. While the rostral cranial NCCs develop the frontonasal skeleton and the skull vault, NCCs from each rhombomere, take distinct pathways to populate different pharyngeal arches (PA). NCCs from rhombomeres 1 and 2 migrate into the first pharyngeal arch and the frontonasal process. This structure gives rise to the incus and malleus of the ears, the mandible, and the maxilla. The frontonasal process gives rise to tissues in the upper half of the face, including the forehead, nose, eyes, and philtrum. NCCs from rhombomeres 3 and 4 migrate into the second pharyngeal arch, which gives rise to the stapes bone of the middle ear, the styloid process of temporal bone, and a part of the hyoid bone. The third arch gives rise to structures related to the hyoid bone and upper pharynx, while the fourth arch forms certain muscles and cartilages of the larynx and lower pharynx (Fig. 1) (Helms, 2003; *Carlson*, 2014; Clifford, *et al*, 2013).

Figure 1



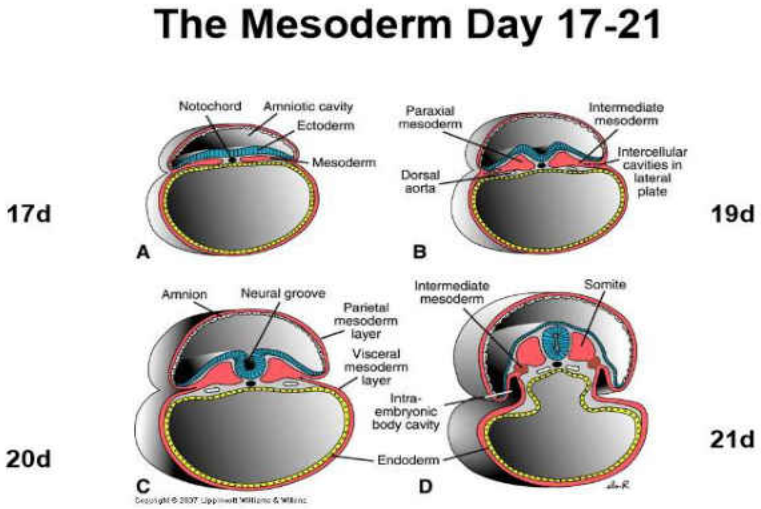
Cranial NCC migration and NCC-derived cartilage and bones: (A) NCCs go through epithelial-mesenchymal transition and migrate ventrolaterally from rhombomeres (R) to populate pharyngeal arches (PA). NCCs in R3 and R5 merge with streams of NCCs from neighboring rhombomeres. Bones and cartilage derived from each PA are listed. (B) Facial and frontal bones are derived from NCCs. Posterior skull base and vault are mostly derived from somatic mesoderm.

Adapted from Clifford *et al*, 2013.Primer on the metabolic bone diseases and disorders of mineral metabolism.

Development of the axial and appendicular skeleton: the intra-embryonic mesoderm of each side of the forming notochord and neural tube thickens to form a longitudinal column of para-axial mesoderm. By the end of the third week of the embryonic development, the para-axial mesoderm divides into paired bodies called somites, located bilaterally to the neural tube. At the fourth week, ventromedial cells migrate toward the notochord to form sclerotome

(mesenchyme). Proteins secreted by notochord and neural tube floor plate induce sclerotome formation. Once induced, sclerotome cells express the transcription factor PAX1 that initiates a cascade of cartilage and bone forming genes for vertebral column development. The first pair of somites develops a short distance posterior to the cranial end of the notochord, and the rest of the somites develop caudally. Around 38 pairs of somites form during the somite period of development, from days 20 to 30. The final number is 42 to 44 pairs. Each somite becomes differentiated into ventromedial sclerotome (for vertebrae and ribs), myotome (muscles) and dermatome (skin). In addition to the paraxial region, the mesoderm forms lateral somatic plates that form all cartilages and bones of appendicular skeleton. By the end of week four, limb buds are visible and each one consists of a mass of mesenchyme derived from the somatic mesoderm, covered by a layer of ectoderm. At the tip of each limb bud, ectodermal cells form an apical ectodermal ridge, which promotes growth and development of the limbs in the proximo-distal axis (Moore, 2008) (Fig. 2 and 3).

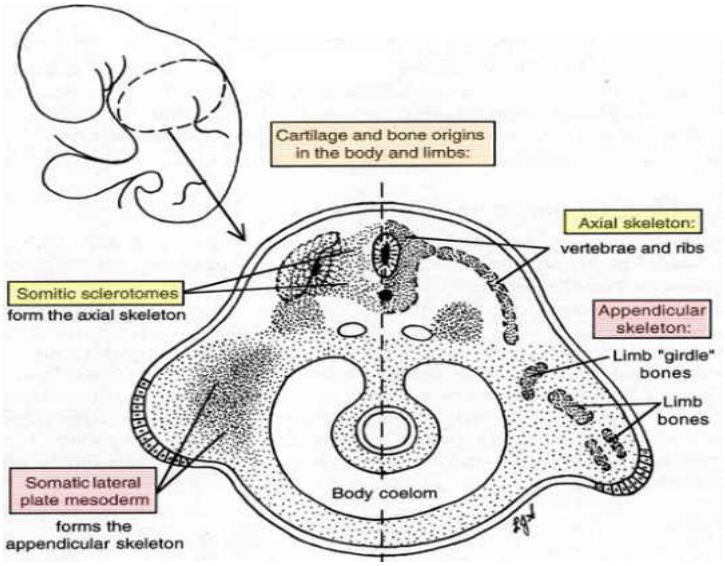
Figure 2



A third week human blastocyst with three germ layers: movement of the mesodermal cells between 17 and 21 days of development.

Adapted from Copyright © 2007 Lippincott Williams & Wilkins

Figure 3



Skeletal development vertebral column and limbs: origin of axial and appendicular skeleton from scleretome and lateral plate mesoderm.

Adapted from Mark, H. Skeletal development: vertebral column and limbs, University of Toledo

1.2 Bone tissue

1.2.1 General features

Bone is one of the most metabolically active connective tissues in the vertebrate organisms. It is a highly vascularized and well innervated mineralized tissue. Bone provides structural support and facilitates mobility by providing levers for muscle attachment. Bone also protects vital structures, serves as reservoirs for minerals and acid-base homeostasis, and is a vital site for hematopoiesis (Buck, *et al*, 2011; Fernandez, *et al*, 2006). Each particular bone of any organism constantly experiences modeling during life to adapt to changing biomechanical forces, as well as remodeling to remove old, micro-damaged bone and replace it with new, mechanically stronger bone to help preserve bone strength (Clarke, 2008).

The two main structural types of bones are cortical and cancellous or trabecular bone. The ratio of cortical to trabecular bone is different for each bone and skeletal site within a specific bone. For example, this ratio is 50:50 in the human femoral head (Clarke, 2008). Cortical bone, with a porosity of 5-10%, provides bone its compressive strength as well as a maximum resistance to torsion and bending (Buck, *et al*, 2011). Cancellous bone has a high metabolic activity and remodeling rate. It can adapt readily to mechanical stimuli and changes in loading forces (Buckwalter, *et al*, 1995). Cortical and trabecular bone are normally formed in a lamellar pattern in which collagen fibrils are tightly packed in sheets with uniform distribution of osteocytes and bone matrix. The mechanism

by which osteoblasts lay down collagen fibrils in a lamellar pattern is unknown, but lamellar bone has a significant strength as a result of the alternating orientations of collagen fibrils (Buck *et al*, 2011; Fernandez *et al*, 2006; Clarke, 2008). The arrangement of these lamellae determines whether the bone is cortical or cancellous. In the cortical bone, lamellae are concentric and parallel to the long axis of the bone. They surround central Haversian canals forming the major structural unit of cortical bone: the osteon. Multiple cell processes, or canaliculi, from osteocytes extend in a radial pattern from the central canal, allowing diffusion of nutrients through the bone matrix (Jepsen, 2009; Buck *et al*, 2011). Cancellous or trabecular bone is formed by a network of bone lamellae, delimiting areolar cavities inside which the bone marrow is found. In this bone, lamellae are arranged in semicircular shapes called packets, and this kind of structure gives cancellous bone remarkable surface area which is an important feature in the rate of bone graft incorporation (Clarke, 2008; Jepsen, 2009).

The periosteum is a fibrous connective tissue sheath that covers the external surface of bone and is attached to the outer cortex via thick collagenous fibers called “Sharpey’s fibers”. It contains blood vessels, nerve fibers, and osteoblasts and osteoclasts. Additionally, it provides an attachment site for some ligaments and tendons. The periosteum is a structure with two layers: a dense, hypocellular outer layer that continues into joint capsules and interconnects adjacent bones and an inner layer, the cambium, which contains osteoprogenitor cells and a vascular plexus. The endosteum is a membranous structure covering the inner surface of cortical bone, trabecular bone, and the blood vessel canals

(Volkmann's canals) present in bone. The endosteum is relatively cellular containing osteoprogenitor cells, as well blood vessels (Clarke, 2008; Buckwalter *et al*, 1995; Buck *et al*, 2011). Both cortical and trabecular bone contain specialized cells, organic matrix and mineral phase (Fernandez *et al*, 2006).

1.2.2 Bone cells

Several cell types can be found in bone (Table 1). Bone cells are located within the bone tissue itself or in the conjunctive stroma of the bone marrow, which is rich in mesenchymal stem cells. These cells differentiate into osteoprogenitor cells that form the osteoblasts and osteocytes, while osteoclasts arise from hematopoietic stem cells (Buck *et al*, 2011; Fernandez *et al*, 2006).

Table 1 Bone cells

BONE MARROW STROMA	BONE TISSUE
Hematopoietic stem cells	Osteoblasts
Mesenchymal stem cells	Pre-osteoblasts
Adipocytes	*Osteocytes*
Macrophages	Osteoclasts
Mastocytes	Pre-osteoclasts
Endothelial cells	Linfoid cells

Adapted from Buck *et al*, 2011; Fernandez *et al*, 2006

Osteoblasts originate from the mesenchymal stem cells of the bone marrow, endosteum, periosteum, and perivascular pericytes (Canfield *et al*, 2000). Osteoblast precursors change shape from spindle-shaped osteoprogenitors to large cuboidal differentiated osteoblasts on bone matrix surfaces after pre-osteoblasts stop proliferating. Active mature osteoblasts that synthesize bone

matrix have large nuclei, enlarged Golgi structures, and substantial rough endoplasmic reticulum (Clarke, 2008). Osteoblasts synthesize the organic matrix or osteoid material at a rate of 2 to 3 μm per day, and express a characteristic enzyme, alkaline phosphatase (ALP), which orchestrates mineralization at a rate of 1-2 μm per day. They can also express other osteoblastic markers such as bone sialoprotein, osteopontin, and osteonectin during the process of osteoblastic differentiation. It is accepted that they: (i) synthesize the collagen and non-collagen proteins of the organic bone matrix, (ii) direct the arrangement of the extracellular matrix fibrils, (iii) contribute to the mineralization of the osteoid material, due to the alkaline phosphatase, (iv) mediate in the resorption carried out by the osteoclasts, through the synthesis of specific cytokines, and (v) synthesize growth factors (Fernandez *et al*, 2006). Usually after 10 weeks, osteoblasts can disappear through apoptosis, become transformed into bone lining cells or into osteocytes (15%) (Aubin and Liu, 1996). Therefore, flattened bone-lining cells are thought to be quiescent osteoblasts that form the endosteum on trabecular and endosteal surfaces and underlie the periosteum on the mineralized surface. Osteoblasts and lining cells are found in close proximity and joined by adherents junctions. Cadherins are calcium-dependent transmembrane proteins that are integral parts of adherent junctions and together with tight junctions and desmosomes join cells together by linking their cytoskeletons (Shin, 2000).

Osteoclasts are large multinucleated cells, rich in mitochondria and vacuoles responsible for bone resorption, located in shallow depressions on bone surfaces

called Howship lacunae (Buck *et al*, 2011; Fernandez *et al*, 2006; Clarke 2008). These cells originate from the bone marrow hematopoietic stem cells known as 'Granulocyte-Macrophage Colony-Forming Units' (GM-CFU), which are precursors of macrophages and monocytes (Fernandez et *al*, 2005; Compston *et al*, 2013). In the process of osteoclastogenesis, marrow stromal cells and osteoblasts play a critical role since they secrete two essential cytokines: macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor-kB ligand (RANKL), which is a ligand situated on the surface of the osteoblasts and pre-osteoblasts (Teitelbaum *et al*, 2003; Fernandez et al, 2006; Clarke, 2008; Clifford *et al*, 2013). RANKL, previously called osteoclast differentiation factor (Simone *et al*, 1997), is a transmembrane cytokine belonging to the tumor necrosis factor family (TNF), and interacts with its receptor, RANK, expressed by pre-osteoclasts. This interaction initiates osteoclastic differentiation and activity to promote resorption. M-CSF is required for the proliferation, survival, and differentiation of osteoclast precursors, as well as osteoclast survival and cytoskeletal rearrangement required for bone resorption (Clarke, 2008; Fernandez et al, 2006). In addition, osteoprotegerin (OPG) is a soluble protein secreted by osteoblasts and pre-osteoblasts that binds RANKL with high affinity to inhibit its action at the RANK receptor (Cohen, 2006). When OPG and RANKL bind together, the union between RANK and RANKL is inhibited, and thus the osteoclastic differentiation is also inhibited. For this reason OPG, RANK and RANKL are important regulators of osteoclastogenesis (Fernandez *et al*, 2006). The other factors and cytokines that regulate osteoclast

formation and activity, include interleukin-1, interleukin-6, parathyroid hormone, 1,25-dihidroxyvitamin D, and calcitonin (Boyle *et al*, 2003; Blair and Athanasou, 2004). The membrane of osteoclasts has two special characteristics: a ruffled border, where resorption takes place, and a clear area rich in microfilaments, with integrins that serve as an anchor to the matrix. When the resorption process starts, the osteoclasts move towards the area to be resorbed and then immediately adhere to the mineralized bone surface with the ruffled border and sealing the edges of the area with the integrins. The β1 family of integrin receptors in osteoclasts, particularly αvβ3, recognizes the Arg-Gly-Asp sequence in the collagen and other proteins of the osteoid matrix. At this level the pH is acidic since osteoclasts secrete hydrogen ions, generated by carbonic anhydrase II and proteolytic enzymes such as collagenases, metalloproteases, cathepsin K, and glucuronidase.These enzymes initiate bone resorption by the solubilization of, first the organic and, then the mineral matrix. Osteoclasts create a sealing zone that surrounds and isolates the acidified resorption compartment from the surrounding bone surface (Ross and Teitelbaum, 1995; Vaananen *et al*, 2000). Bone resorption can be blocked by disruption of either the ruffled border or the actin ring created by the fibrillar actin cytoskeleton of the osteoclast. When the osteoclasts are actively resorbing, they form podosomes, which attach to bone matrix, rather than focal adhesions as formed by most cells. Podosomes are composed of an actin core surrounded by αvβ3 integrins and associated cytoskeletal proteins (Clarke, 2008).

Osteocytes represent terminally differentiated osteoblasts and function within syncytial networks to support bone structure and metabolism. In the adult skeleton, osteocytes account for 90–95% of all bone cells, compared to 4–6% osteoblasts and approximately 1–2% osteoclasts (Clarke, 2008; Clifford *et al*, 2013). Once the matrix is mineralized, some osteoblasts remain trapped within vacuoles called lacunae, becoming transformed into osteocytes. Osteocytes maintain connection with each other and cells on the bone surface through dendritic processes generally radiating towards the bone surface and the blood supply. The dendritic processes travel through the bone in tiny canals called canaliculi while the cell body is encased in lacunae. This interconnection allows osteocytes to function as a network of sensory cells that respond to mechanical loading through this extensive network (Fernandez et al, 2006; Clarke, 2008; Clifford *et al*, 2013). Osteocytes are linked metabolically and electrically through gap junctions composed primarily of connexin 43 (Plotkin *et al*, 2002). Connexins are integral cellular proteins that maintain gap junctions between cells to allow direct communication through intercellular channels. Gap junctions are required for osteocyte maturation, activity, and survival (Clarke, 2008).Osteocytes have long been thought to control biologic activity of bone since they transduce stress signals from bending or stretching of bone into bone resorption or formation (Fernandez et al, 2006; Clarke, 2008; Buck and Dumanian, 2011; Clifford *et al*, 2013). Signaling mechanisms involved in mechanotransduction include prostaglandin E2, cyclo-oxygenase 2, various kinases, Runx2, and nitrous oxide (Clarke, 2008). It has also been shown that osteocytes have another important

function: to regulate phosphate homeostasis; therefore, the osteocyte network may also function as an endocrine gland (Clifford *et al*, 2013). Until recently, the markers described for osteocytes were limited to low- or no-alkaline phosphatase, high casein kinase II, high osteocalcin protein expression, and high CD44 as compared to osteoblasts. At the present time, osteocyte markers such as E11/gp38, phosphate-regulating neutral endopeptidase on the chromosome X (Phex), dentin matrix protein 1 (DMP1), sclerostin, FGF23, and ORP150 are well known (Clifford *et al*, 2013) (Table 2)

Table 2 Osteocyte markers

Marker	Expression	Function
E11/gp38	Early embedding cell	Dendrite formation
CD44	More highly expressed in osteocytes compared to osteoblasts	Hyaluronic acid receptor associated with E11 and linked to cytoskeleton
Fimbrin	All osteocytes	Dendritic branching
Phex	Early and late osteocytes	Phosphate metabolism
OF45/MEPE	Late osteoblasts through osteocytes	Inhibitor of bone formation/regulator of phosphate metabolism
DMP1	Early and mature osteocytes	Phosphate metabolism and mineralization
Sclerostin	Late embedded osteocyte	Inhibitor of bone formation
FGF23	Early and mature osteocytes	Induces hypophosphatemia
ORP150	Mature osteocytes	Protection from hypoxia

Adapted from Clifford *et al*, 2013

1.2.3 Bone matrix

Bone matrix represents 90% of the composition of the bone volume. It consists of four major components: inorganic or mineral matrix (65%), organix matrix (20%), and lipids and water (< 15%) (Clarke, 2008).

Organic matrix, secreted by osteoblasts, is predominantly type I collagen (90%) (Table 3) with trace amounts of types III and V and FACIT collagens at certain stages of bone formation that may help define collagen fibril diameter. FACIT collagens are members of the family of Fibril-Associated Collagens with Interrupted Triple Helices, a group of non-fibrillar collagens that serve as molecular bridges that are important for the organization and stability of extracellular matrices. The presence of small amounts of collagen type III has been found, related to Sharpey's fibers. It is believed that collagen has no great affinity for calcium, for this reason other proteins are involved in mineral deposition (Fernandez et al, 2006; Clarke, 2008; Buck and Dumanian, 2011).

Osteoblasts also synthesize and secrete non-collagenous proteins which make up 10 to 15% of total bone protein. The non-collagenous proteins are divided broadly into several categories, including serum-derived proteins, proteoglycans, glycosylated proteins, SIBLINGs (Small Integrin-Binding Ligands N-Glycosylated proteins), gla-containing proteins, and growth factors (Fernandez *et al*, 2006; Clarke, 2008; Clifford *et al*, 2013).

(i) Serum-derived proteins include, mainly, albumin and α_2-HS-glycoprotein. These proteins have good affinity for hydroxyapatite, and therefore are able to bind to bone matrix.

(ii) Proteoglycans are large molecules and make up 10% on the non-collagenous proteins, and bone matrix contains several members of this family such as versican (chondroitin-sulphate), hyaluronan (glycosaminoglycan), decorin, biglycan, perlecan, osteoadherin, lumican, aspirin, and fibromodulin among others.

(iii) Glycosylated proteins with various functions are abundant in bone. During bone formation, it is distinctive the synthesis of high levels of alkaline phosphatase, thus it is considered a good marker of osteoblast activity. This enzyme liberates inorganic phosphate from phosphoric esters, and is necessary for mineralization. The most abundant non-collagenous protein produced by bone cells is osteonectin, and it plays a role in the regulation of cellular adhesion between the matrix and the cells as well as is important for normal bone mineralization.

(iv) Bone cells produce at least 12 proteins that may mediate cell attachment. Among them, they are five proteins that are phosphorylated and/or sulfated, and contain the RGD tripeptide (Arg-Gly-Asn), also called SIBLINGs: osteopontin, bone sialoprotein, dentin matrix protein 1 (DMP1), dentin sialophosphoprotein (DSPP), and matrix extracellular phosphoprotein (MEPE). These glycoproteins are

essential to bone regeneration and remodeling processes because the Arg-Gly-Asn sequence is recognized by osteoblast and osteoclast integrins. They also act as bone cell surface receptors, allowing the adhesion of the cells to the extracellular matrix, and activating signals. Other proteins that participate in cell attachment are fibronectin, vitronectin, fibrillin and thrombospondins.

(v) Four bone matrix non-collagenous proteins can be distinguished in the group of Gla-containing proteins: matrix gla protein (MGP), osteocalcin, periostin, and protein S. Osteocalcin is a matrix protein produced by osteoblasts and platelets. In human bone, osteocalcin is concentrated in osteocytes, and its release may be a signal in the bone turnover cascade. Its measurements in serum have proved valuable as a marker of bone turnover in metabolic disease states.

(vi) Growth factors include the bone morphogenetic proteins, transforming growth factors β family, interleukin-1, and interleikn-6, for example. These factors all play important roles in bone osteogenesis, mineralization, and remodeling (Table 3).

Table 3 Osteoid matrix

COLLAGEN	**Type I, III, V, X**
SERUM-DERIVED PROTEINS	• Albumin • α_2HS glycoprotein
PROTEOGLYCANS	• Aggrecan • Versican • Decorin • Biglycan • Asporin • Fibromodulin • Osteoadherin • Lumican • Perlecan
GLYCOSYLATED PROTEINS	• Alkaline phosphate • Osteonectin • Periostin • Tetranectin • Tenascin-C • Tenascin-X • Secreted phosphoprotein
SIBLINGs AND OTHER GLYCOPROTEINS WITH CELL ATACHMENT ACTIVITY	• Osteopontin • Bone sialoprotein • DMP-1 • Dentin sialophosphoprotein • MEPE • Thrombospondins • Fibronectin • Vitronectin • Fibrillin
GLA-CONTAINING PROTEINS	• Matrix Gla protein • Osteocalcin • Periostin • Protein S
GROWTH FACTORS	• IGF-I, -II • TGF-β

Adapted from Clifford *et al*, 2013. Primer on the metabolic bone diseases and disorders of mineral metabolism.

The inorganic bone matrix accounts for 99% of the body's storage of calcium, 85% of the phosphorous, and 40-60% of the magnesium and sodium stores. Inorganic matrix is mainly in the form of hydroxyapatite and provides the majority of bone strength, stiffness, and resistance to compressive forces. Removal of the inorganic matrix makes bone soft, malleable, and spongy (Buck, 2011). The extracellular mineralized matrix is now considered as something more than simply a reservoir of calcium and phosphorous, since it constitutes a reserve of proteins that participate in the regulation of cellular differentiation and in the integrity and function of bone tissue (Young, 2003).

1.2.4 Bone growth, modeling, and remodeling

Bone growth occurs longitudinally and radially by the process of endochondral ossification and appositional bone growth, respectively. Longitudinal growth occurs at the growth plates, where cartilage proliferates in the epiphyseal and metaphyseal areas of long bones, before subsequently undergoing mineralization to form primary new bone. Appositional bone growth arises at the level of the periosteum, with subsequent resorption of old bone at the level of the endosteum (Clarke, 2008; Buck, 2011).

By the process of modeling, the bones change their shape in response to physiologic influences or mechanical forces. Modeling of the cranium, for example, is thought to be transmitted via mechano-transduction signals from underlying brain growth (Stool and Vig, 2003). Bones may enlarge or change axis by subtraction or addition of bone to the appropriate surfaces by

independent action of osteoblasts and osteoclasts in response to biomechanical forces. Bones normally widen with age in response to periosteal apposition of new bone and endosteal resorption of old bone (Clarke, 2008). Wolff's law states that bones change shape to accommodate stresses placed upon them (Sommerfeldt and Rubin, 2001). In fact, bone resorption occurs if stress does not happen and is reinforced where stress forces are applied. An example is the resorption of an edentuolous mandible from the lack of the normal forces of mastication (Buck, 2011).

Bone remodeling is the process that takes place to maintain bone health and strength as well as mineral homeostasis. The remodeling process resorbs old bone and forms new bone to prevent accumulation of bone micro-damage (Clarke, 2008; Buck, 2011). The bone remodeling unit is composed of a tightly coupled group of osteoclasts and osteoblasts that sequentially carry out resorption of old bone and formation of new bone. The remodeling cycle is composed of four sequential phases; activation, that includes fusion of multiple mononuclear cells to form multinucleated preosteoclasts; resorption, mediated by osteoclasts and takes only approximately 2 to 4 weeks during each remodeling cycle; reversal, where preosteoblasts are recruited to begin new bone formation, and formation that takes approximately 4 to 6 months to be completed. Osteoblasts synthesize new collagenous organic matrix and regulate mineralization of matrix by releasing small, membrane-bound matrix vesicles that concentrate calcium and phosphate and enzymatically destroy mineralization inhibitors such as pyrophosphate or proteoglycans (Anderson, 2003).

Remodeling begins at birth and continues through adulthood to the time of death (Clarke, 2008; Buck, 2011).

1.3 Mesenchymal stem cells (MSCs)

1.3.1 History

Although the early work of Tavassoli and Crosby (Tavassoli and Crosby, 1968) clearly set up proof of an inherent osteogenic potential associated with bone marrow (BM), the specific identity of any cell functioning as a progenitor of differentiated bone cells could not be outlined. Few years later, Friedenstein *et al* (Friedenstein, 1970), in a series of studies, verified that the ability of bone marrow cells of generating new bone marrow when transplanted into a different site, was associated with a secondary subpopulation of BM cells. These cells were distinct from the majority of hematopoietic cells by their rapid adherence to tissue culture vessels and by the fibroblast-like appearance of their progeny in culture, indicating their origin from the stromal compartment of BM (Bianco *et al*, 2008). These investigators also demonstrated that seeding of BM cell suspensions at clonal density resulted in the establishment of discrete colonies initiated by single cells. These colonies represented the colony-forming unit fibroblastic (CFU-F). Additional study reviews by Friedenstein (Friedenstein, 1990) of *in vivo* transplantations, led to the conclusion that the progeny a single BM stromal cell could generate multiple skeletal tissues (bone, cartilage, adipose tissue, and fibrous tissue). Friedenstein and Owen called this cell a BM stromal

stem cell (Owen and Friedenstein, 1988). Consequently, these initial studies revealed that a second type of stem cell could be present in the BM and, specifically, in the hematopoiesis-supporting stroma. In 1999, Pittenger *et al* (Pittenger *et al*, 1999) published an additional similar work and the concept of a non-hematopoietic stem cell in BM start being repeated worldwide. The term mesenchymal stem cell, proposed previously by Caplan in 1991 (Caplan, 1991) as an alternative to stromal or osteogenic stem cell, earned wide acceptance.

1.3.2 Biological characteristics of MSCs

Stem cells are defined as clonogenic, undifferentiated cells characterized by their ability to self-renew and give rise to terminally differentiated cells of multiple lineages (Shanti *et al*, 2007; Deng *et al*, 2008; Eckfeldt *et al, 2005*). Stem cells have been isolated and characterized from embryonic, fetal, and adult tissues (Shanti *et al*, 2007). Due to ethical, political and technical issues, the use of embryonic and fetal stem cells is still controversial, so using adult or postnatal stem cells has become more accepted (Shanti, RM *et al*, 2007; Deng *et al*, 2008; Keller, 2005). A variety of tissues can serve as source for the different type of adult stem cells (Ratajczak *et al*, 2014; Sousa *et al*, 2014; Shanti *et al*, 2007) (Table 4).

Table 4 ADULT STEMM CELLS

Stem Cell	Source (location)	Tissue differentiation potential
Hematopoietic	Bone marrow Mobilized peripheral blood Umbilical cord blood	Blood cell types, endothelial, myoblasts, and hepatocytes
Epithelial	Epidermis Intestinal lining	All cells of epithelium crypts and all cells of epidermal cells
Neural	Subventricular zone lining the lateral ventricles Subgranular zone, part of the dentate gyrus of the hippocampus	Neurons, oligodendrocytes, and astrocytes
Mesenchymal	Bone marrow, muscle, trabecular bone, adipose tissue, dermis, umbilical cord blood, periosteum, blood, synovial membrane, periodontal ligament, and deciduous teeth	Adipocyte, chondrocyte, myoblast, osteoblast, cardiomyocyte, hepatocytes, neuron, astrocyte, endothelial, fibroblast, and stromal cells
Olfactory	Human olfactory mucosa cells, which are found in the lining of the nose	Neurons and glia

Adapted from Ratajczak *et al*, 2014; Sousa *et al*, 2014; Shanti *et al*, 2007

The term mesenchymal stem cell is based on the premise that the cells can differentiate into a variety of mesodermal tissues including bone, cartilage, and adipose (Si, YL *et al*, 2011). In line with this concept, an important feature of MSCs is their ability to differentiate into several mesenchymal lineages such as osteoblasts, chondrocytes, adipocytes, myoblasts, and tenocytes (Pittenger *et al*, 1999; Deng *et al*, 2008; Pittenger, 2008). There is also evidence that MSCs could have the potential to differentiate into cells of ectodermal lineage such as neurons, as well as endodermal lineage such as hepatocytes (Jiang *et al*, 2002; Lee *et al*, 2004; Tomita *et al*, 2007). Moreover, an increasing number of evidence suggests that MSCs have immunomodulatory properties, anti-inflammatory

effects, and secrete various growth factors and cytokines (Si, YL *et al*, 2011; Shanti *et al*, 2007; Pittenger, 2008; Chamberlain *et al*, 2007). The underlying mechanisms responsible for the immunosuppressive and anti-inflammatory effects of MSCs are not completely understood yet (Si *et al*, 2011; Shanti *et al*, 2007). Nonetheless, evidence states that MSCs lack immunogenicity based on their immune phenotype. They express low levels of major histocompatibility complex-I (MHC-I), do not express MHC-II or costimulatory molecules that include CD40, CD80, and CD86 (Le Blanc and Ringden, 2007; Tse *et al*, 2003). Consequently, MSCs will not activate allogeneic or xenogeneic lymphocytes (Si et *al*, 2011). In addition, MSCs are able to suppress the activation and proliferation of both T and B lymphocytes (Jones *et al*, 2007; Corcione *et al*, 2006). MSCs secrete growth factors and cytokines which exhibit autocrine and paracrine activities (Pittenger, 2008) that may modulate inflammation, apoptosis, fibrosis of damaged tissues and tissue regeneration (Si et *al*, 2011). Some of these include vascular endothelial growth factor (VEGF), stem cell factor (SCF-1), macrophage colony stimulating factor (M-CSF) and interleukins (IL-1, -6, -7, -8, -11, -14, and -15), stromal cell-derived factor (SDF-1) (Haynesworth *et al*, 1996; Reese *et al*, 1999; Pittenger, 2008). There are still no uniformly accepted markers to confirm homogeneity of MSCs (Si et *al*, 2011; Shanti *et al*, 2007; Chamberlain *et al*, 2007; Chen and Tuan, 2008). For this reason, the International Society for Cell Therapy has issued the minimal set of standard criteria to identify MSCs (Dominici *et al*, 2006). These include: (i) the ability to adhere to plastic surfaces under standard culture conditions; (ii) positive

expression of CD73, CD90, and CD105; (iii) lack of expression of CD14, CD19, CD 31, CD34, CD45, and human leucocyte antigen-DR (HLA-DR) surface molecules; (iv) multipotent ability to differentiate into bone, cartilage, and adipose tissue; and (v) immunomodulatory functions.

In addition to the bone marrow, MSCs are also found in almost all postnatal organs and tissues, including periosteum, adipose tissue, periodontal ligament, dermis, deciduous teeth, vascular pericytes, trabecular bone, umbilical cord and umbilical cord blood as well as amniotic membrane (Bianco, 2008; Rebelatto *et al*, 2008; Seo *et al*, 2004; Miura *et al*, 2003; Markov *et al*, 2007; Brighton *et al*, 1992; Mageed *et al*, 2007; Alviano *et al*, 2007; Si et *al*, 2011). Bone marrow, adipose tissue, umbilical cord blood, and umbilical cord are usually considered as the main sources of MSCs for tissue regeneration and engineering (Si et *al*, 2011). Apart from being the first documented source of MSCs, bone marrow has proven to be reproducible and convenient site in all species for harvesting MSCs. (Si et *al*, 2011; Pittenger, 2008). While MSCs from different tissues display similar basic biological features, there are considerable disparities among them such as difference in the expansion potential under identical culture conditions (Kern *et al*, 2006), and age-related functional properties. Furthermore, the existence of site-specific variation in bone cell responses has been suggested in the literature. Diverse studies have proposed that MSCs derived from craniofacial and axial/appendicular bones are phenotypically and functionally distinctive based on their different embryological origins (Akintoye *et al*, 2006; Gronthos *et al*, 2006). The jaw bones arise embryologically from neural crest cells of the

neuroectoderm germ layer, while the axial and appendicular bones arise from the mesoderm (Akintoye *et al*, 2006; Aghaloo *et al*, 2010; Chai and Maxson, 2006). In addition, the mandible and maxilla, are formed by intra membranous ossification and, in the case of the mandible, secondary cartilage at its proximal end contributes endochondral components at later stages of development. Meckel's cartilage participates, to a limited extent, in the formation of the mandible, but two secondary cartilages (coronoid and condylar) contribute also to the mandible (Clifford *et al,* 2013). Furthermore, the jaw bone could develop non-odontogenic bone pathologies such as osteoclast-like giant cells and fibrous lesions found in cherubism (Ueki *et al*, 2001) and hyperparathyroid jaw tumor syndrome that do not occur in non-oral bones (Simonds *et al*, 2002). Additionally, osteonecrosis of the jaws caused by bone antiresorptives such as bisphosphonates and denosumab (Ruggiero et al, 2004) affect only the maxilla and mandible, suggesting different homeostatic mechanisms between the jaws and long bones. In an earlier study on skeletal site-dependent responsiveness of bone cells, Malpe *et al* (Malpe *et al*, 1997) assessed their responsiveness to insulin-like growth factors (IGFs), which are important regulators of bone metabolism**.** They concluded that there are skeletal site-dependent differences in the production of IGF system components and suggest that the regulation of bone metabolism may vary at various skeletal sites.

Akintoye *et al* (Akintoye *et al*, 2006) investigated skeletal site-specific phenotypic and functional differences between orofacial (maxilla and mandible) and axial (iliac crest) human BMSCs in same individuals *in vitro* and *in vivo*. The results

suggested that orofacial BMSCs are unique cell populations, and that the differences between both types of cells are skeletal site-dependent, possibly related to distinctive embryological origins and adjustment to functional demands at each skeletal site.

Aghaloo *et al* (Aghaloo *et al*, 2010) hypothesized that rat mandible vs. long-bone marrow-derived cells possess different osteogenic potential. By using bone marrow stromal cells derived from rat mandible and from rat tibiae, these investigators compared the *in vitro* osteoblastic differentiation and *in vivo* bone formation capacity of both cell types. They reported that there is an amplified osteogenic potential and augmented capacity of mandibular BMSCs to induce bone formation *in vitro* and *in vivo*.

1.3.3 Clinical applications of MSCs

MSCs are viable cell populations for tissue engineering, regenerative medicine, and autoimmune disease therapy because of their multipotent capacity, ease of culture expansion and low immunogenicity (Chamberlain *et al*, 2007; Meirelles *et al*, 2008; Si *et al*, 2011).

a) MSCs in cardiovascular conditions

Some studies have demonstrated that MSCs could have an important function in myocardial infarctions and ischemic cardiomyopathies (Psaltis *et al*, 2008; Ohnishi *et al*, 2007). This therapeutic capacity could be completed by different functions such as direct differentiation into cardiac tissue (Gojo *et al*, 2003); secretion of cytokines and growth factors (Kinnaird *et al*, 2004); through

immunosuppressive properties that might decrease inflammation of damaged myocardial tissue (Du *et al,* 2008); and through stimulation of endogenous repair (Paul *et al*, 2009). In the study by Gojo *et al* (Gojo *et al*, 2003), it was demonstrated that the injection of adult MSCs into healthy adult myocardium could produce cardiomyocytes, endothelial cells, and pericytes or smooth muscle cells, revealing that cultured MSCs have the capacity to engraft into healthy tissue and can differentiate into several cell types *in vivo*.

a) MSCs in diabetes

Therapeutic efficacy of MSCs in diabetes mellitus has been mentioned in some reports. In the study by Chao *et al* (Chao *et al*, 2008), for example, MSCs from Wharton's jelly of the human umbilical cord were successfully differentiated into mature islet-like cell clusters with the ability of producing insulin *in vitro* and *in vivo*. Working with mice, Ezquer *et al* (Ezquer *et al*, 2008) demonstrated that the systemic administration of bone marrow-derived MSCs could control hyperglycemia and prevents renal damage in type I diabetes. Nonetheless, the mechanisms by the MSCs could have this therapeutic effect is still unclear. Some authors (Xie *et al*, 2009) have proposed that MSCs differentiate directly into functionally competent new β-cells.

b) MSCs in neurological disorders

According to some studies, MSCs could have neuroprotective effects in central nervous system injuries and progressive degenerative diseases. This role has been described for spinal cord injuries (Himes *et al*, 2006); Parkinson's disease (Park *et al*, 2008); autoimmune encephalomyelitis (Zhang *et al*, 2006), and

multiple system atrophy (Lee and Park, 2009), among others. Interestingly, it is unrevealed if MSCs could differentiate into neural cells *in vivo*. Blandini *et al* (Blandini *et al*, 2010) showed that human MSCs *in vitro* expressed some neural markers including nestin, β III tubulin and microtubule-associated protein 2 (MAP-2), but did not express a glial or specific neuronal markers. However, after these cells were transplanted into rats, they lost positivity for nestin and expressed a glial-like phenotype. Hofstetter *et al* (Hofstetter *et al*, 2002) found that rat MSCs injected into rats with spinal cord injuries, formed guiding strands in the injured spinal cord facilitating regeneration.

c) MSCs in graft-versus-host disease (GVHD)

As a result of the immunomodulatory properties of MSCs, infusions of this cell type have been used to treat GVHD developed in patients with allogeneic hematopoietic stem cell transplantations (Si *et al*, 2011). Le Blanc *et al* (Le Blanc *et al,* 2004) transplanted haploidentical mesenchymal stem cells in a patient who had progressive severe GVHD that was unresponsive to all types of therapy. They reported remarkable decrease of symptoms in this patient. Later, in a multicenter, phase II experimental study, Le Blanc *et al* (Le Blanc *et al*, 2008) treated 55 patients with steroid-resistant, severe, acute GVHD with mesenchymal stem cells. More than 50% of the patients had a complete response and nine showed improvement.

d) MSCs in bone/cartilage defects

Degenerative bone diseases such as osteoarthritis (OA), rheumatoid arthritis (RA), and osteogenesis imperfecta (OI) have found great treatment options in

MSCs. Properties of MSCs such as the ease of isolation and expansion and the multipotential differentiation capacity, especially the chondrogenic differentiation property of MSCs, make MSCs the cell type of choice for articular cartilage tissue engineering that intends to replace and regenerate the diseased structure in joint diseases. Moreover, their potent immunosuppressive and anti-inflammatory functions can be harnessed for therapeutic application in degenerative joint diseases mentioned above (Chen and Tuan, 2008). Cartilage tissue engineering has used the chondrogenic differentiation potential of MSCs loaded on a three dimensional (3-D) scaffold as replacement tissue for cartilage repair (Chen and Tuan, 2008). In addition, MSCs have been used directly in cell therapy for *in situ* repair of OA cartilage. The study performed by Murphy *et al* (Murphy *et al*, 2003), treated induced OA in goats with autologous MSCs in hyaluronan solution. Their results demonstrated inefficient engraftment of MSCs to articular cartilage. They concluded that the favorable effect of MSCs, on cartilage protection and on OA progression, was probably due to induction of endogenous progenitor cells. These cells were responsible to regenerate meniscus that, in turn, retarded cartilage degeneration associated with OA. This study, and others (Augello *et al*, 2007; Noth *et al*, 2008), have suggested that MSC-based graft exert a therapeutic effect in arthritis, possibly through their trophic effect and their anti-inflammatory and immunosuppressive actions, which can significantly affect the local environment and resident endogenous tissue progenitor cells in carrying out the regenerative function (Chen and Tuan, 2008). A T-cell-mediated systemic disease like RA is characterized by articular cartilage damage (Si *et al*, 2011),

and the potential therapeutic value of MSCs in its treatment has been evaluated in some studies. Zheng *et al* (Zheng *et al*, 2008) showed that bone marrow-derived MSCs and MSC-differentiated chondrocytes could suppress type II collagen-reactive T-cell responses in RA. This suggests that MSCs could be a potential candidate for RA treatment in future if this is further confirmed *in vivo*. Horwitz *et al* (Horwitz *et al*, 2002) demonstrated the viability of bone marrow-derived mesenchymal cells therapy in a group of six children with severe OI. They infused allogeneic cells and five patients showed engraftment in one or more sites, including bone, skin, and marrow stroma, and had an acceleration of growth velocity during the first 6 months post-infusion. Despite the outcomes of all studies mentioned above, caution should be exercised as this field of research is still developing and conflicting results have been reported in different systems from different labs (Chen and Tuan, 2008; Si *et al*, 2011).

e) Applications in maxillofacial surgery

MSCs have shown to be an ideal cell source for maxillofacial tissue engineering. When these cells are used with scaffolding materials that possess suitable biological and physical properties, tissue regeneration from cell-based therapies can produce desirable clinical outcomes (Shanti *et al*, 2007). MSCs have been also used to deliver genes or gene products such as bone morphogenetic proteins for bone repair (Chang *et al,* 2003) or the use of bone marrow-derived MSCs as vehicles for chemotherapeutics (e.g. Interferon-β) into tumors (Studeny *et al*, 2002). A number of delivery vehicles loaded with MSCs have been employed to heal critical-sized segmental bone defects. An example of this is the

study by Bruder *et al* (Bruder *et al*, 1998) who examined the effect of cultured autologous MSCs on the healing of critical-sized segmental defects in the femora of adult female dogs. The cells were loaded onto porous ceramic of hydroxyapatite and beta-tricalcium phosphate ceramic. It was found a greater amount of bone in the implants that had been loaded with mesenchymal stem cells compared with the implants that had not been loaded with cells. In pediatric patients, cell-based tissue engineering, preferably using autologous cells, presents a promising, alternative method for skull bone reconstruction (Shanti *et al*, 2007). A 7-year-old girl with widespread calvarial defects after severe head injury was successfully treated with autologous adipose-derived stem cells that were grafted to the calvarial defects *(*Lendeckel *et al*, 2004). The stem cells were kept in place using autologous fibrin glue. Mechanical fixation was attained by two large, resorbable macroporous sheets acting as a soft tissue barrier at the same time. After 3 months, postoperative computed tomography scans showed new bone formation and near complete calvarial continuity. Certainly, more advances in the engineering of craniofacial bone are necessary, as well as development of resorbable scaffolds that will replicate tissue shape and form while degrading in a controlled manner (Shanti *et al*, 2007). Another maxillofacial use of MSCs is in temporomandibular joint (TMJ) reconstruction. As the TMJ is susceptible to diverse degenerative pathologies, cell-based tissue engineering approaches using MSCs for the replacement of mandibular condyles offer an important therapeutic option (Shanti *et al*, 2007). In the literature some reports (Alhadlaq *et al*, 2003; Tuli *et al*, 2004) have described different approaches for

the ex *vivo* development of articular tissue component, such as mandibular condyle. However, a significant amount of research is still needed before tissue-engineered mandibular condyles can be placed for clinical uses (Shanti *et al*, 2007).

1.3.4 Isolation of MSCs

MSCs can be obtained from multiple tissues but bone marrow offers the most readily available source. Most of the information about MSCs, specifically biological properties and characteristics, is from bone marrow-derived cells (Shanti *et al*, 2007). *In vitro* MSCs expansion is necessary for regenerative and immunotherapeutic approaches since adult bone marrow contains low percentage of MSCs and a significant number of cells is required for the specialized therapies (Sotiropoulou *et al* , 2006). Effective isolation and expansion of MSCs depends on several factors such as culture medium, starting and passaging cell-plating density, culture surfaces, addition of supplementary factors, and the effects of donor age and cryopreservation (Colter *et al*, 2000; Sekiya *et al*, 2002; Caterson *et al*, 2002; Pittenger, 2008; Sotiropoulou *et al* , 2006). Many different formulations of growth media have been used in experimental and clinical protocols to isolate and growth MSCs (Pittenger, 2008; Sotiropoulou *et al*, 2006).

Numerous methods have been proposed for qualitative assessment of MSCs isolated for clinical use varying from simple colony-forming assays to more

complex morphological characterizations. To mention some of these approaches, in the study by DiGirolamo *et al* (DiGirolamo *et al*, 1999) the replicative potential of human marrow stromal cells was evaluated by a simple colony-forming assay in which samples from early passages were plated at low densities of about 10 cells per cm^2. On the other hand, Smith *et al* (Smith *et al*, 2004) proposed a more sophisticated morphological analysis that may be useful as a rapid method to characterize small stem-like cells from a number of adult tissues. Sotiropoulou *et al* (Sotiropoulou *et al*, 2006) investigated the optimal culture conditions for isolation and expansion of human MSCs. Among several growth media, they concluded that those based on α-Minimum Essential Medium (α-MEM) are more suitable for both isolation and expansion of multipotent MSCs. In cell culture processes, the addition of L-glutamine to the medium has been considered a problem, as reported in some studies. This compound is susceptible to both chemical and metabolic deamination, producing ammonia which can be inhibitory to cell growth. Therefore, glutamine-containing dipeptides such as alanyl-glutamine and glycyl-glutamine have been considered as potential substitutes for glutamine in culture medium due to their stability (Christie and Buttler, 1994; Sotiropoulou *et al*, 2006). The other major component of MSCs isolation and growth media is fetal bovine serum (FBS). Most media preparations usually use 10% fetal calf serum to provide a mixture of undefined growth factors, cytokines, and attachment factors. FBS contains, particularly, platelet-derived growth factor (PDGF), basic fibroblast growth factor (b-FGF or FGF-2), and epidermal growth factor (EGF) as well as small amounts of other growth factors. It has been

established that serum-free defined media lack attachment factors to aid MSC attachment and cell yields tend to be low (Pittenger, 2008). Regarding passaging cell-plating density, Sotiropoulou *et al* (Sotiropoulou *et al*, 2006) found that initial plating densities of 5,000 to 10,000 cells/cm^2 resulted in much higher numbers of the starting MSC-enriched adherent population. These results are consistent with previous reports that have evaluated parameters for MSCs expansion and displayed that plating MSCs at low density benefits proliferation and stemness preservation (Sekiya *et al*, 2002; Colter *et al*, 2001; Prockop et al, 2001). Sotiropoulou *et al* (Sotiropoulou *et al*, 2006) also stated that an additional factor that influences the expansion of human MSCs is the quality of plastic surface used for their adhesion. These investigators used culture flasks from four different companies and demonstrated that the quality of cells produced did not differ among the different types of flasks. In addition, this study assessed effect of b-FGF concentrations on MSCs proliferative capacities. This study supports previous reports (Tsutsumi *et al*, 2001; Hori *et al*, 2004) that isolation and proliferative potential of MSCs are dose-dependent.

1.3.5 MSCs model organisms

MSCs have important applications not only in human regenerative medicine but also in veterinary medicine. Animal models are widely used to study the properties and potential of stem cells providing valuable information for future applications in human medicine (Ribitsch *et al*, 2010). Currently, the focus of

attention in veterinary medicine and research is the use of MSCs from either extra embryonic or adult tissues.

Most of the conventional MSC research has been performed by using cells isolated from humans and murine models. Nonetheless, MSCs have also been isolated from unconventional model organisms, such as cat (*Felis catus*), dog (*Canis familiaris*), chicken (*Gallus gallus*), duck (*Anas platyrhyncha*), goat (*Capra hircus*), buffalo (*Bubalus bubalis*), cattle (*Bos taurus*), rabbit (*Oryctolagus cuniculus*), pig (*Sus scrofa*), sheep (*Ovis aries*), horse (*Equus caballus*) and guinea pig (*Cavia porcellus*) (Calloni *et al*, 2014).

a) Cat (*Felis catus*)

Martin *et al* (Martin *et al*, 2002) isolated, for the first time, feline MSCs from bone marrow. Later, other reports have described isolation of MSCs from adipose tissue (Webb *et al*, 2011), umbilical cord blood (Jin *et al*, 2008), and fetal fluid and membranes (Iacono *et al*, 2012). Cat MSCs exhibit a morphology similar to MSCs isolated from mice and humans, characterized by the expression of classic MSC-associated markers (CD9, CD44, CD90 and CD105) and the absence of the surface proteins CD14, CD34 and CD45 (Calloni *et al*, 2014).

b) Cattle (*Bos Taurus*)

Studies have reported isolation of bovine MSCs from bone marrow and umbilical cord (Bosnakovski *et al*, 2005; Mauck *et al*, 2006). They express the cell surface markers CD29, CD73, CD90 and CD105 and present a fibroblast-like morphology. Generally, most of these bovine MSCs studies have investigated *in*

vitro culture systems to achieve and analyze chondrogenic differentiation (Calloni *et al*, 2014).

c) Rabbit (*Oryctolagus curriculus*)

Different tissues have served as a source of rabbit MSCs: bone marrow, adipose tissue, peripheral blood, synovium, periosteum, placenta and fetal liver (Moreno *et al*, 2010; Hui *et al*, 2005; Lee *et al*, 2012). Morphology of the cells resembles the classic fibroblast-liike shape and can differentiate in vitro into chondroblasts and epithelial-like cells (Li *et al*, 2012: Wan *et al*, 2006). Other reports have demonstrated *in vivo* and *in vitro* differentiation to osteoblasts, adipocytes and corneal epithelial cells (Wan *et al*, 2006; Gu *et al*, 2009).

d) Sheep (*Ovis aries*)

Ovine MSCs have been isolated from bone marrow, particularly from the iliac crest region, adipose tissue, amniotic fluid, dental pulp and periodontal ligament (Niemeyer *et al,* 2010; Martinez-Lorenzo *et al*, 2009; Shaw *et al*, 2011). However, in 2003 MSCs were isolated for the first time from sheep umbilical cord (Murphy *et al*, 2003). Rentsch *et al* (Rentsch et al, 2010) reported that ovine MSCs and human-derived MSCs have similar proliferative characteristics and differentiated into the same lineages. Other studies have reported specifically *in vitro* and *in vivo* adipogenic and osteogenic differentiation (Niemeyer *et al*, 2010; Rentsch *et al*, 2010).

1.4 Canine mesenchymal stem cells (cMSCs)

1.4.1 Why characterize cMSCs?

To optimize clinical applications of stem cells, it is paramount to test safety and efficacy in large-animal models of preclinical studies. Canine models are known to accurately predict clinical outcomes in adult stem cell transplantation and are, therefore, likely to act as accurate preclinical models for stem cell therapies. In fact, long-term outcomes of organ or hematopoietic transplantation in dogs have accurately predicted outcomes in humans (Csaki *et al*, 2007; Hayes *et al*, 2008; Volk *et al*, 2012)

Using dogs as dependable preclinical models in the development of cellular transplantation therapies has important advantages over some other laboratory animals. Canines experience external and environmental elements that are associated with different pathologies such as cancer, obesity, and traumatic injuries. Also the clinical presentation and progression of these diseases are similar to their equivalents in humans (Parker *et al*, 2010; Volk *et al*, 2012). Distinctive treatment options, imaging, and repeated biological sampling are, especially, possible in dogs due to their size and availability of vital veterinary infrastructure. These circumstances plus continuing clinical progresses in companion animal care have increased sensitivity to detect adverse side effects of new therapies that would otherwise reduce risks to humans (Volk *et al*, 2012).

Clearly, canine model has a significant value for translational studies that can advance human medicine and also enhance veterinary therapies. For further

advancements of cMSC-based regenerative medicine and tissue engineering, it is essential to gain more insight into their differentiation capacity, define donor characteristics, refine ex *vivo* expansion strategies, and evaluate the tissues formed by these cells at the biochemical, ultrastructural and immunomorphological levels.

1.4.2 What is known about cMSCs?

Canine mesenchymal stem cells (cMSCs) can be obtained from numerous sources such as bone marrow, adipose tissue, umbilical cord blood, umbilical cord matrix, umbilical cord vein, periodontal ligament, dental pulp, amniotic fluid, and amniotic membrane (Vieira *et al*, 2010; Reich *et al*, 2012; Volk *et al*, 2005; Volk *et al*, 2012; Dissanayaka *et al*, 2011; Wang *et al*, 2012; Seo *et al*, 2009; Zucconi *et al*, 2010; Uranio *et al*, 2011; Kisiel *et al*, 2012). The greatest volume of the studies on cMSCs has been performed using cells from bone marrow and adipose tissue. The procedure for obtaining bone marrow in dogs is usually easy and relatively non-invasive. Commonly used donor sites are the proximal humerus, proximal femur or the tuber coxae (Crovace *et al*, 2008; Fortier and Travis, 2011). Adipose tissue is also considered an attractive source for MSCs, mainly, due to the accessibility of the tissue at various sites in the body (Stewart & Stewart, 2011), and ability to collect it during routine canine surgery or liposuction techniques (Vieira et al, 2010). Depending on the source from which the cells are isolated, canine MSCs can be passaged around 6 to 11 times (Martinello *et al*, 2011). In the literature, there are various reports that have

reported about characterization of cMSCs based on their morphology, immunophenotype, and gene expression (Table 5).

Table 5

Table 1. Overview of the current data on canine mesenchymal stem cells.

Study	Source	Cellular protein markers	Gene expression markers	Differentiation potential
Guercio et al. 2012	Adipose tissue	–	NanoG Oct4 Sox2	Osteogenic Chondrogenic Adipogenic
Hodgkiss-Geere et al. 2012b	Bone marrow	Positive: CD44, STRO-1 Negative: CD34, CD45	–	Chondrogenic
Kang et al. 2012	Bone marrow Adipose tissue Umbilical cord blood Wharton's jelly	Positive: CD44, CD73, CD90, CD105 Negative: CD14, CD34, CD45	–	Osteogenic
Kisiel et al. 2012	Bone marrow Adipose tissue Muscle Periosteum	Positive: CD90, CD44 Negative: CD34, CD45, CD146	NanoG Oct4 Sox2	Osteogenic Adipogenic
Takemitsu et al. 2012	Bone marrow Adipose tissue	Positive: CD29, CD44, CD90 Negative: CD34, CD45, SSEA-3, SSEA-4, TRA-1-60, TRA-1-81	NanoG Oct3/4 Sox2	Osteogenic Adipogenic
Martinello et al. 2011	Adipose tissue	Positive: CD90, CD44, CD140a, CD117 Negative: CD34, CD45	–	Adipogenic Osteogenic Myogenic
Vieira et al. 2010	Adipose tissue	Positive: CD44, CD29, CD90 Negative: CD14, CD34, CD45, CD117	–	Osteogenic Chondrogenic Adipogenic Myogenic
Neupane et al. 2008	Adipose tissue	–	NanoG Oct4 Sox2	Osteogenic Chondrogenic Adipogenic
Csaki et al. 2007	Bone marrow	Positive: CD105, CD90 Negative: CD45, CD34	–	Osteogenic Chondrogenic Adipogenic
Kamishina et al. 2006	Bone marrow	Positive: CD90, MHC-I Negative: CD34, CD45, MHC-II	–	Neurogenic

Note: '–': no data available.

Adapted from de Bakker *et al*, 2014. Veterinary Quarterly, 2014

Morphologically the cMSCs display the typical fibroblast-like shape, but with some variations such as elongated and cuboidal outlines (Csaki *et al*, 2007; De Schauwer *et al*, 2011). Depending on the source of the cells, several studies have showed that cMSCs have a variable surface marker profile. These reports showed positive and simultaneous expression of several markers such as CD29,

CD44, CD90, and MHC-I, while being negative for CD34, CD45, CD14, CD105, and MHC-II, among others (Table 5).

Mathieu *et al* (Mathieu *et al*, 2009) emphasized the importance of using specific anti-canine antibodies in cell surface marker characterization due to the lack of cross-reactivity between the dog cell surface markers and human antibodies. This fact could explain some negative results for classic MSCs markers in canine cells. Interestingly, some trials have demonstrated that cMSCs secrete various cytokines that allow them to inhibit leucocyte proliferation. Kang *et al* (Kang *et al*, 2008) demonstrated that canine adipose-derived MSCs expressed soluble factors such as transforming growth factor beta, IL-6, IL-8, vascular endothelial growth factor, hepatocyte growth factor, and others. These factors were associated with immunomodulatory effects of the cMSCs. As human MSCs, cMSCs also express pluripotency-associated transcription factors NanoG, Oct4, and Sox2 (Table 5).

Generally, one important characteristic of MSCs is their osteogenic, chondrogenic, and adipogenic potential (Pittenger *et al*, 1999). This differentiation capacity has also been demonstrated in cMSCs isolated from bone marrow and adipose tissue. In addition, some authors have investigated cMSCs harvested from other anatomical parts such as amniotic membrane, umbilical cord blood, Wharton's jelly, muscle, periosteum (Csaki *et al*, 2007; Neupane *et al*, 2008; Vieira *et al*, 2010; Volk *et al*, 2012; Kisiel *et al*, 2012; Park *et al*, 2012; Kang *et al*, 2012; Guercio *et al*, 2013)

Volk *et al* (Volk *et al*, 2012) studied canine bone marrow-derived MSCs obtained from humerus, femur, tibia, an iliac crest and the effects of donor characteristics (age and harvest site) and *ex vivo* expansion on the differentiation potential of the cells. Osteogenesis, chondrogenesis, and adipogenesis were, particularly, evaluated. The authors found that advancing age had a negative effect on colony-forming unit-fibroblastic as well as osteogenic potential. Site of harvest was also found to have substantial effects on MSC properties.

Csaki *et al* (Csaki *et al*, 2007) verified the *in vitro* multilineage differentiation potential of isolated adult canine bone marrow MSCs from femur, at the ultrastructural and immunomorphological levels. They demonstrated that the cells had proliferative capacities and, under appropriate culture conditions could differentiate well into functional osteoblasts, adipocytes and chondrocytes during *in vitro* development.

Park *et al* (Park *et al*, 2012) isolated and characterized MSCs from six different canine amniotic membrane tissues. They demonstrated that the amniotic membrane-derived MSCs proliferated actively, showed adherence to plastic culture surface and their morphology was similar to those typical MSCs with a spindle, fibroblast-like shape. Additionally, the cells displayed multipotent differentiation capacity of osteogenesis, adipogenesis, neurogenesis, and chondrogenesis *in vitro*.

Kisiel *et al* (Kisiel *et al*, 2012) firstly, isolated and characterized canine muscle-derived MSCs and periosteum-derived MSCs. Secondly; they compared the proliferation potential of MSCs from these two potential donor sites with two

conventional canine sources; bone marrow and adipose tissue. These investigators were able to demonstrate that plastic-adherent cells, with the distinctive fibroblastic phenotype, were isolated and expanded from all four donor tissues. Furthermore, the cells expressed surface markers CD90 and CD44, and were negative for CD34 and CD45. Positive expression of pluripotency-associated transcription factors Sox2, Oct4, and NANOG was also noticed. In terms of differentiation ability, muscle-derived MSCs appeared to have the greatest adipogenic potential compared with the other tissue-derived MSCs. Osteogenic differentiation was achieved in all four MSC types demonstrated by the expression of alkaline phosphatase, Runx2, osterix, and osteopontin, however the study does not indicate the tissue that exhibited higher or lower expression of these bone markers.

The authors reported that their attempts at differentiating canine MSCs into the chondrogenic lineage were unsuccessful based on morphological and histochemical assessments. Periosteum was a superior tissue source in providing the greatest number of MSCs per gram of tissue when the cells were grown to 80% to 100% confluence in passage 1, suggesting that periosteum derived cMSCs may be useful in allogeneic applications.

Osteogenic differentiation has been demonstrated by morphological changes of the cells under induction, which have adopted polygonal appearance containing nodular aggregates that stained positively with von Kossa. Ultrastructural cellular changes, translated into a bigger number of cell organelles, and a well-organized extracellular matrix have been observed through transmission electron

microscopy. Additionally, cultures grown under osteogenic conditions have deposited a mineralized matrix that has stained with Alzarin red S. To complement mineralization assays, mRNA levels or protein expression of osteogenic markers such as Runx2, collagen type I, bone sialoprotein, osteonectin, osterix, osteopontin, and osteocalcin have been assessed (Kadiyala *et al*, 1997; Csaki *et al*, 2007; Neupane *et al*, 2008; Vieira *et al*, 2010; Volk *et al*, 2012).

Adipogenic differentiation has been confirmed by the presence of round- shape cells with cytoplasmic lipid vacuoles stained with Oil Red O technique. Under electron microscopy the newly formed adipocytes have confirmed the accumulation of lipid droplets in their cytoplasm with well-developed rough endoplasmic reticulum and mitochondria. Characterization of the adipogenic extracellular matrix by immune-electron microscopy has revealed abundant amounts of collagen type I and adiponectin, the most abundant protein in adipose tissue. Based on real-time PCR and Western blotting, cells under adipogenesis induction have displayed significant amounts of adiponectin, upregulation of the adipocyte-specific transcription factor peroxisome proliferative-activated receptor γ (PPARγ), lipoprotein lipase (LPL), fatty acid binding protein-4 (FABP4), and β1-integrin. PPARγ is important for adipocyte differentiation and stabilizing the metabolic function of differentiated adipocytes (Lazar *et al*, 2002; Csaki *et al*, 2007; Neupane *et al*, 2008; Vieira *et al*, 2010; Volk *et al*, 2012)

Chondrogenic differentiation has been characterized by the accumulation of glycosaminoglycans (GAG) evidenced by Alcian blue staining in the differentiated cMSCs. Analysis through transmission electron microscopy has shown newly formed chondrocytes with round shape, and containing high amounts of glycogen, numerous cell organelles, and augmented quantities of euchromatin in nuclei. Immuno-transmission electron microscopy has disclosed that newly formed extracellular matrix contained abundant amount of collagen type II and cartilage specific proteoglycans (CSPG). Western blot analysis of whole cell extracts have confirmed high amounts of collagen type II, CSPG, and activation of the cartilage specific transcription factor sex-determining region Y box 9 (Sox9) (Csaki *et al*, 2007; Neupane *et al*, 2008; Vieira *et al*, 2010; Volk *et al*, 2012).

As illustrated above, osteogenic, adipogenic, and chondrogenic differentiation have been mostly reported for cMSCs. Nonetheless, the potentials of these cells to differentiate into other lineages such as myogenic (Vieira *et al*, 2010; Martinello *et al*, 2011) or neurogenic lines (Kamishina *et al*, 2006; Seo *et al*, 2009; Park *et al*, 2012; Oda *et al*, 2013) have also been documented.

Vieira *et al* (Vieira *et al*, 2010) reported isolation, characterization, and multilineage differentiation potential of canine adipose-derived MSCs, obtained from subcutaneous adipose tissue by liposuction and biopsy procedures. Besides demonstrating the cMSCs were able to differentiate into adipogenic, chondrogenic, and osteogenic cells, they also showed differentiation ability into myogenic lineage. After 10 days in myogenic medium, adipose-derived MSCs

formed multinucleated structures. Myogenic differentiation was confirmed by the expression of myosin measured through immunofluorescence, and gene expression levels of myogenin, dystrophin, and MyoD only in induced cells.

Oda *et al* (Oda *et al*, 2013) used three previously reported methods to differentiate cMSCs, harvested from iliac crest, into neuron-like cells. Then, the cells were characterized according to morphological analysis and expression of neuronal markers. cMSCs under neurogenic induction experienced sequential changes in their appearance, from fibroblastic to neuron-like cells with multiple branching processes. Immunocytochemical analysis showed that the induced cells expressed markers of both immature neurons (nestin, 84.7%) and mature neuronal cells (microtubule-associated protein-2 (MAP2), 95.7%; βIII-tubulin protein, 12.9%; glial fibrillary acidic protein (GFAP), 9.2%). The investigators concluded that, under appropriate *in vitro* conditions, canine bone marrow-derived MSCs can be efficiently differentiated into cells with neuronal phenotypes.

In the study by Park *et al* (Park *et al*, 2012), neurogenesis differentiation was evaluated using amniotic membrane-derived canine MSCs. The authors measured the expression of the neural-associated markers GFAP, βIII-tubulin, and MAP2 by immunostaining and real-time PCR. Induced cMSCs expressed GFAP assessed at both protein and gene levels and in non-induced cells. The specific neural markers βIII-tubulin and MAP2 were expressed in cells cultured under neural differentiation conditions.

1.4.3 What is unknown about cMSCs?

Full understanding of cMSCs biology is yet to be conclusively elucidated. More detailed knowledge of differentiation and manipulation of cMSCs into other tissues are crucial to their application for MSC-based therapies in veterinary medicine, and indirectly in human MSC-based therapies.

Few studies have been published targeting certain factors that may have significant effects on differentiation capacity and culture expansion of cMSCs. Precise definition of optimal donor age may have significant impact when decisions have to be made on choice of autologous or allogeneic MSC therapies. If autologous cells are to be used for clinical trials in older individuals, it is very important to determine if the age of the donor will influence the outcomes of the therapy (Volk *et al*, 2012). MSC-based therapies require a significant number of cells; therefore, the knowledge of ex *vivo* cell expansion, and associated variables should be completely clarified. Volk *et al* (Volk *et al*, 2012) studied the influence of cell passage on the osteogenic capacity of cMSCs, and found that this diminishes with increasing passage. The authors suggested that the shortening of MSC telomere length may explain this diminished differentiation capacity. Beside culture expansion, it is important to also assess post-expansion cell yield per gram of donor tissue and specific characteristics of induction media used for cMSCs culture (Volk *et al*, 2012; Kisiel *et al*, 2012; de Bakkler *et al*, 2014).

As previously stated, a number of studies have reported isolation and *in vitro* differentiation capacity of cMSCS, but not many trials have followed up to identify

their cell surface markers and mRNA expression profiles. Furthermore, the mechanisms by which cMSCs act to repair individual tissues *in vivo* are still unclear. More preclinical or clinical studies are necessary to define if the cMSCs function through direct differentiation into specific tissue lines, immunomodulatory action and secretion of growth factors, or both.

Skeletal site-specific properties of cMSCs from the same subject and their site-dependent effects on tissue regeneration have not been clearly defined. Previous studies focusing on bone marrow-derived as well as adipose-derived cMSCs suggest that MSC frequency and differentiation capacity may also be influenced by the specific site of tissue harvest, but these studies have compared response of MSCs from long bones, with the absence, to our knowledge, of trials making comparisons between cells from the orofacial region and axial/appendicular skeleton ((Csaki *et al*, 2007; Neupane *et al*, 2008; Vieira *et al*, 2010; Kisiel *et al*, 2012; Volk *et al*, 2012).

Although many of the tendon/ligament injuries present in humans are also frequently diagnosed in dogs (de Bakker *et al*, 2013), regenerative MSC-based therapies for this kind of lesions, either traumatic or degenerative, have not been completely investigated in canine medicine (de Bakker *et al*, 2014).

1.4.4 Therapeutic applications of cMSCs

The use of MSCs as an alternative treatment option for several canine diseases such as spinal cord injuries, bone defects/ degenerative diseases, cardiovascular pathologies, metabolic diseases, and others has been reported in the literature.

Trials on healing of spinal cord injury have found cMSCs to be sustainable therapies. Jung *et al* (Jung *et al*, 2009) determined the efficacy of autologous and allogeneic bone marrow-derived MSC transplantation in experimentally-induced spinal cord injury of dogs. By using three groups of 10 beagle dogs, they injected autologous MSCs to the first group, allogeneic MSCs to the second, and no MSCs to the third one. They observed that both autologous and allogeneic groups showed an improvement in the neurological signs of pelvic limbs compared with the control group. These findings were corroborated with magnetic resonance imaging, histopathological examinations, and immunofluorescence analysis. It was concluded that autologous and allogeneic MSCs transplantation can be clinically helpful therapies for spinal cord injuries. Lim et al (Lim et al, 2007) used adult mongrel dogs to evaluate the effects of allogeneic umbilical cord blood (UCB)-derived MSCs and recombinant methionyl human granulocyte colony-stimulating factor (rmhGCSF) on spinal cord injuries performed using balloon compression methods at the first lumbar vertebra. One week after the induction of the neuronal lesions, UCB-MSCs were directly injected into the injured site of the spinal cord and rmhGCSF was administered subcutaneously. The dogs were divided in 5 groups: no treatment, saline treatment, UCB-MSCs, rmhGCSF, and UCB-MSCs plus rmhGCSF (UCBG). The results were evaluated after 2, 4, and 8 weeks after transplantation. The investigators found no significant differences between the UCB-MSC and UCBG groups, and between the no treatment and saline groups. In addition, there was no evidence of regeneration of spinal cord tissue by magnetic resonance imaging

and histology, but significant evidence of functional and sensory improvement after allogeneic UCB-MSCs transplantation was observed. Moreover, they noticed newly formed neuronal tissues in the injured structures of the spinal cord in the UCB-MSC and UCBG groups. In summary, they determined that the outcomes of this study showed that transplantation of UCB-MSCs resulted in recovered nerve function in dogs after a spinal cord injury.

Treatment of bone defects in dogs have been described in the literature based, mainly, on associations of cMSCs and different scaffolds. Sun *et al* (Sun *et al*, 2011) harvested and cultured bone marrow-derived MSCs from the iliac crest of beagle dogs. The cells were pre-osteodifferentiated and seeded into a chitosan/collagen I/β-glycerophosphate (β-GP) composite hydrogel to promote osteogenesis. After 28 days, scanning electronic microscopy observations indicated good spreading of bone marrow MSCs and mineral nodules were observed in this hydrogel scaffold. The *in vivo* phase consisted in subcutaneous injection of the chitosan/collagen/β-GP hydrogel loaded with pre-osteodifferentiated dog-bone marrow MSCs into nude mouse dorsum. After 4 weeks, partial bone formation was detected in the hydrogel which indicated that chitosan/collagen/β-GP hydrogel composite could induce osteodifferentiation in cMSCs without exposure to a continual supply of external osteogenic factors. In conclusion, the authors stated that this hydrogel composite should be useful as a bone regeneration scaffold. Yoshioka *et al* (Yoshioka *et al*, 2012) created bilateral bone defect in the upper incisor regions of beagle dogs, and evaluated bone regeneration achieved by transplantation of cMSCs derived from iliac bone

marrow mixed with carbonated hydroxyapatite (CAP) particles. Six months after the transplantation, absolute closure of the jaw cleft was attained on the experimental side. Occlusal X-ray and histological examinations revealed that the regenerated bone on the experimental side was almost equivalent to the original bone contiguous to the jaw cleft. The researchers suggested that the application of MSCs with CAP particles can become a new treatment modality for bone regeneration for patients with congenital anomalies in the orofacial region such as cleft lip and palate.

The role of cMSCs in regeneration of muscular tissues, particularly cardiomyocytes, and therefore in heart diseases has also been studied. Some breeds of dogs, particularly the Cavalier King Charles Spaniel, are affected by cardiac diseases such as endocardiosis or dilated cardiomyopathy that contribute to more than 50% of mortalities in these dogs (Bonnett *et al.*, 2005; Hodgkiss-Geere *et al*, 2012). Studies like the one performed by Hodgkiss-Geere *et al* (Hodgkiss-Geere *et al*, 2012) suggests that cMSCs-based therapy might provide benefits to these heart pathologies. These investigators analyzed adult canine cardiac stem cells taken from canine cardiac tissue, specifically from the right/ left atria and ventricles immediately post-mortem. They were able to isolate, characterize, and explore the cells ability to differentiate into cardiac myocytes. The cells were exposed to four differentiation protocols and demonstrated the following marker profile: stem cell marker c-kit and early cardiac differentiation markers GATA 4 and flk-1, positive; the cardiomyocyte marker cardiac troponin T and another early cardiac differentiation marker, NKx2.5, low. Gene expression

studies demonstrated that cardiac directed differentiation was partially achieved, with up-regulation of cardiac troponin T and NKx2.5, and down-regulation of c-kit and endothelial lineage markers. However the cells did not express the ryanodine receptor or β_1-adrenergic receptors and did not contract spontaneously. Based on these results, the authors concluded that the canine heart has a reliable and reproducible resident population of adult stem cells, and that, even though, complete differentiation was not achieved and key components of the contractile machinery were not detected, the study could achieve a comprehensive characterization of canine cardiac stem cells and serves as a foundation for further studies about optimizing conditions needed for cardiac differentiation. In an earlier study, Silva *et al* (Silva *et al,* 2005) showed that, in a canine chronic myocardial ischemia model, the intramyocardial injections of bone marrow-derived MSCs resulted in differentiation of those cells into smooth muscle and endothelial cells that translated to increased vascularity and improved cardiac function. In conclusion, they suggested that, with further investigation, the MSC transplantation might become an alternative therapy for ischemic heart failure.

The combination of genetic engineering and cell transplantation provides a novel promise for diabetes treatment. Some reports in the literature have investigated these optional therapies using cMSCs. In a diabetes study, by Zhu *et al* (Zhu *et al*, 2011), bone-derived Beagle canine mesenchymal stem cells were isolated, expanded, and transfected with a recombinant retroviral plasmid containing human insulin and enhanced green fluorescent protein (EGFP). Then the cells

were transplanted into the livers of diabetic Beagle dogs by arterial intervention technique. EGFP was used as the radiotracer to detect the insulin secretion, the colonization of bone marrow derived-MSCs (BMSCs), and the long-term effects of BMSCs on experimental animals. The variations of body weight, blood glucose, serum insulin levels, and plasma C-peptide were determined after autotransplantation. An increase in the body weight, a decrease in blood glucose levels, and a reduction in the need for insulin injections were reported, but no β-pancreatic cell regeneration was observed. As a general conclusion, the authors expressed that experimental diabetes could be relieved effectively by intrahepatic autotransplantation of BMSCs expressing human insulin, which implies a new strategy of gene therapy for type I diabetes.

Continuing preclinical and clinical trials are necessary in canine medicine. Dogs are considered to be a superior animal model for humans; therefore, advanced state-of-art research in this field will benefit both dogs and humans.

2 RESEARCH AIMS

2.1 Purpose

Investigation of mesenchymal stem cell-based therapies such as bone tissue engineering procedures and regenerative medicine has gained increasing importance in both human and veterinary medicine. There are many properties of mesenchymal stem cells (MSCs) that make their use an attractive option for clinical applications. The body of studies on MSCs has focused on cells isolated

from humans and murine models. Among other organisms, dogs are recognized to be a suitable model for MSC studies due to their anatomical, biochemical, physiological, and pathophysiological characteristics. Increasing veterinary clinical trials involving canine subjects will provide unique more opportunities to assess both the efficacy and safety of adult stem cell therapies that can be translated to human medicine. Nevertheless, detailed knowledge of biology of canine MSCs (cMSCs) has not been completely elucidated. The effect of many factors such as anatomical site, passage number, culturing protocols, and donor characteristics of cells from canine origin still remain unclear. Further understanding of cMSC biology will provide valuable information to refine cell-based therapies such as donor graft selection for bone regeneration in veterinary as well as human medicine.

The main purpose of this study was to characterize cMSCs isolated from beagle dogs based on proliferative and multipotent differentiation properties.

2.2 Specific aims

This study intends to characterize cMSCs through the following specific objectives;

1) To evaluate *in vitro* expansion and proliferative potential of cryopreserved cMSCs from two different anatomical sites: orofacial region (mandible) and appendicular bone (femur).
2) To investigate and compare *in vitro* differentiation potential of cMSCs into distinct cellular lineages, namely osteogenic, adipogenic, chondrogenic,

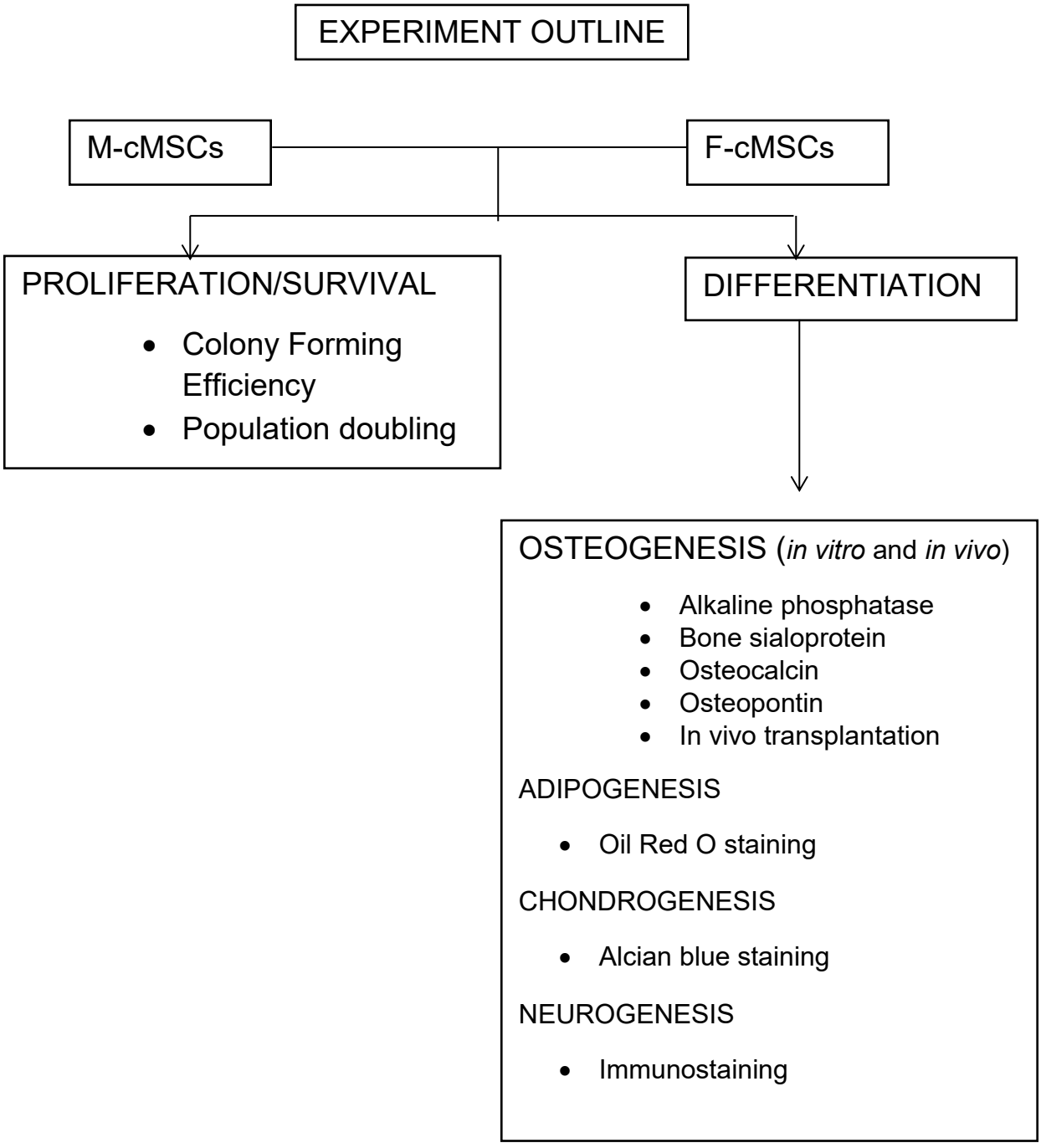
and neurogenic, from two different anatomical sites: orofacial region (mandible) and appendicular bone (femur).

3) To demonstrate and compare *in vivo* osteogenic differentiation of cMSCs from two different anatomical sites: orofacial region (mandible) and appendicular bone (femur).

4) To test whether mandible-cMSCs (M-CMSCs) demonstrate superior proliferative and multipotent differentiation properties than femur-cMSCs (F-cMSCs) from same animals.

3 MATERIAL AND METHODS

3.1 Experiment outline



3.2 Sample and cell culture

Canine MSCs from the mandibular body and proximal femur of 2 Beagle dogs (ages: 3 weeks, 2 females) were previously isolated and cryopreserved in Dr. Akintoye's laboratory.

The primary cMSCs were further expanded in culture using growth medium consisting of α-MEM supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 100 U/ml Penicillin, 100 mg/ml streptomycin sulfate and 2 mM glutamine (Gibco, Life technologies, NY) and maintained at 37^0C, in humidified atmosphere of 5% CO_2 and air. Non-adherent cells were washed away, after 24 hours, with phosphate-buffered saline (PBS) and the medium was changed every 3-4 days. At 80% of confluence, the cMSCs were detached with 0.5% trypsin (Invitrogen-Life Technologies, Carlsberg CA and split as detailed in experiments outlined below. Overall, the cells used in this study were within passage 6 or lower.

3.3 Cell proliferation

The proliferation rate of cMSCs was assessed by growth curve analysis (Akintoye *et al*, 2006). Cells were plated at 9.5 x 10^3 cells/cm^2 in 6-well plates (Coming Life Sciences, Acton, MA) in α-MEM growth medium, which was changed twice weekly. Cells were released on days 1, 3, 6, 9, 12, and 15 and counted using hemocytometer to plot a growth curve.

3.4 Colony forming efficiency (CFE) assay

Colony forming efficiency assay was performed as previously described (Akintoye *et al*, 2006; Volk *et al*, 2012). Primary cMSCs were cultured in triplicate 25 cm^2 plastic culture flasks at 10^1, 10^2 and 10^3 cells/flask with non-osteogenic growth medium. Cells were fixed on day 14 with 100% methanol, subsequently stained with methyl violet (Sigma-Aldrich, St Louis, MO) and aggregates of 50 or more cells were counted as colonies.

3.5 Life span measurements

Long term survival of cMSCs was assessed by population doublings (PD) as previously described (Akintoye *et al*, 2006). Cells were plated at 1 x 10^6 cells/flask, and PD was calculated from generation number after repeated cell passage at 1:10 split ratio until the cells attained replicate senescence.

3.6 Canine telomerase activity assay

The presence of canine telomerase reverse transcriptase (cTERT) was determined by Western blotting of nuclear extracts isolated with Nuclei EZ Prep Isolation Kit according to the manufacturer's protocol (Sigma-Aldrich). Nuclear extracts were obtained at different passages during the population doubling experiment above. Culture dishes were washed with Dulbecco's Phosphate Buffered Saline and the cells were harvested by using Nuclei EZ lysis buffer. The entire cell lysates were centrifuged at 3000 rpm, and the clear supernatant was aspirated, conserving the nuclei pellet on ice. The supernatant contains

cytoplasmic components and was saved for later analysis. Nuclei pellets were resuspended in Nuclei lysis buffer, and centrifuged again. After this, the nuclei pellets were resuspended in Nuclei EZ storage and frozen at -80 °C to be used in the next steps. Equal amounts of nuclear extracts were used to evaluate expression levels of monoclonal antibody to cTERT. The blots were probed with rabbit polyclonal telomerase reverse transcriptase antibody (Novus Biologicals) at 1:1000.This primary antibody was followed by anti-rabbit (1:2000) as secondary antibody. Probing of blots with anti β-actin (1:2000) served as loading control. Immunoreactive bands were analyzed digitally with Kodak Image Station 4000MM.

3.7 In vitro osteogenic differentiation

Osteogenic differentiation was performed as previously described (Volk *et al*, 2005; Volk *et al*, 2012). Canine MSCs were cultured at 1 x 10^4 cells/cm^2 in 10 sixty mm dishes (Corning Life Sciences, Acton, MA) with α-MEM growth medium without osteogenic inducers until they reached confluence. Half of the dishes (n=5 dishes) were pre-coated with poly-L-lysine (Sigma-Aldrich) to enhance cell attachment under long-term culture. At confluence, the cells in coated dishes were exposed to osteogenic medium containing supplements of 100 ng/ml of human bone morphogenetic protein-2 (BMP-2, GenScript, Piscataway, NJ, USA) and 100 μM L-Ascorbic acid 2-phosphate (10^{-4} M) for 7 and 14 days. Medium was changed twice weekly. Cells in the other set of dishes (n=5) were cultured in α-MEM growth medium without inducers and used as control. At 7 and 14 days

protein lysate and RNA were collected in parallel experimental culture dishes. Total protein amount from lysates was determined using the Bicinchoninic acid protein assay (Pierce™ BCA Protein Assay Kit). Equal (50 µg) protein amount was loaded on a 4 – 20% gradient gel and transferred on nitrocellulose membrane for western blotting. The membranes were probed with the following primary antibodies: rabbit anti-bone sialoprotein (BSP) polyclonal antibody (Bioss Inc.) at 1:200; rabbit anti-osteocalcin (OCN) polyclonal antibody (Bioss Inc.) at 1:200; rabbit anti-osteopontin (OPN) antibody (Rockland Inc.) at 1:500, and rabbit anti-alkaline phosphatase (ALP) antibody (Novus Biologicals) at 1:800. Primary anti β-actin (1:1000) and anti-α-tubulin (1:200) served as loading controls. Furthermore, the primary antibodies were reacted with anti-mouse or anti-rabbit secondary antibodies at concentrations ranging from 1:1000 – 1:3000. Digital analysis of immunoreactive bands was performed using with Kodak Image Station 4000MM (Molecular Imaging Systems, Carestream Health, Rochester, NY).

3.8 Isolation of RNA and Real-Time Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from osteogenenically-induced and control cells using TRI Reagent® (Sigma-Aldrich). First strand cDNA was prepared with first strand SuperScript™ Double-Stranded cDNA Synthesis Kit (Invitrogen Life Technologies, Carlsbad, CA) using an oligo-dT primer. Two microliters of first

strand cDNA was added to a total volume of 50 µl PCR buffer containing: 1.5 mM $MgCl_2$, 200 µM dNTP, 2.5 U Taq DNA polymerase (Promega, Madison, WI) and 200 nM of each primer set. Real-time PCR was performed with 7300 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA) using the STBR Green Master Mix (Applied Biosystems, Foster City CA) and the following custom-designed primers:

Canine BSP - forward 5'-TTGCTCAGCATTTTGGGAATGG-3';

Canine BSP – reverse 5'-AACGTGGCCGATACTTAAAGACC-3';

Canine Osteocalcin – forward 5'-CTG GTCCAGCAGATGCAAAG-3';

Canine Osteocalcin – reverse 5'-CCGCTTGGACACGAAGGTT-3';

Canine ALP - forward 5'-TTCAAACCGAGACACAAGCAC T-3';

Canine ALP – reverse 5'-GGGTCAGTCACGTTGTTCCTGT-3';

Canine Osteopontin - forward 5'-CGAGTCTGATGAATCCGATGA A-3';

Canine Osteopontin – reverse 5'-TTGGGTTGCTGGAATGTCAGT-3'.

Gene expression levels were normalized to the housekeeping gene:

Canine β2 microglubulin - forward 5'-TCACGACACCCAGCAGAGAA-3';

Canine β2 microglubulin – reverse 5'-GGAACCCTGACACGTAGCAGTT-3'.

3.9 In vivo osteogenesis by transplantation into immunocompromised host

Bone regenerative capacity of femur and mandible cMSCs was evaluated using the mouse model of *in vivo* bone formation in immunocompromised hosts as described by Akintoye *et al* (Akintoye *et al*, 2006). The animal protocol was approved by the University of Pennsylvania Office of Regulatory Affairs.. Non-

induced and osteogenically induced mandible and femur MSCs were transplanted into separate subcutaneous pockets of three different animals as follows: 2×10^6 cMSCs were attached to 40 mg spheroidal hydroxyapatite/tricalcium phosphate (particle size 0.5–1.0 mm, Zimmer, Warsaw, IN) and transplanted into separate subcutaneous pockets aseptically created in 4-week-old immunocompromised nude female mice (NIH-III NU/NU, Charles River Laboratories, Wilmington, MA). Transplants were harvested at 6, 8 and 12 weeks, fixed in 4% paraformaldehyde for 48 hours, decalcified in 10% EDTA (pH 8.0) and embedded in paraffin. Five-micrometer sections were deparaffinized, stained with hematoxylin/eosin, and semi-quantitative bone formation was scored, microscopically, by four blinded independent observers as previously described (Akintoye *et al*, 2006). Bone scores, that were performed by the four observers, ranged from 0 (no bone observed within the transplant), 1 (minimal amount of bone), 2 (weak bone formation occupying only a small portion of the transplant), 3 (moderate bone formation occupying a significant portion but less than 50% of the transplant) and 4 (abundant bone formation, occupying more than 50% of the transplant).

3.10 Adipogenic differentiation

Adipogenic differentiation was induced as previously described (Akintoye *et al*, 2006; Volk *et a*l, 2012). cMSCs were cultured at 1.8 x 10^3 cells/cm^2 in 4-well chamber slides (Coming Life Sciences, Acton, MA) using α-MEM growth medium without adipogenic inducers. At approximately 100% confluence, the growth

medium was switched to adipogenic medium containing supplements of 10^{-8} M dexamethasone, insulin (1 µg/ml), 1-methyl-3-isobutylxanthine (IBMX, 5×10^{-8} M), indomethacin (10^{-4} M), and fetal bovine serum (FBS) 10% for 15 days; medium was changed twice weekly. Similar culture plates without exposure to adipogenic medium served as control. At 15 days, the cells were rinsed with 2x phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde for 10 minutes at room temperature, stained with 0.3% Oil Red O for 1 hour, and counterstained with 1% Fast green dye for 10 minutes. The slides were mounted with coverslip and lipid droplets were identified and quantified microscopically. Using Image J, lipid droplets were counted for the cell types, femur and mandible, non-induced and induced cells, and then the number of droplets per cell was calculated.

3.11 Chondrogenic differentiation

Chondrogenesis differentiation assay was performed as previously described (Csaki *et al*, 2007; Park *et al*, 2012; Volk *et al*, 2012). Chondrogenic differentiation was induced using the pellet method. cMSCs were cultured in 75 cm² flasks at 75 x 10^4 cells/cm² until they reached 80-90% confluence. Then, the cells were trypsinized and counted with hemocytometer. 2 x 10^6 cells were transferred into various 15 ml polypropylene tubes. The cells were centrifuged to a pellet form, and supernatant was aspirated without disturbing the pellets. Thereafter, the pellets were washed with PBS, then cultured in chondrogenic medium consisting of alpha-MEM. supplemented with 10^{-8} M dexamethasone, ITS^+ 1%, L-Ascorbic acid 2-phosphate (10^{-4} M), transforming growth factor-beta

3 (TGF-β3) 10ng/ml, β-glycerophosphate 10mM, glutamine 2 mM, penicillin-streptomycin sulfate 100 U, and pyruvate 2 mM. Cell pellets cultured with growth medium without chondrogenic inducers were used as control. Chondrogenic medium was replenished every 2-3 days. The pellets were harvested after 4 and 8 weeks for histological analysis, Pellets were fixed with 4% paraformaldehyde for 12 hours, and processed for paraffin embedding. 5 µm sections were, stained with Alcian blue solution, counterstained with nuclear fast red solution, dehydrated, and mounted with coverslip for histological evaluation.

3.12 Neural differentiation

Canine MSCs were cultured at 4 x 10^3 cells/cm^2 in 8-well chamber slides coated with collagen (Corning® BioCoat™) with normal α-MEM growth medium without inducers until they reached confluence. Thereafter, 4 chambers were exposed to neurogenic medium, and the other 4 were kept in normal growth medium as the control group. Neurogenically induced cells were pre-incubated for 24 hr. with α-MEM medium supplemented with 20% fetal bovine serum (Atlanta biological, Lawrenceville, GA), 100 U/ml Penicillin, 100 mg/ml streptomycin sulfate, 2 mM glutamine (Gibco, Life technologies, NY), and 10 ng/ml β-fibroblast growth factor (β-FGF, 10 ng/ml) (BD Biosciences) while control cells were still retained in α-MEM growth medium without β-FGF. The pre-induction medium was then removed, and the cells were washed with PBS and transferred to neuronal induction medium composed of: α-MEM supplemented with 20% fetal bovine serum, 100 U/ml Penicillin, 100 mg/ml streptomycin sulfate, 2 mM glutamine

(Gibco, Life technologies, NY), 2% dimethyl sulfoxide (DMSO; Sigma-Aldrich), 10 ng/ml fibroblast growth factor (FGF, 10 ng/ml), 200 *μ*M butylated hydroxyanisole (BHA; Sigma-Aldrich), 10 μM Forskolin (Sigma), 25 mM KCl, 2 mM Valproic acid (Calbiochem), and 5 μg/mL insulin. Cells were incubated for 24 hours, 4, 7, and 14 days at 37ºC in a humidified 5% carbon dioxide environment. Neural differentiation was evaluated using morphological analysis and immunostaining. Early neuronal expression was assessed with anti-nestin polyclonal antibody (LifeSpan BioSciences, Inc.), while late neuronal expression was assessed with anti-beta III tubulin (Bioss). Cells were fixed with 4% paraformaldehyde, incubated in 0.1% TritonX-100 for 5 minutes, blocked with 3% goat serum for 30 minutes, and incubated overnight with primary antibodies: 1:200 dilution of anti-nestin and 1:200 anti-beta III. After washing, the samples were incubated with 1:500 dilution of fluorescent-labeled secondary antibody goat anti-rabbit Alexa Fluor 555 (Life Technologies). Nuclei were visualized with 1 ųg/ml of Hoeschst 33342. Specimens were serially excited and images were captured on the microscope.

3.13 Statistical analysis

All experiments were performed at least three times; each cell type (induced and control) were tested in triplicates, and the resulting data was averaged prior to subsequent analysis. The results were expressed as mean ± standard deviation. Comparison of responses between mandible-cMSCs and femur-cMSCs was measured by the paired t-test analysis and values of $p<0.05$ were considered

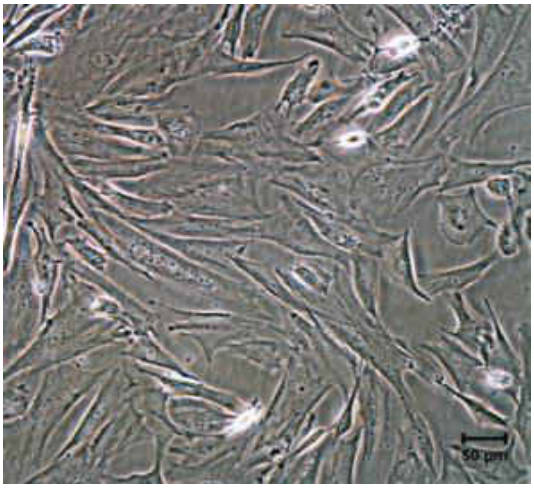
statistically significant. Cell proliferation was tested by analyzing slopes of linear regression lines of mandible and femur cells. A value of $p<0.05$ was also considered statistically significant.

4 RESULTS

4.1 Cell culture of cMSCs

The primary F-cMSCs and M-cMSCs expanded in culture displayed characteristic polymorphic, fibroblast-like morphology in monolayer culture, as is shown by M-cMSCs (Figure 4). Within about 5 to 6 days, the M-cMSCs were usually 80-90% confluent, while F-cMSCs were comparatively at 60-70%.

Figure 4



20x

Figure 4: Representative image of M-cMSCs monolayer showing characteristic fibroblast-like morphology.

4.2 Cell proliferation

When cMSCs were plated at low densities of 95,115 cells/cm^2 and counted at days 1, 3, 6, 9, 12, and 15, M-cMSCs displayed significant higher proliferative rates until day 9 compared with those of F-cMSCs. A test of slopes demonstrated that the differences between both slopes, mandible and femur, were very significant (p= 0.006). Additionally, while F-cMSCs proliferation plateaued at day 9, the M-cMSCs continued to grow exponentially before plateauing by day 12 (Fig. 5).

Figure 5

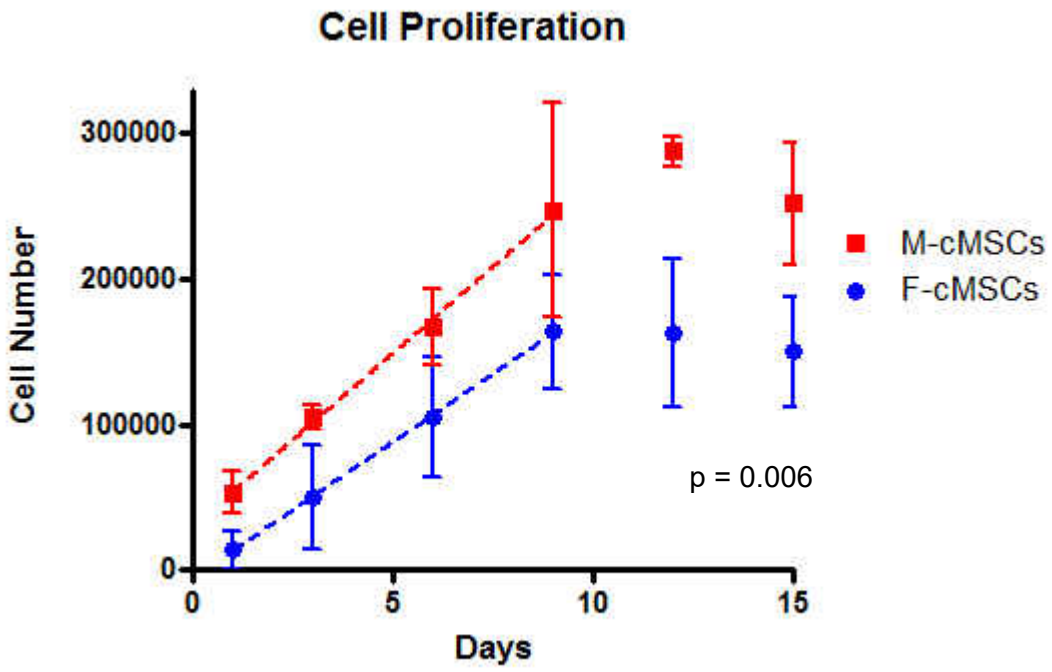


Figure 5 Cell proliferation: the slope representing the number of mandible cells until day 9 was significant different (p = 0.006) compared with that of femur cells, demonstrating that M-cMSCs had higher proliferative rate than F-cMSCs. After day 9, the proliferative capacity of both cell types started decreasing.

4.3 Colony forming efficiency assay

Cells isolated from femur and mandible were apparently similar in terms of their ability to form colonies, which were visualized and counted after being stained with methyl violet (Figure 6).

Figure 6

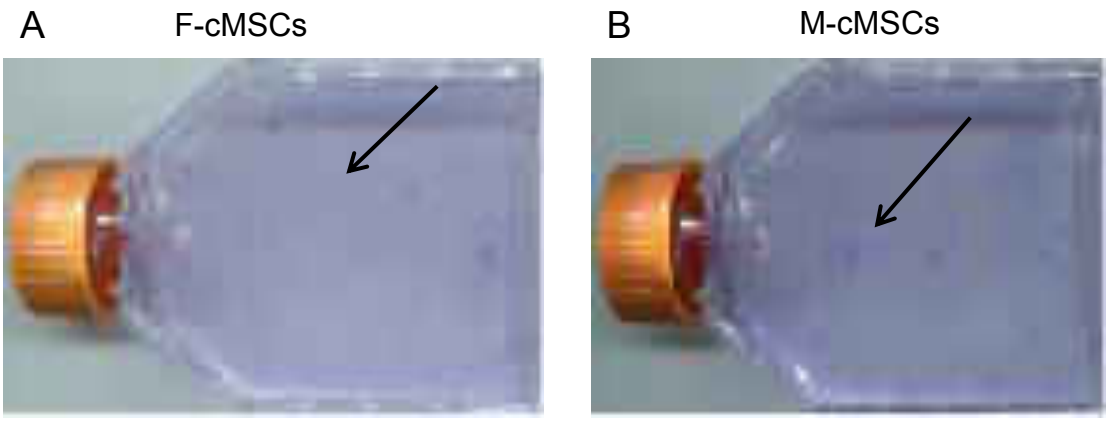


Figure 6 Colonies in plastic flasks A: F-cMSCs B: M-cMSCs. Arrows indicate stained cell colonies of both cell types.

Mean colony forming efficiency per 10^5 nucleated cells was not significantly different between F-cMSCs and M-cMSCs (Figure7)

Figure 7

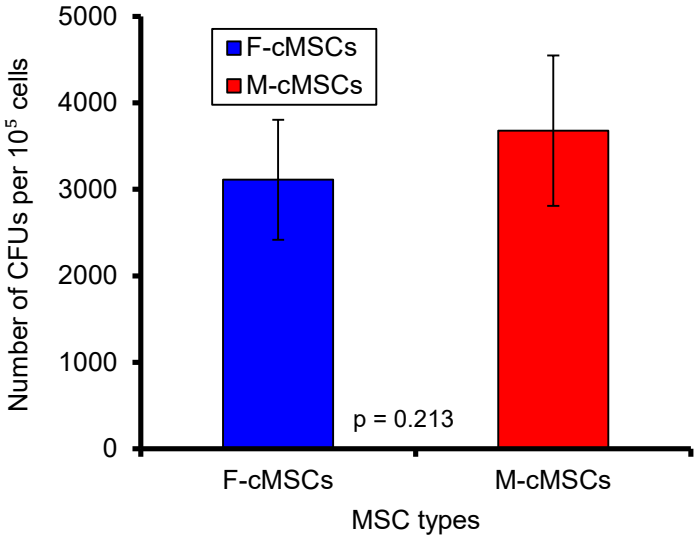


Figure 7 Mean colony forming units per 10^5 nucleated cells was not significantly different between F-cMSCs and M-cMSCs.

4.4 Population doubling and telomerase activity

Life span of cMSCs assessed by population doublings (PDs) capacity demonstrated that M-cMSCs were able to survive until passage 12, which relates with a total of 77 days after repeated passaging, but F-cMSCs reached replicative senescence at passage 6, which occurred 10 days earlier than M-cMSCs (Fig. 8). Since this experiment was performed in duplicates, a statistic analysis was not possible to be performed. However, qualitatively and as mentioned above, mandible cells survived more days compared with femur cells.

Figure 8

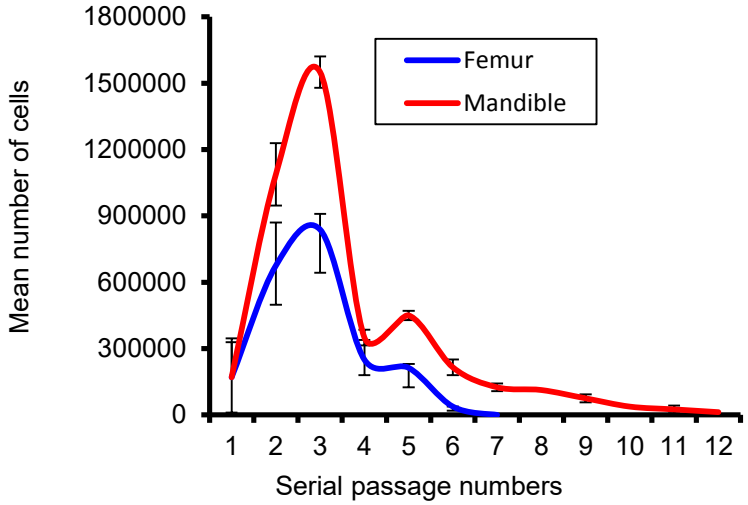


Figure 8 Life span: M-cMSCs were able to survive five more passages relative to F-cMSCs

To complement lifespan assessment canine telomerase reverse transcriptase (cTERT) activity was evaluated by Western blotting of nuclear extracts isolated at different passages of the population doubling experiment. The expression of cTERT progressively decreased as the cells progressed toward senescence as

demonstrated by immunoreactivity and quantitative analysis of the immunoreactive bands. (Fig 9A-B) While F-cMSC TERT was quantitatively higher at baseline and subsequent passages, the expression was more short-lived relative to M-cMSCs (Fig. 9B).

Figure 9A

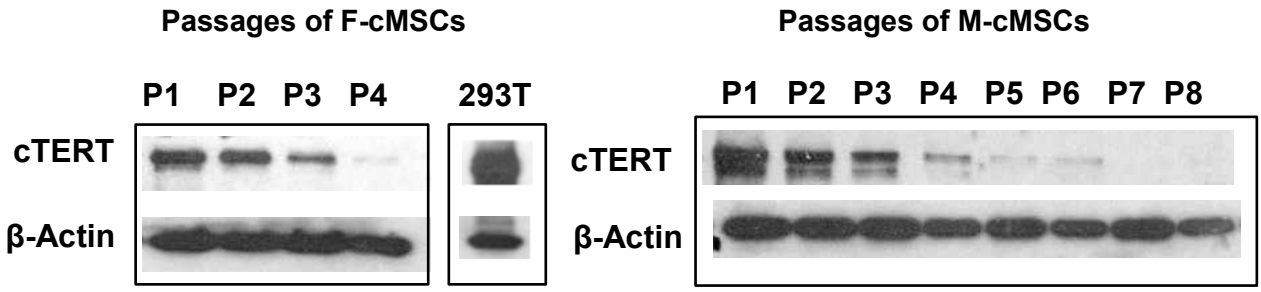


Figure 9A Immunoreactive bands of canine telomerase obtained through Western blot progressively decreased for both, femur and mandible cells with subsequent passages. β-actin served as loading control and 293T cells as control for expression of cTERT.

Figure 9B

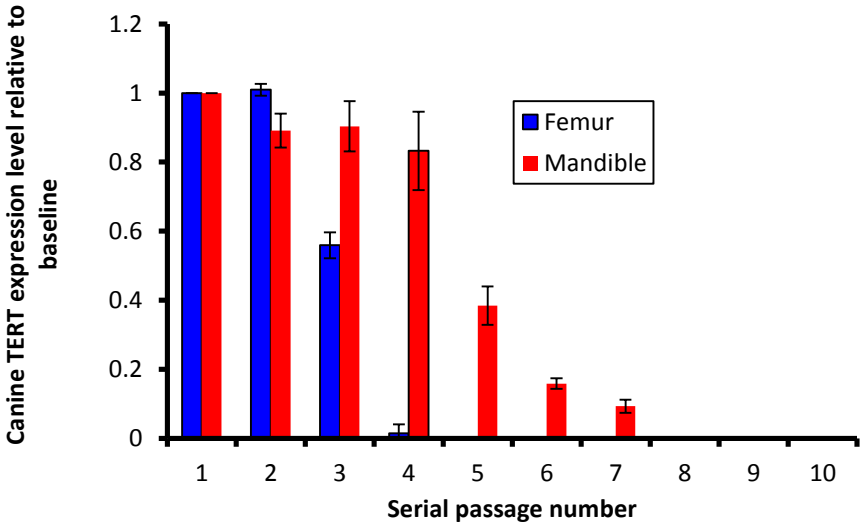


Figure 9B Expression of canine TERT relative to baseline: the expression of cTERT was downregulated as the cells progressed toward senescence. F-cMSCs demonstrated a more short-lived expression compared with m-cMSCs..

4.5 *In vitro* osteogenesis

Time-dependent *in vitro* osteogenic properties of both induced, M-cMSCs and F-cMSCs showed increased expression levels of early osteogenic markers such as alkaline phosphatase (ALP) and bone sialoprotein (BSP) compared with non-induced cells. These findings are representative of, at least 3 different experiments. Interestingly, induced mandible cells displayed active expression of ALP at 7 days of induction, which showed their initial response to osteogenic differentiation. In addition, on day 14 mandible cells exhibited a significant up-regulation of ALP (p= 0.04) compared with femur cells. As expected, both cell types showed maximal ALP expression on day 7 (Figure 10).

Figure 10

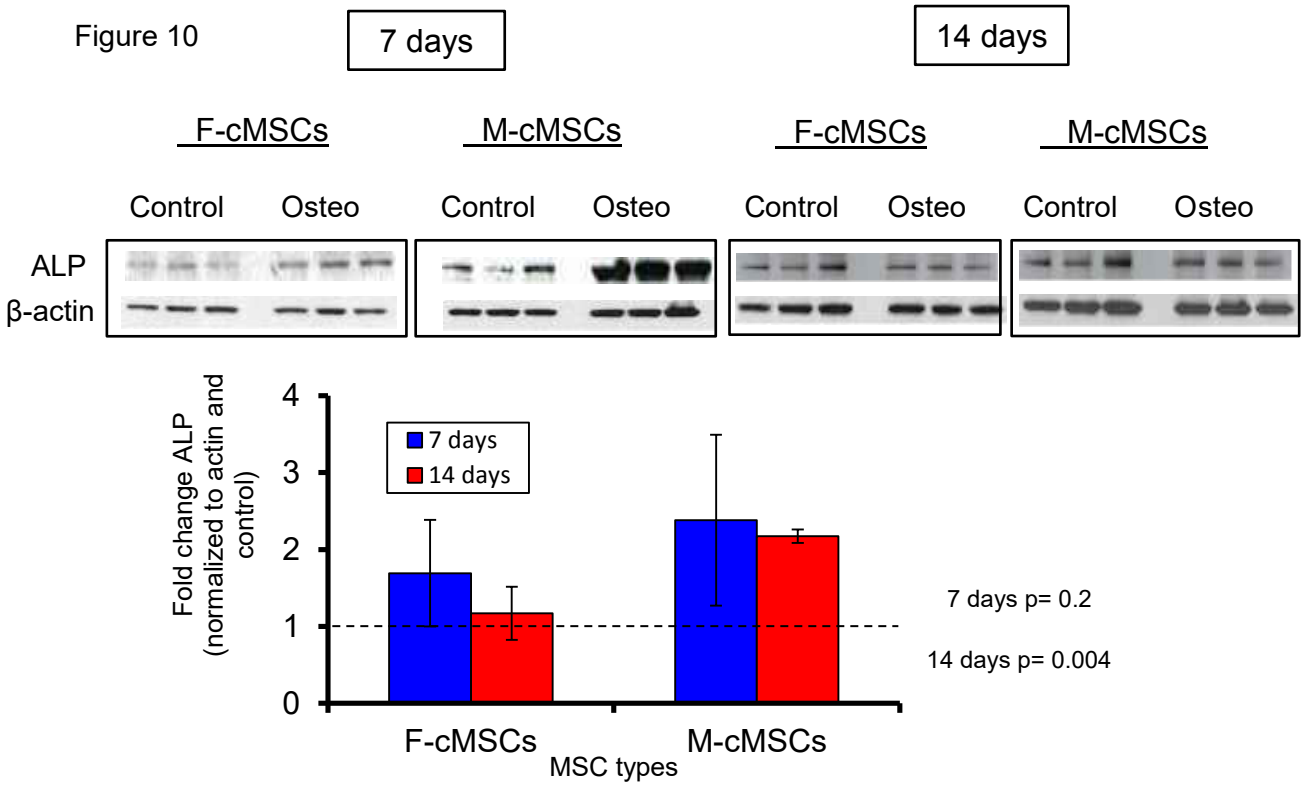


Figure 10 **Alkaline phosphatase** expression at 7 and 14 days of osteogenic induction: Although at 7 days the difference was not statistically significant, M-cMSCs demonstrated higher expression levels of ALP at 7 and 14 day time points compared with F-cMSCs.

In relation to BSP expression at 7 and 14 days of osteogenic induction, there was a higher time-dependent BSP expression in mandible cells relative to femur cells. As with ALP, there was a statistically significant up-regulation (p= 0.05) of BSP in mandible cells at day 14 compared with cells from femoral origin (Figure 11).

Figure 11

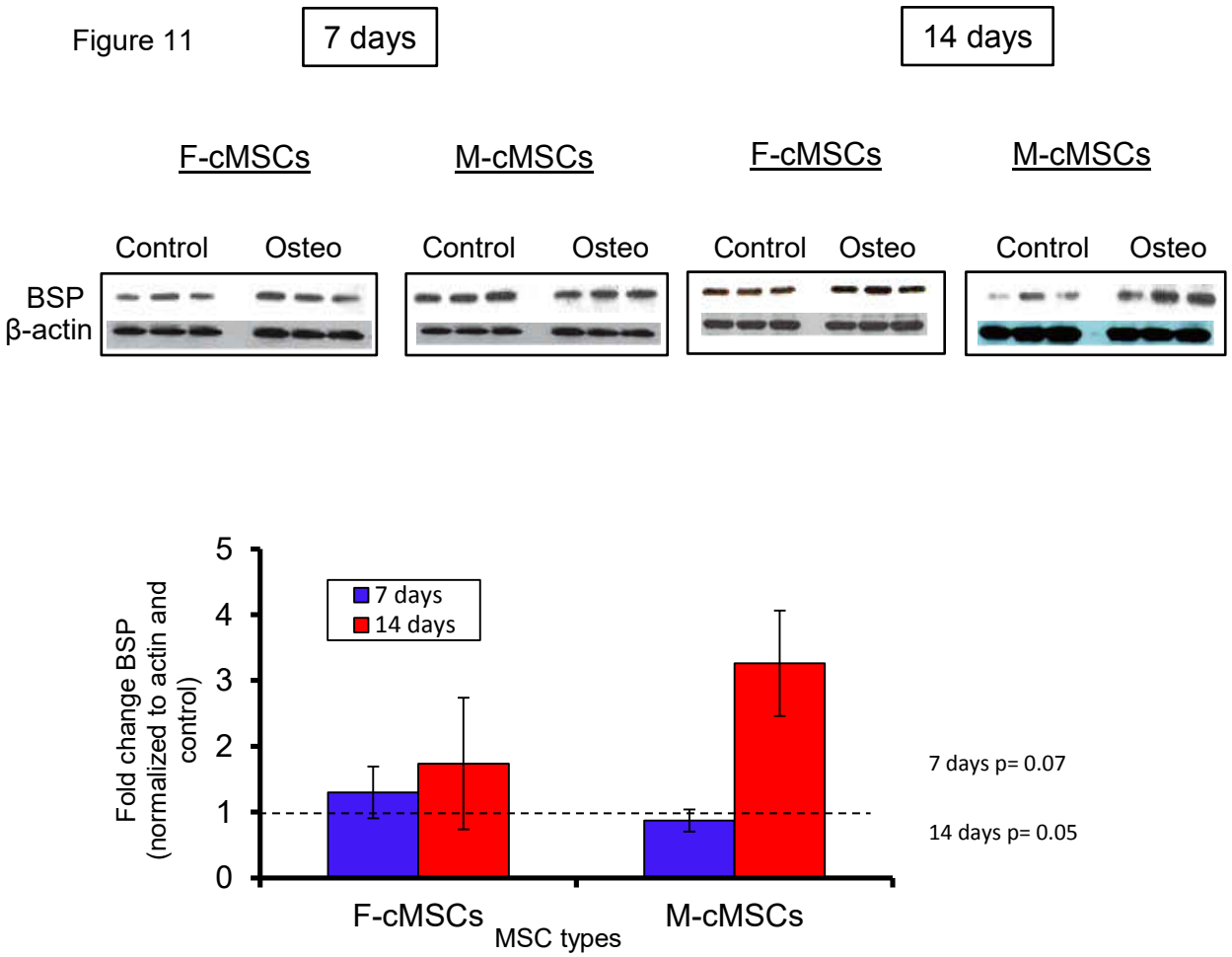


Figure 11 **Bone sialoprotein** expression at 7 and 14 days of osteogenic induction: there was a higher time-dependent BSP expression in M-cMSCs relative to F-cMSCs based on 7 and 14 days.

While there were no differences in osteopontin (OPN) expression levels between the two cell types, osteocalcin (OCN) was not expressed early by F-cMSCs compared to M-cMSCs that consistently demonstrated measurable levels of the late osteogenic marker OCN at both 7 and 14 days. (Figures 12 and 13).

Figure 12

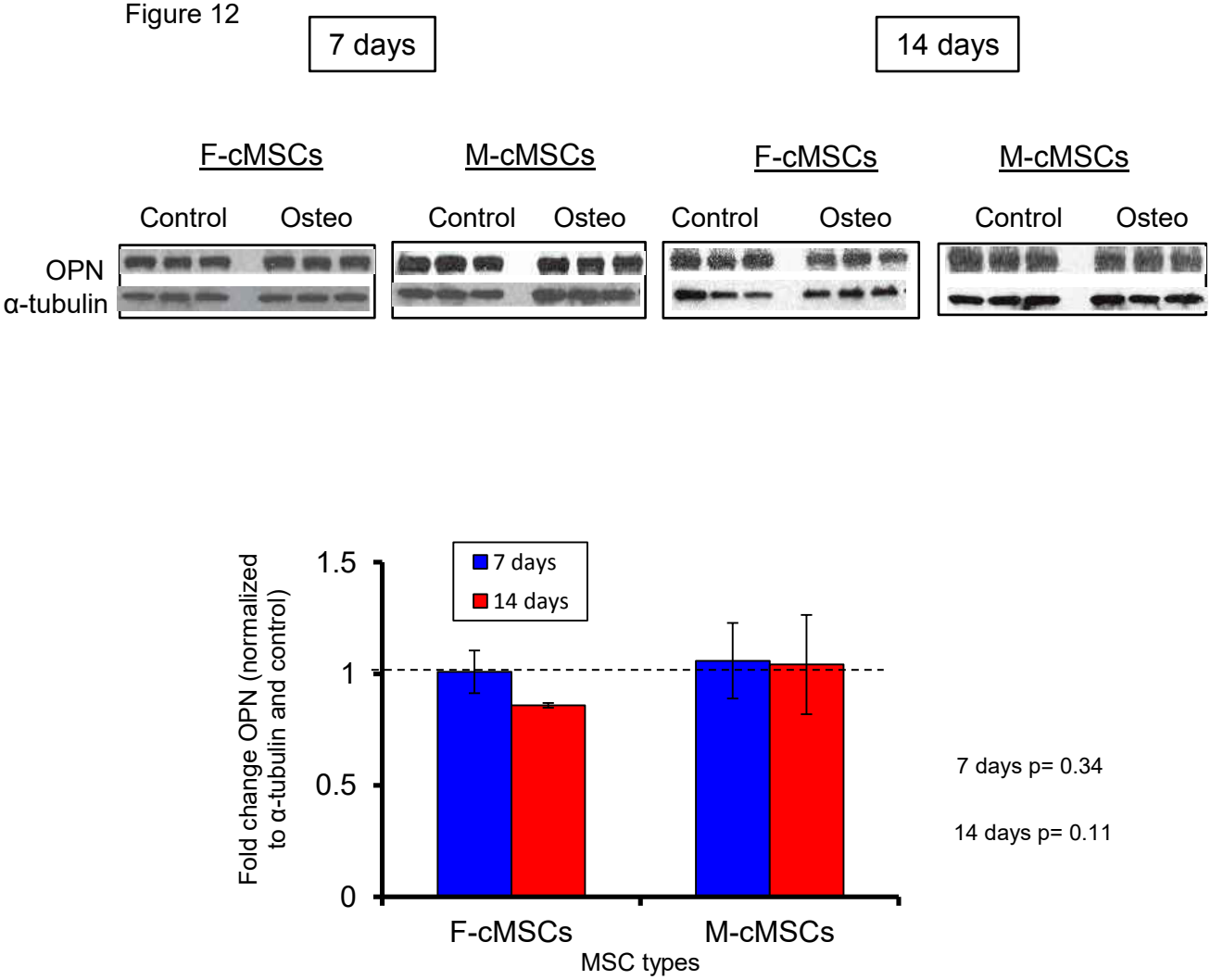


Figure 12 **Osteopontin** expression at 7 and 14 days of osteogenic induction: there was not difference in OPN expression at 7 and 14 days between the two types of cells.

Figure 13

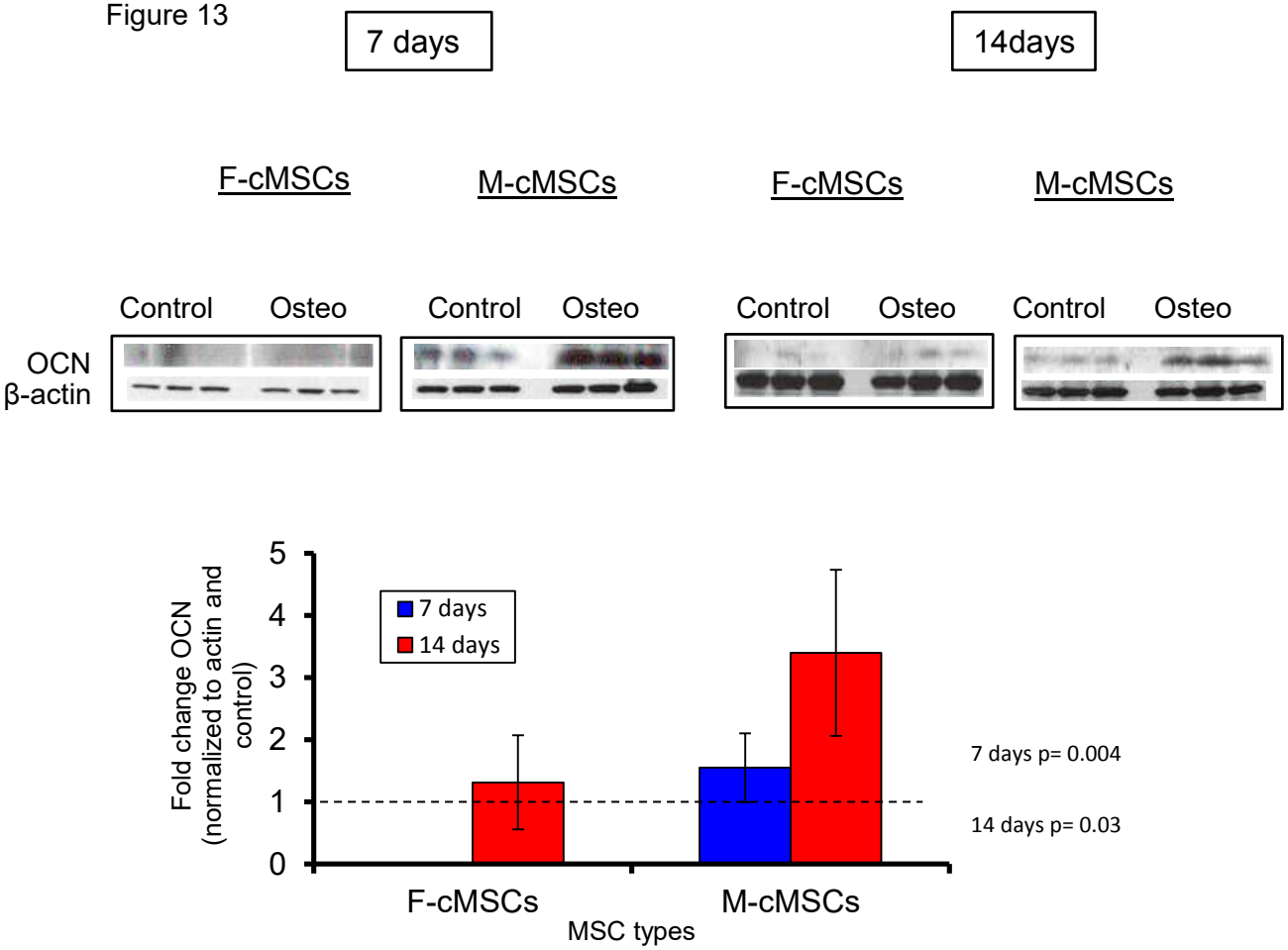


Figure 13 **Osteocalcin** expression at 7 and 14 days of osteogenic induction: while there was no early expression of OCN in F-cMSCs, it was significantly upregulated in M-cMSCs relative to F-cMSCs at 7 and 14 days.

4.6 Reverse transcriptase polymerase chain reaction (RT-PCR)

Gene transcripts of ALP, BSP, OPN and OCN were also assessed by real-time RT-PCR in cMSCs exposed to osteogenic and non-osteogenic conditions. Gene expressions of ALP, BSP, and OCN were significantly up-regulated in mandible compared with femur cells at 7 and 14 days of induction (p values indicated in the respective graphs). These findings were consistent with Western blot results.

Differences between the two cell types were more clearly defined after 14 days of osteogenic stimulation based on significantly upregulated expression levels of ALP, BSP and OCN in M-cMSCs relative to F-cMSCs. Interestingly, OPN gene transcript was only moderately upregulated in M-cMSCs at day 7 (Fig 14 - A, B, C, D).

Figure 14

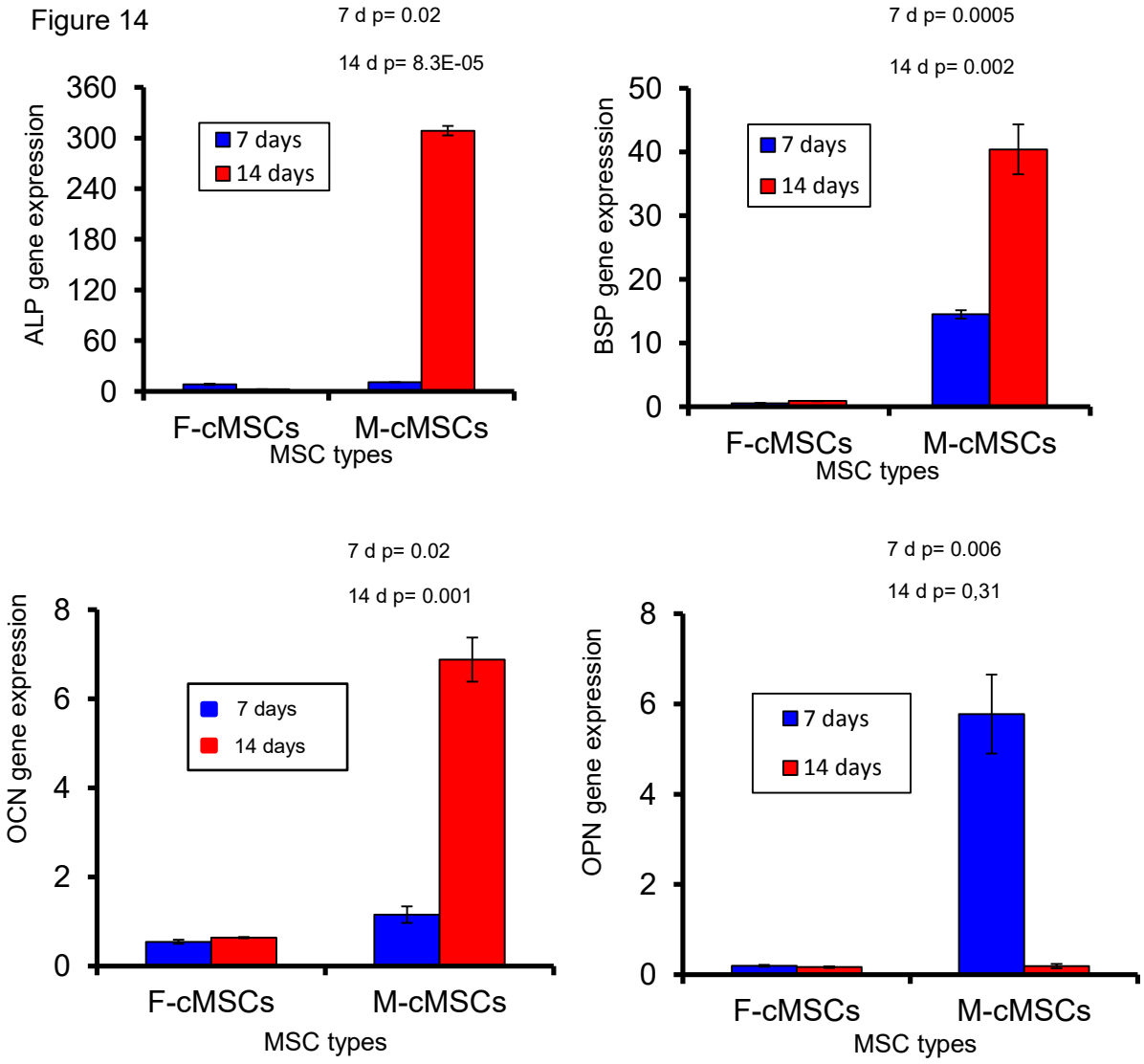


Figure 14 Real time PCR; gene expressions of ALP (A) and BSP (B) were significantly upregulated in M-cMSCs compared to F-cMSCs at 7 and 14 days of induction. In terms of transcription of OCN (C), it was also upregulated in M-cMSCs relative to F-cMSCs at both time periods. However,OPN (D) expression moderately increased at 7 days in M-cMSCs compared to F-cMSCs.

4.7 In vivo osteogenesis

Bone forming capacity of cMSCs assessed by *in vivo* transplantation showed microscopically observable bone nodules in hematoxylin/eosin stained sections after 6, 8, and 12 weeks (Figures 15-20). Semi-quantitative analysis using an established bone scoring system (Akintoye *et al*, 2006) showed that bone formation capacities of M-cMSCs and F-cMSCs were not different between non-induced and osteogenically-induced cells (Fig 21).

Figure 15

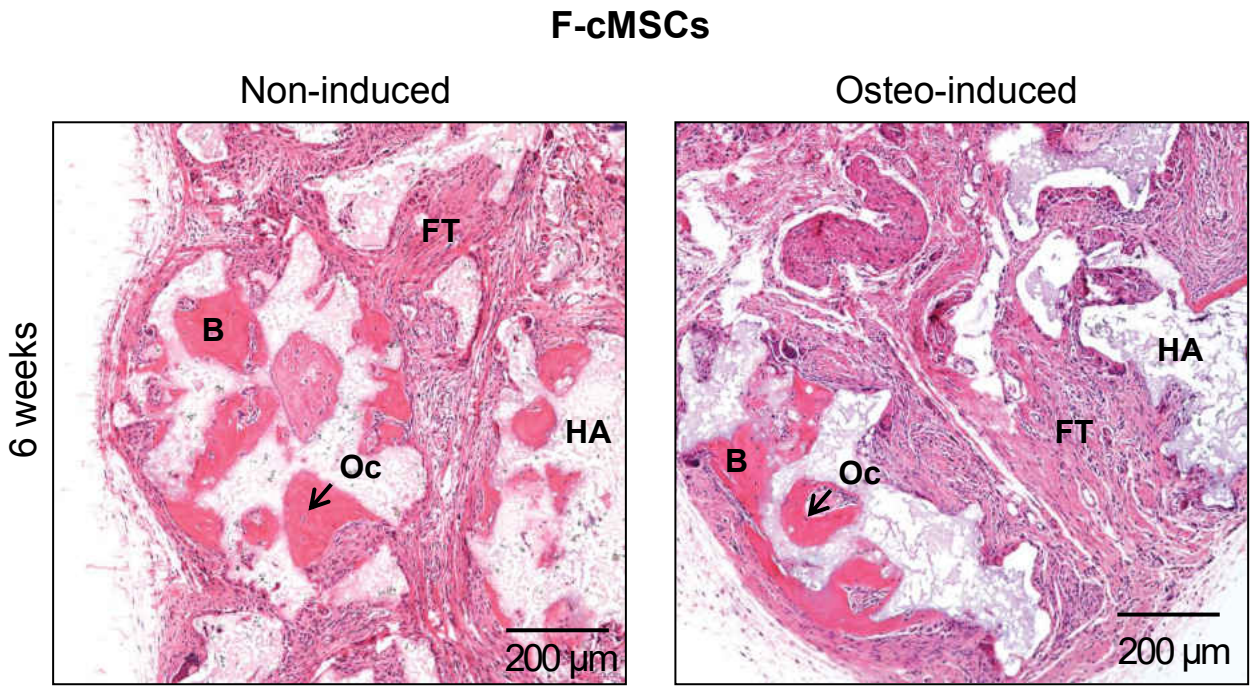


Figure 15 *In vivo* bone regeneration after 6 weeks transplantation: hematoxylin and eosin stained sections of bone formed *in vivo* by non-induced and osteogenically-induced F-cMSCs. Both cell types, non-induced and induced, demonstrated bone formation (woven bone) (B= bone; FT; fibrous tissue; HA: hidroxyapatite/tricalcium phosphate carrier; Oc; osteocyte).

Figure 16

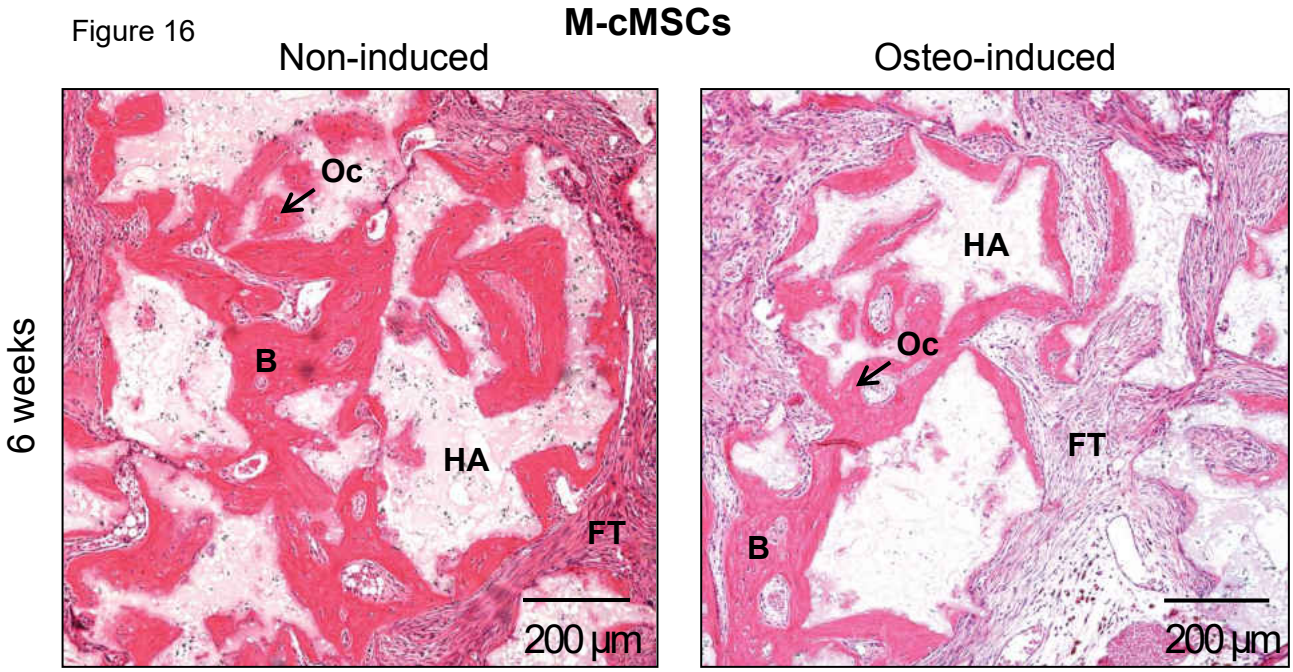


Figure 16 *In vivo* bone regeneration after 6 weeks transplantation: hematoxylin and eosin stained sections of bone formed *in vivo* by non-induced and osteogenically-induced M-cMSCs. Both cell types, non-induced and induced, demonstrated bone formation (woven bone) (B= bone; FT; fibrous tissue; HA: hidroxyapatite/tricalcium phosphate carrier; Oc; osteocyte).

Figure 17

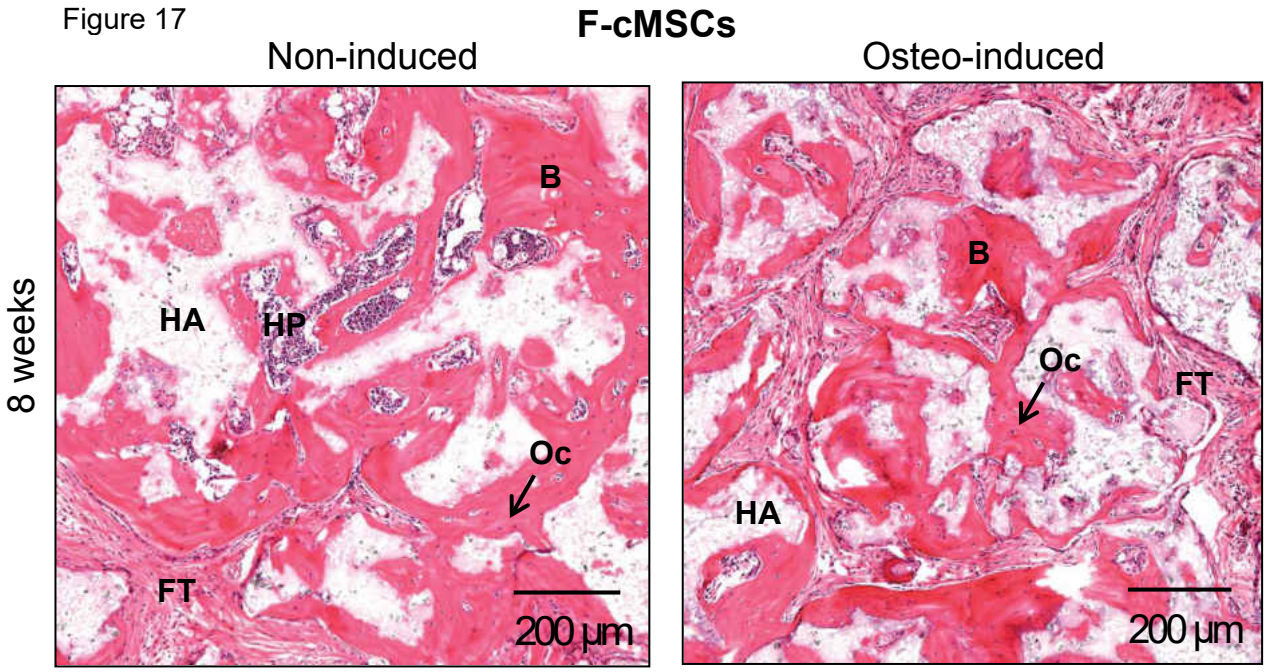


Figure 17 *In vivo* bone regeneration after 8 weeks transplantation: hematoxylin and eosin stained sections of bone formed *in vivo* by non-induced and osteogenically-induced F-cMSCs. Both cell types, non-induced and induced, demonstrated bone formation (woven bone) (B= bone; FT; fibrous tissue; HA: hidroxyapatite/tricalcium phosphate carrier; Oc; osteocyte; HP: hematopoiesis).

Figure 18

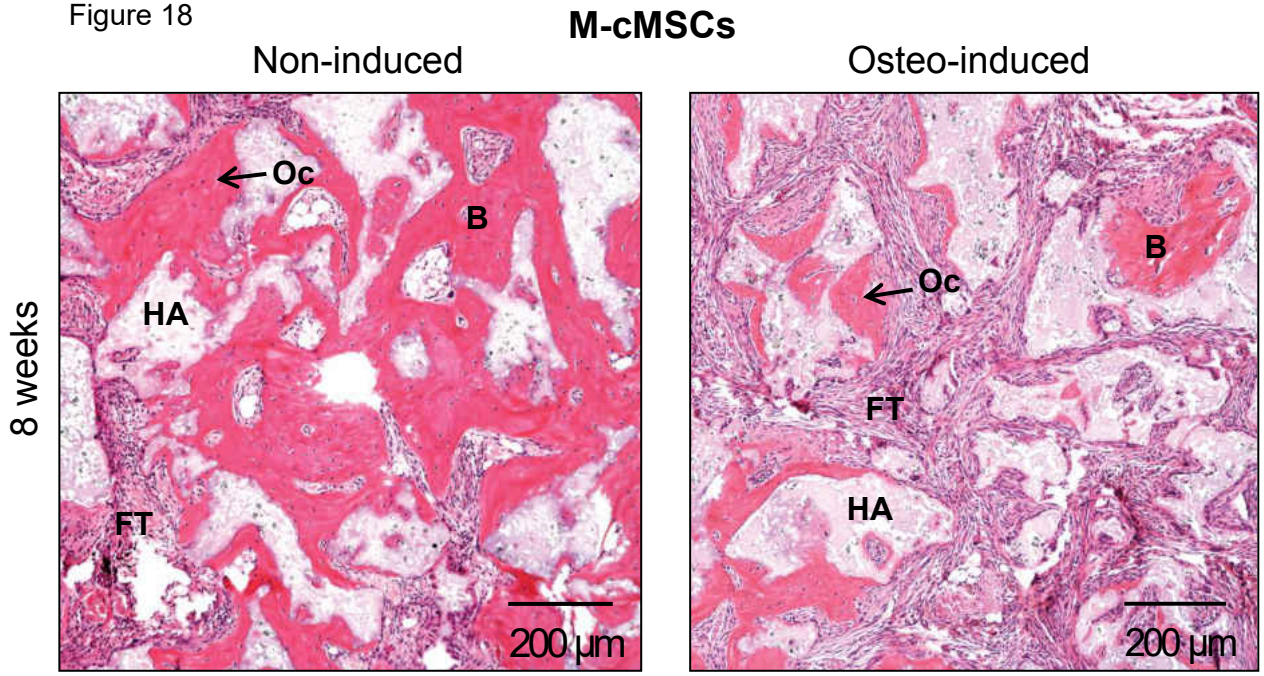


Figure 18 *In vivo* bone regeneration after 8 weeks transplantation: hematoxylin and eosin stained sections of bone formed *in vivo* by non-induced and osteogenically-induced M-cMSCs. Both cell types, non-induced and induced, demonstrated bone formation (woven bone) (B= bone; FT; fibrous tissue; HA: hidroxyapatite/tricalcium phosphate carrier; Oc; osteocyte).

Figure 19

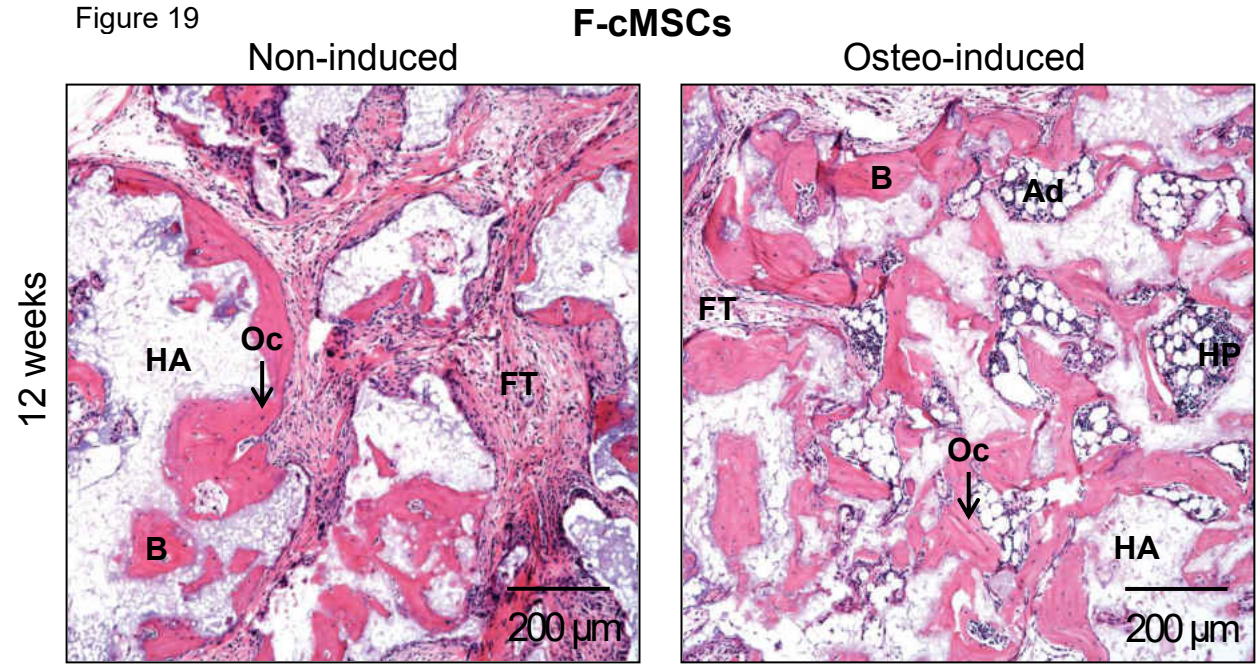


Figure 19 *In vivo* bone regeneration after 12 weeks transplantation: hematoxylin and eosin stained sections of bone formed *in vivo* by non-induced and osteogenically-induced F-cMSCs. Both cell types, non-induced and induced, demonstrated bone formation (woven bone) (B= bone; FT; fibrous tissue; HA: hidroxyapatite/tricalcium phosphate carrier; Oc; osteocyte; HP: hematopoiesis;
Ad: adipose tissue).

Figure 20

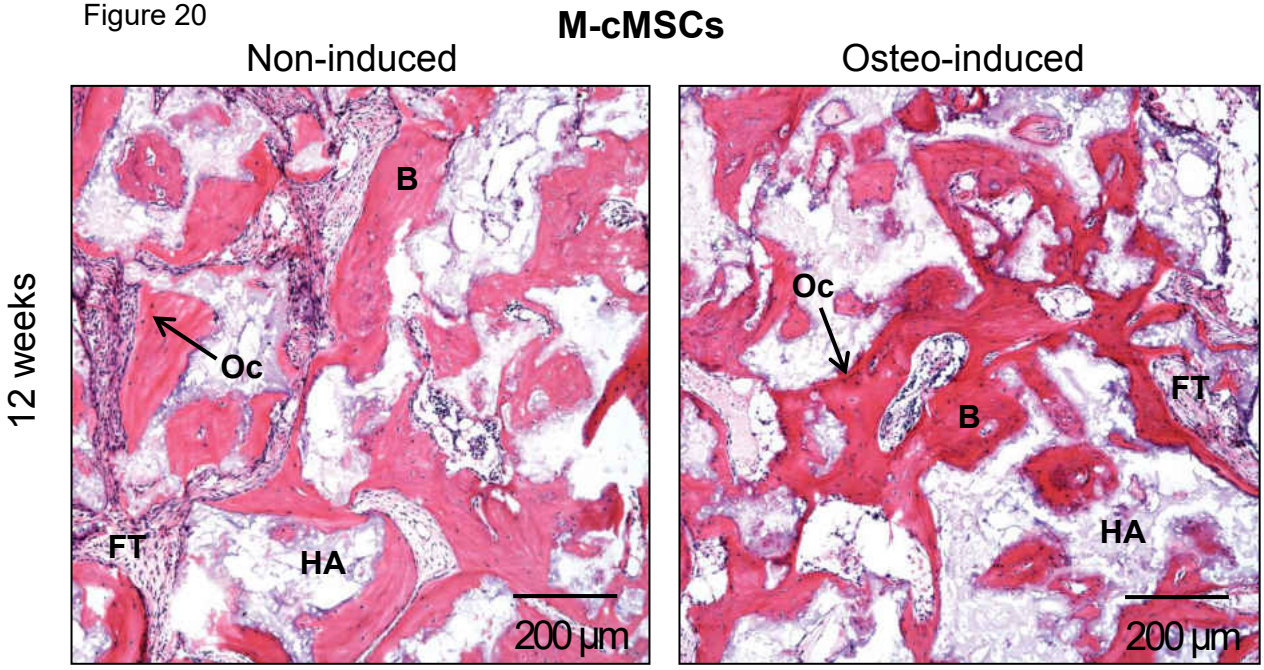


Figure 20 *In vivo* bone regeneration after 12 weeks transplantation: hematoxylin and eosin stained sections of bone formed *in vivo* by non-induced and osteogenically-induced M-cMSCs. Both cell types, non-induced and induced, demonstrated bone formation (woven bone) (B= bone; FT; fibrous tissue; HA: hidroxyapatite/tricalcium phosphate carrier; Oc; osteocyte).

Figure 21

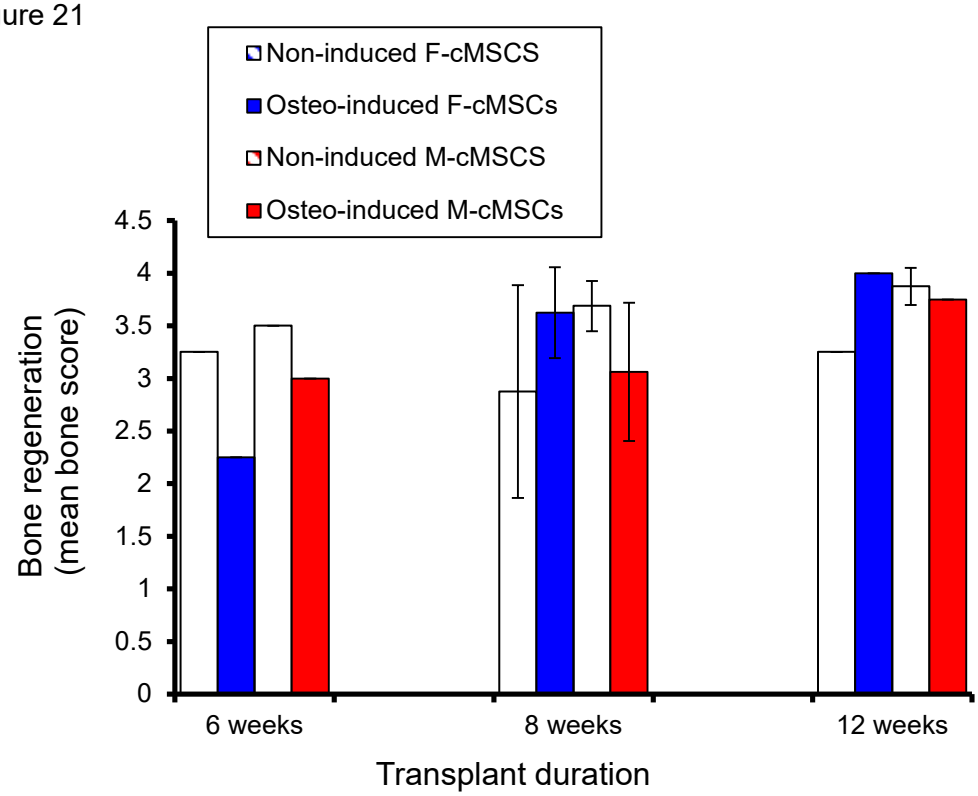


Figure 21 In *vivo* bone regeneration: hematoxylin and eosin stained-sections of bone formed *in vivo* by osteogenically-induced cMSCs transplanted in the subcutis of immunocompromised nude mice. Both types of cells, mandible and femur and non-induced and induced, formed bone independently of the duration of the transplants.

4.8 Adipogenic differentiation

After 15 days of adipogenic induction, non-induced and induced cMSCs were stained with Oil Red O and observed microscopically to assess lipid droplets within cytoplasm. Cell cultures treated with adipogenic induction media were found to contain greater accumulation of lipid-rich vacuoles within cells compared

to the untreated control cells. Oil Red O staining for fat revealed that these vacuoles contain neutral lipids consistent with adipocyte phenotype. By visualization, M-cMSCs showed more numerous lipid clusters and larger in size than those of femur cells. This demonstrates that adipogenic differentiation of the mandible cells was apparently more efficient (Figure 22).

Figure 22

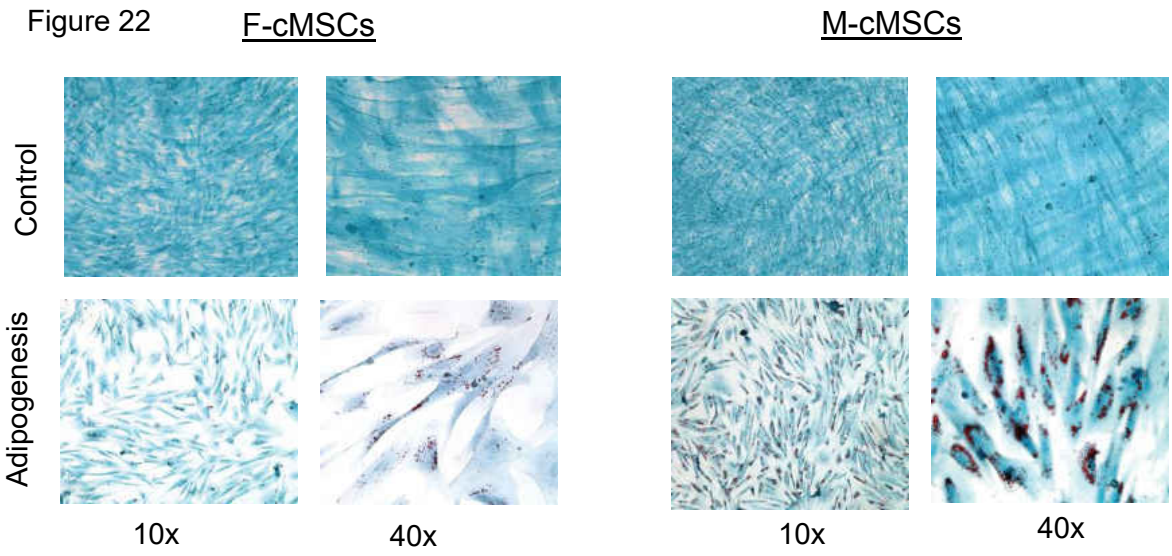


Figure 22 Adipogenesis: Oil Red O staining revealed cytoplasmic lipid inclusions in both cells, F-cMSCS and M-cMSCs, cultured in the presence of adipogenic inducers, as compared to cells cultured under control conditions. More abundant lipid droplets were observed in M-cMSCs relative to F-cMSCS.

After counting the lipid droplets for both cell types, the mean of lipid vacuoles per cell was higher in cells under adipogenic induction, femur and mandible, relative to non-induced cells. Additionally, mandible cells exhibited a significant ($p = 0.007$) higher number of lipid droplets compared with femur cells, which is consistent with the visualization assessment (Figure 23).

Figure 23

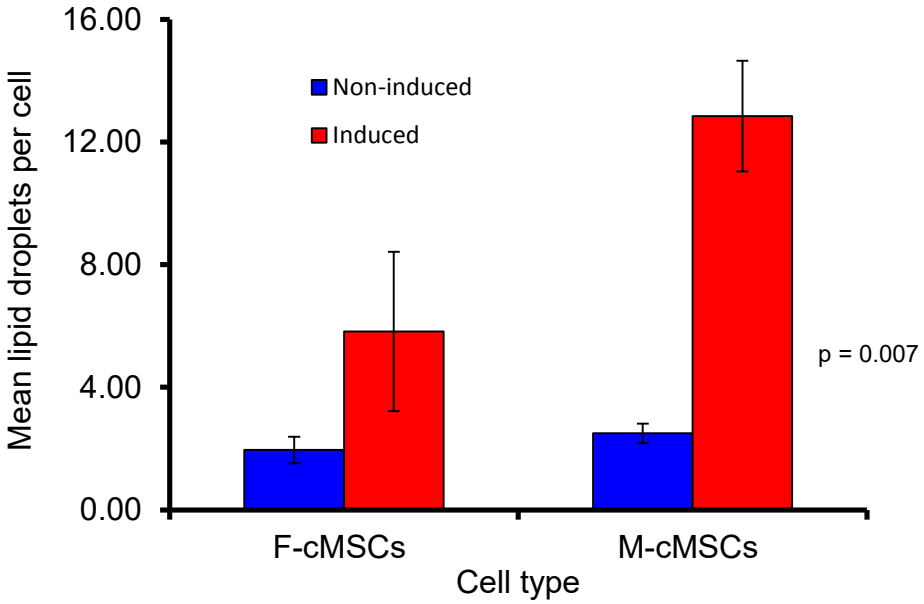


Figure 23 Mean lipid droplets per cell: induced cells, femur and mandible displayed higher number of lipid droplets per cell. Induced M-cMSCs showed significant higher number of lipid vacuoles than F-cMSCs (p= 0.007).

4.9 Chondrogenic differentiation

The chondrogenically-induced and non-induced pelleted cMSCs were assessed histologically using Alcian Blue staining after 4 and 8 weeks of pellet culture. Comparatively, chondrogenically-induced cells displayed significant higher number of chondrocyte-like cells per unit area based on pink to red staining patterns (Figures 24-25). M-cMSCs were more responsive to chondrogenic

induction especially after 4 weeks because the tissue sections showed apparently more chondrocytes per unit area (p = 0.009) (Figure 26).

Figure 24

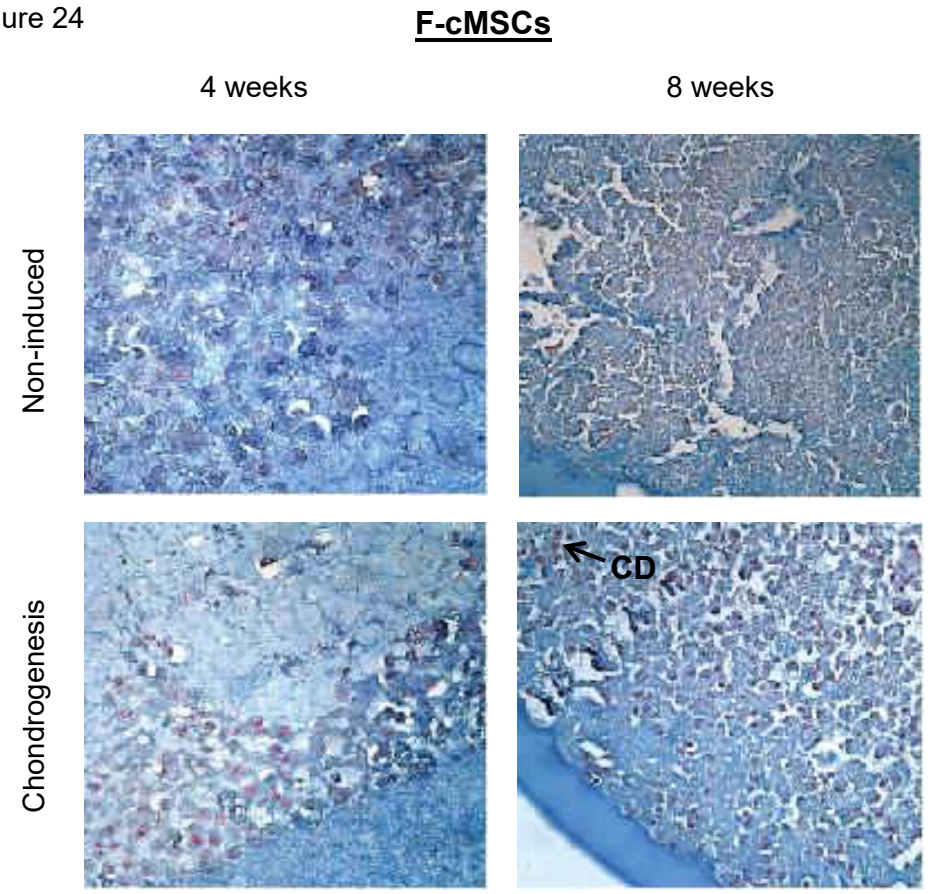


Figure 24 Chondrogenesis differentiation F-cMSCs: microphotographs showed an increased number of chondrocytes stained with alcian blue technique, in cell cultures under chondrogenic induction than cells under non-induced conditions; CD: chondrocyte.

Figure 25

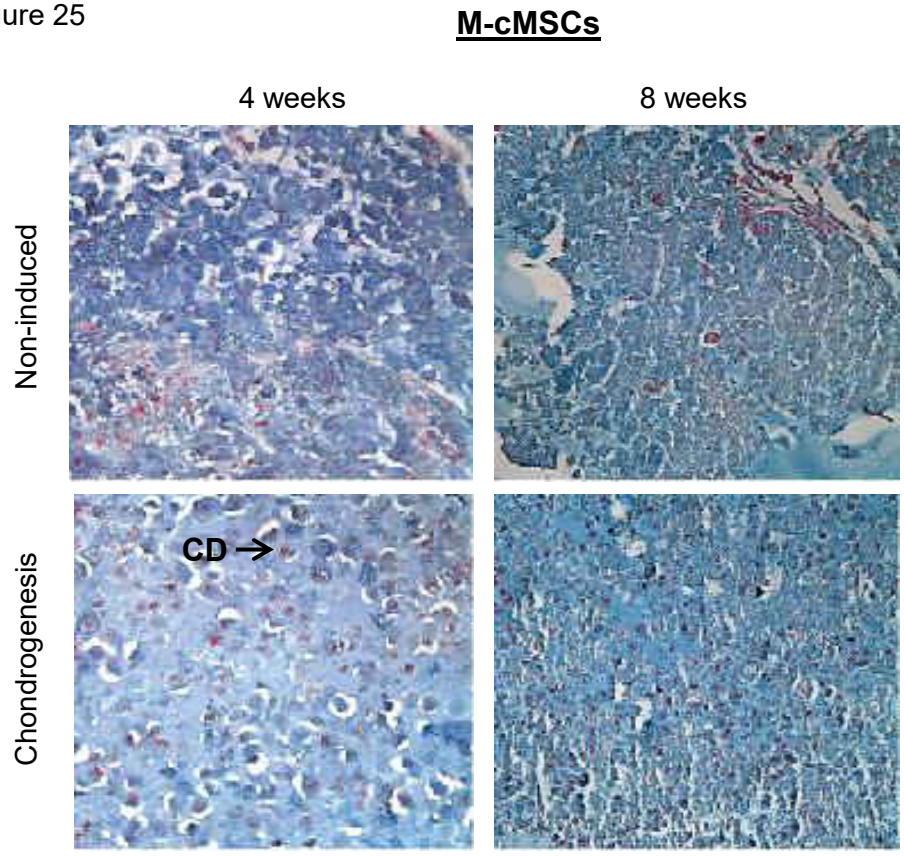


Fig. 25 Chondrogenesis differentiation M-cMSCs: microphotographs showed an increased number of chondrocytes stained with alcian blue technique, in cell cultures under chondrogenic induction than cells under non-induced conditions. In addition, a higher number of chondrocytes was observed in M-cMSCs compared to F-cMSCs; CD: chondrocyte.

Figure 26

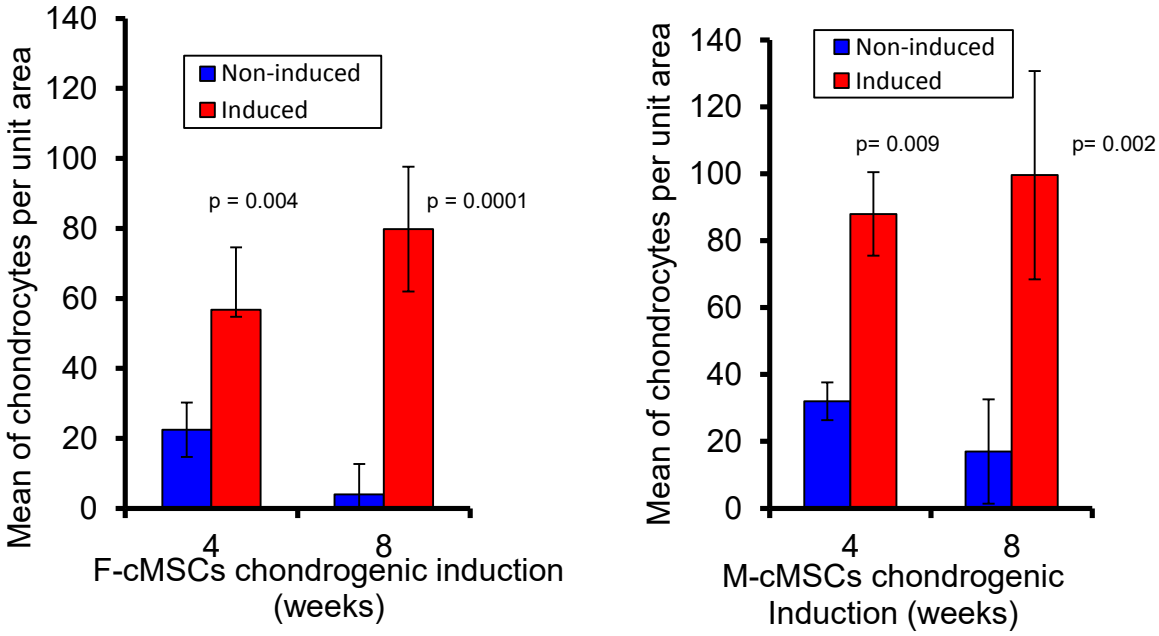


Fig. 26 Mean of chondrocytes per unit area for F-cMSCs and M-cMSCS: both types of cells demonstrated a significant higher number of chondrocytes at 4 and 8 weeks under induced conditions relative to non-induced conditions. At 4 weeks, there was a significant (p=0.009) higher number of chondrocytes in induced M-cMSCs compared to F-cMSCs.

4.10 Neurogenic differentiation

After 24 hours, 4, 7, and 14 days of neurogenic induction, the cMSCs were immunostained with two neuronal markers; nestin and βIII-tubulin. Neurogenic culture medium induced spindle-shaped morphological changes (Figures 27-36) as early as 24 hours post-induction. Long-term neural stimulation further induced cMSCs to acquire long cytoplasmic processes and neuron-like morphology with characteristic dendritic shape (Figures 27-36). The neuronally-induced cells were slightly more reactive to both neuronal markers: nestin and βIII tubulin.

Figure 27

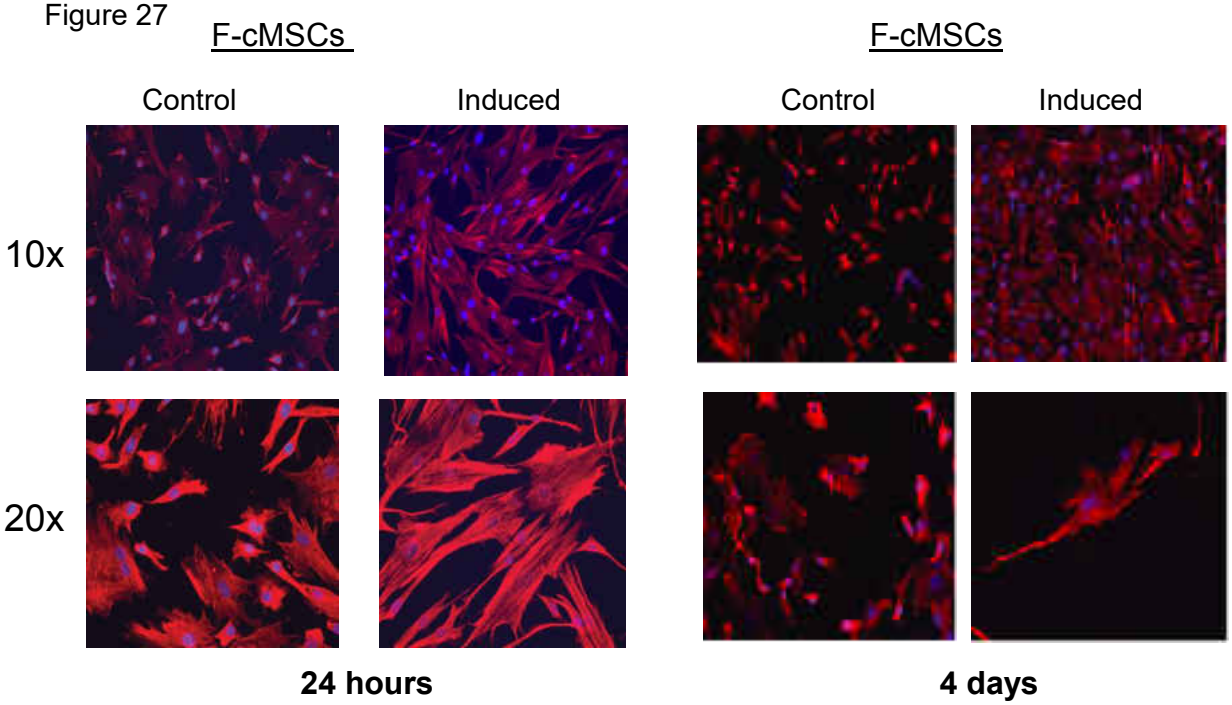


Figure 27 Neurogenesis-nestin 24 hours and 4 days induction: after neural induction, induced F-cMSCs changed morphologically into a spindle shape. The induced cells acquired more long fibroblastic neuronal extensions, mimicking a dendritic shape, than control cells.

Figure 28

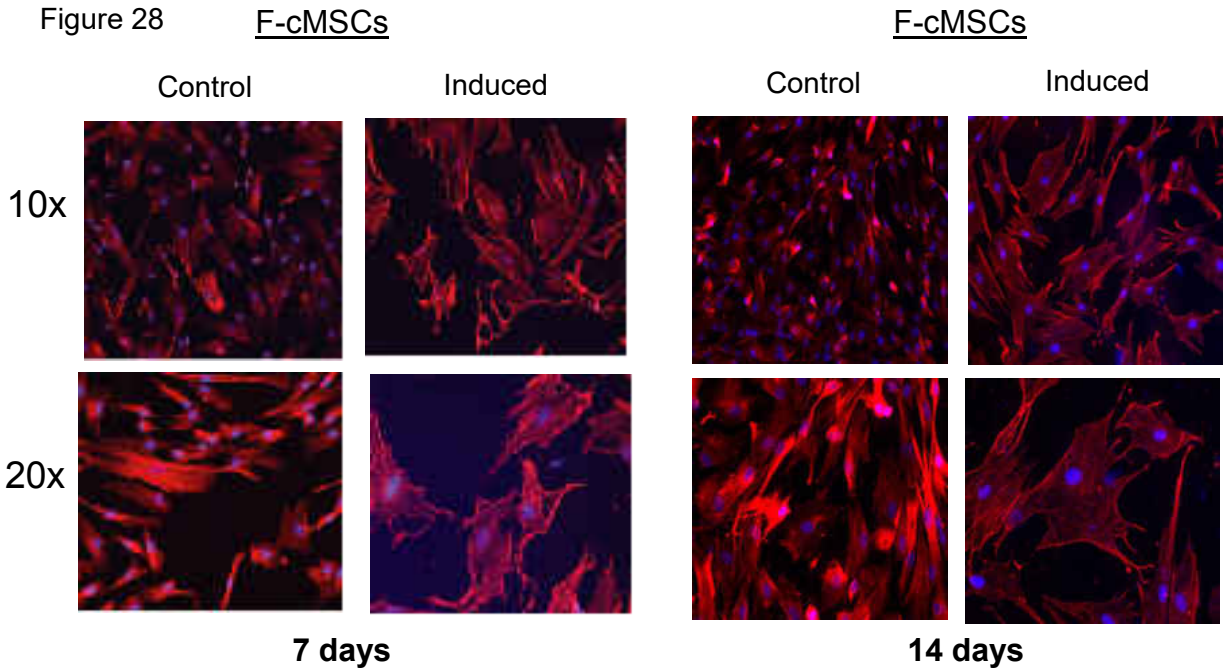


Figure 28 Neurogenesis-nestin 7 and 4 days induction: Induced F-cMSCs continued to undergo spindle-shaped morphological changes. There was similar expression of nestin by non-induced and induced cells.

Immunostaining of non-induced and induced F-cMSCs with no primary antibody (anti-nestin) included showed no expression or extreme decreased expression of nestin (Figure 29).

Figure 29

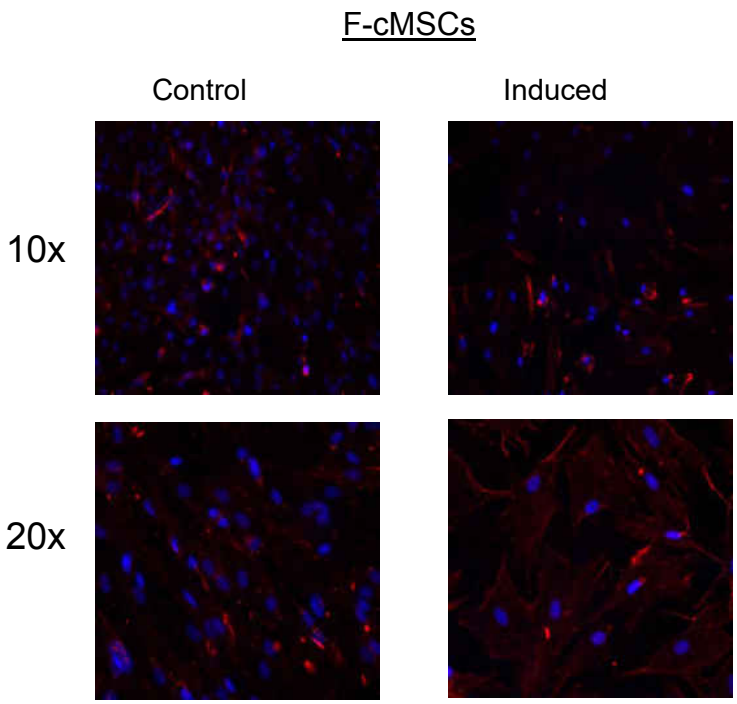


Figure 29 Negative control of neurogenesis-nestin in non-induced and induced F-cMSCs: immunofluorescence microphotographs showing no expression or extreme decreased expression of the primary antibody anti-nestin.

Figure 30

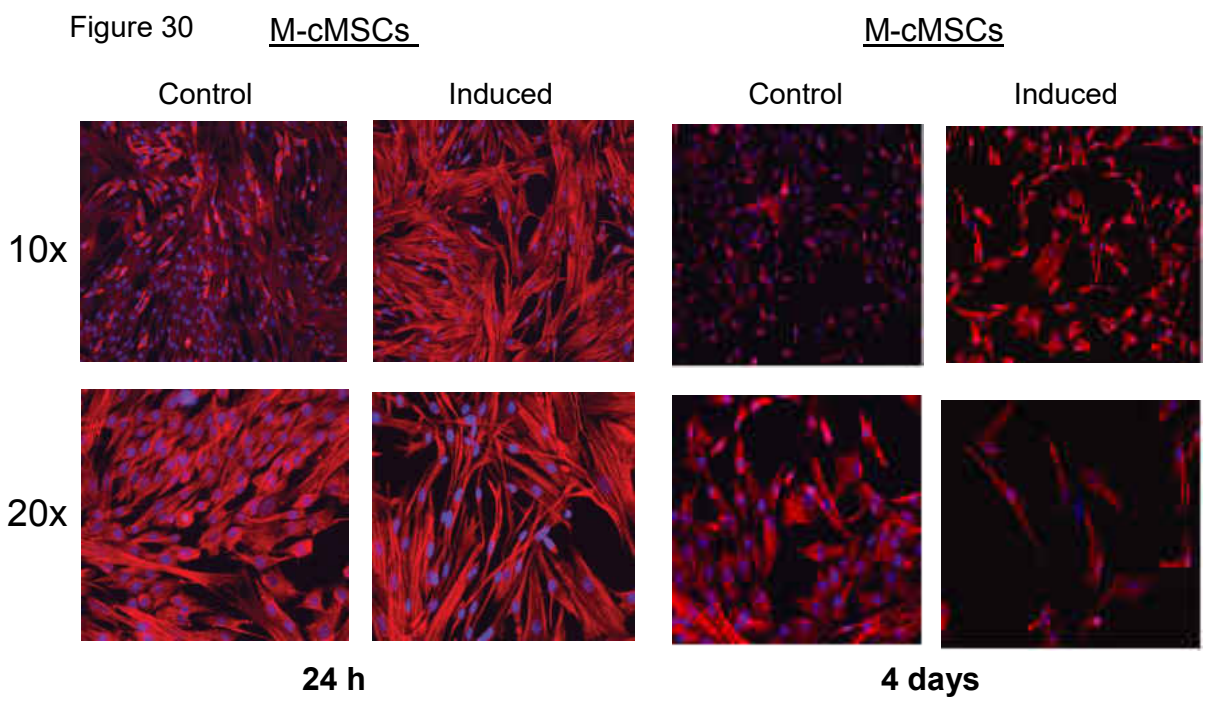


Figure 30 Neurogenesis-nestin 24 hours and 4 days induction: after neural induction, induced M-cMSCs changed morphologically into a spindle shape. The induced cells acquired more long fibroblastic neuronal extensions, mimicking a dendritic shape, than control cells.

Figure 31

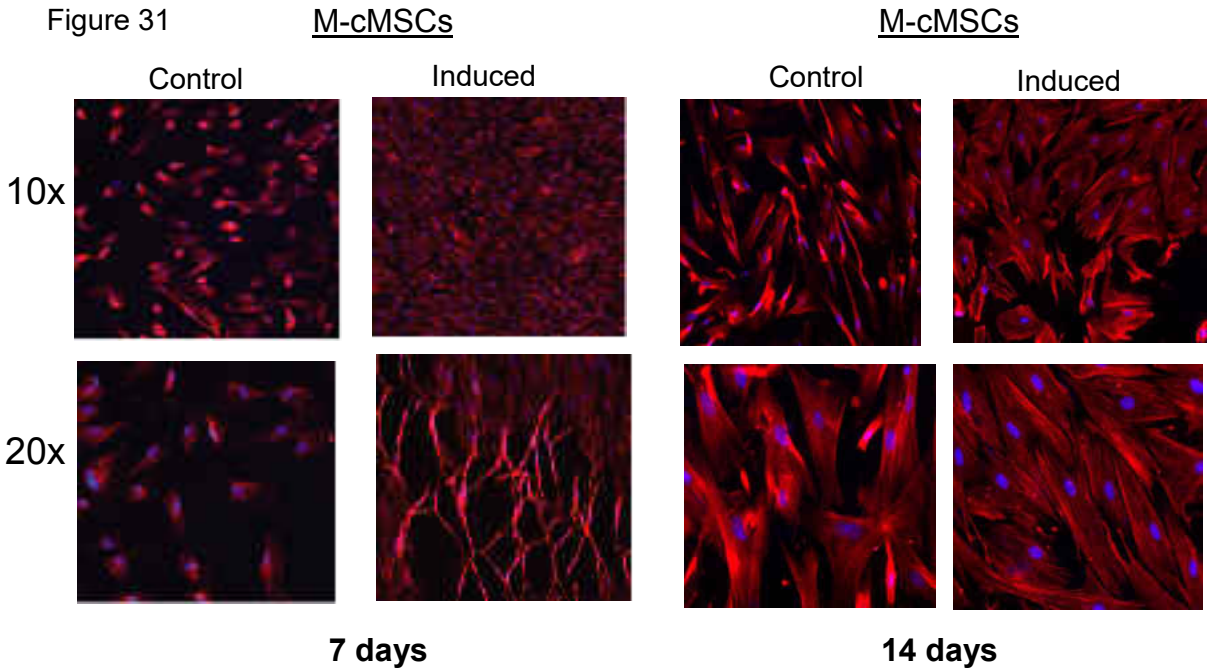


Figure 31 Neurogenesis-nestin 7 and 14 days induction: Induced M-cMSCs continued to undergo spindle-shaped morphological changes. There was similar expression of nestin by non-induced and induced cells..

Immunostaining of non-induced and induced M-cMSCs with no primary antibody (anti-nestin) included showed no expression or extreme decreased expression of nestin (Figure 32).

Figure 32

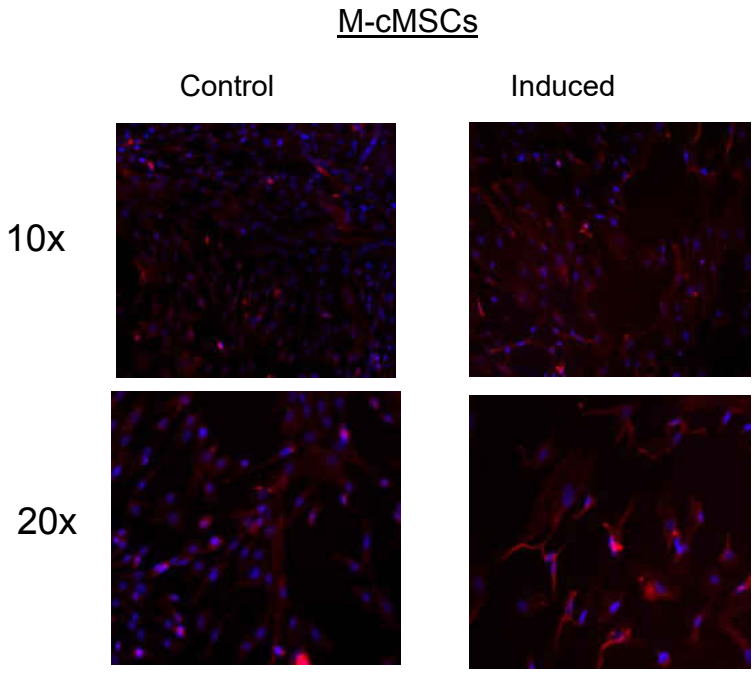


Fig. 32 Negative control of neurogenesis-nestin in non-induced and induced M-cMSCs: immunofluorescence microphotographs showing no expression or extreme decreased expression of the primary antibody anti-nestin.

Figure 33

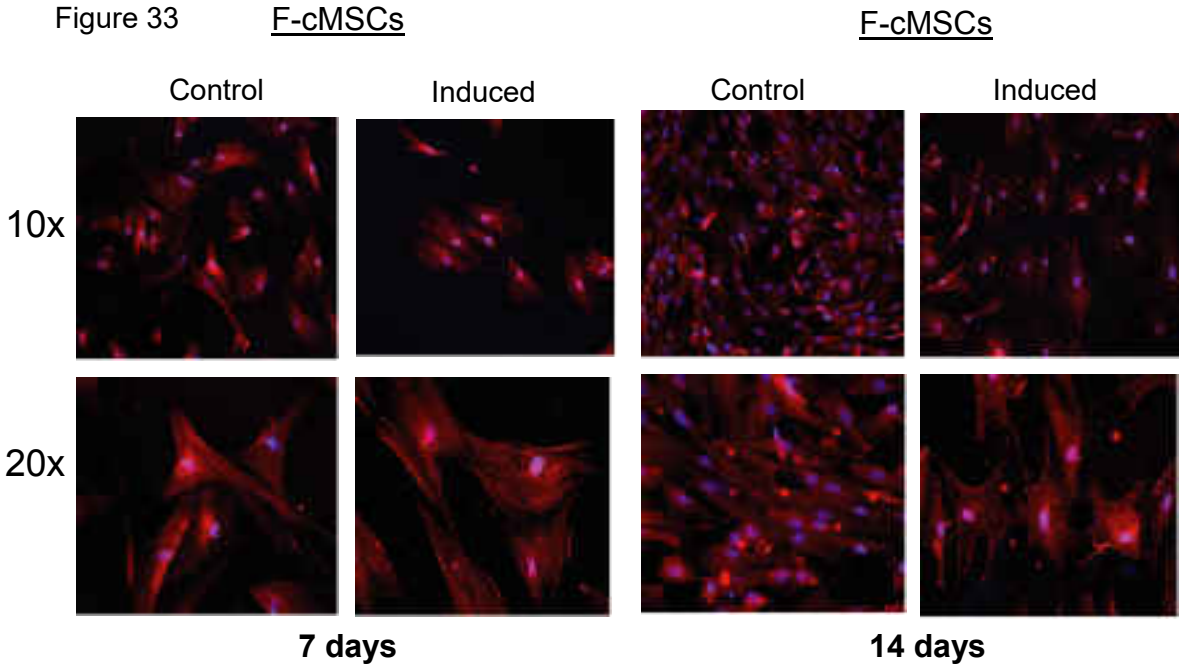


Figure 33 F-cMSCs neurogenesis βIII-tubulin 7 and 14 days induction: immunofluorescent microphotographs of F-cMSCs stained with primary antibody against βIII-tubulin showed that induced cells adopted more neural-like features than control cells. However, control and induced cells demonstrated similar expression of βIII-tubulin.

Immunostaining of non-induced and induced F-cMSCs with no primary antibody (anti- βIII-tubulin) included showed no expression or extreme decreased expression of βIII-tubulin (Figure 34).

Figure 34

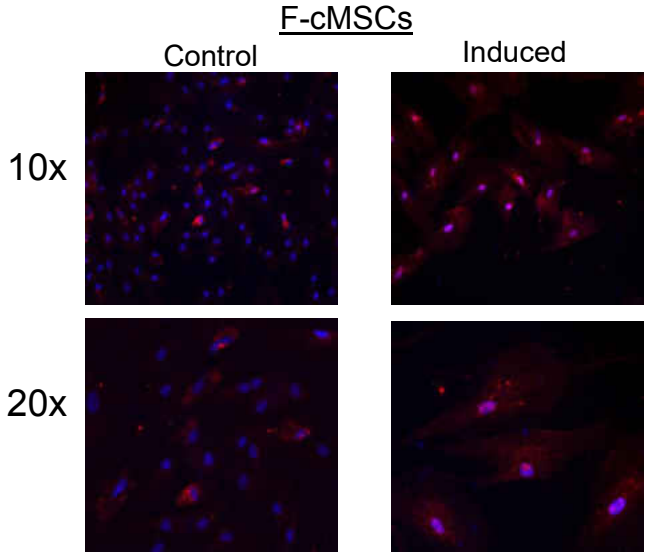


Figure 34 Negative control of neurogenesis- βIII-tubulin in non-induced and induced F-cMSCs: immunofluorescence microphotographs showing no expression or extreme decreased expression of the primary antibody anti-βIII-tubulin.

Figure 35

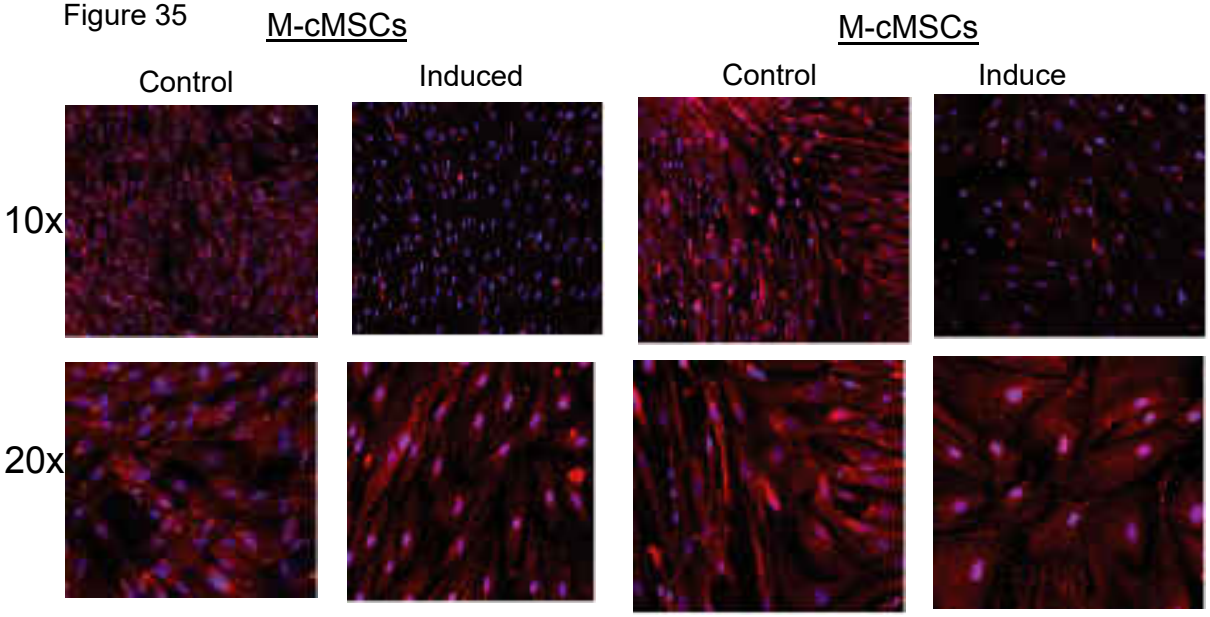


Figure 35 M-cMSCs neurogenesis βIII-tubulin 7 and 14 days induction: immunofluorescent microphotographs of M-cMSCs stained with primary antibody against βIII-tubulin showed that induced cells adopted more neural-like features than control cells. However, control and induced cells demonstrated similar expression of βIII-tubulin.

Immunostaining of non-induced and induced M-cMSCs with no primary antibody (anti- βIII-tubulin) included showed no expression or extreme decreased expression of βIII-tubulin (Figure 36).

Figure 36

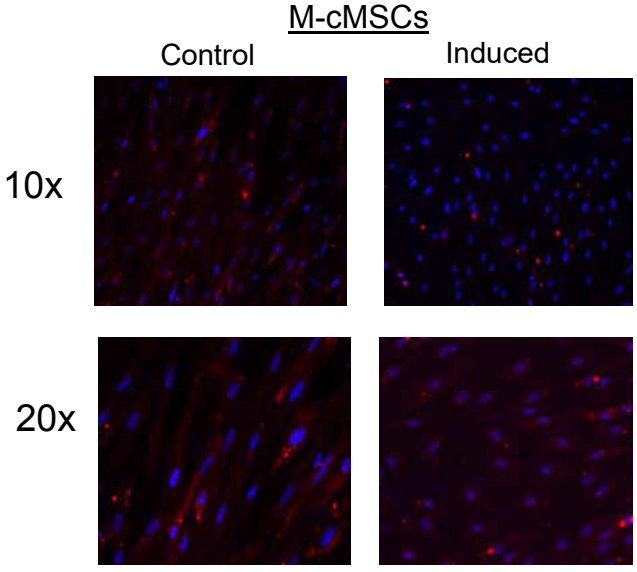


Figure 36 Negative control of neurogenesis- βIII-tubulin in non-induced and induced M-cMSCs: immunofluorescence microphotographs showing no expression or extreme decreased expression of the primary antibody anti-βIII-tubulin.

5 DISCUSSION

Safety and efficacy of new MSC-based therapies for humans must be assessed using two animal species: rodents, usually as first group, and a non-rodent large animal as second group (de Bakker *et al*, 2014). As stated, MSCs can be isolated from unconventional model organisms such as dogs, cats, goats, rabbits, cattle, sheep, horses, guinea pigs (Calloni *et al*, 2014). Based on their anatomical, pathological, biochemical and physical characteristics, dogs (Canis familiaris) are recognized to be reliable and attractive models to assess MSC-based regenerative medicine and tissue engineering. These advancements result in great benefits for translational studies in human medicine, as well as the obvious impact on cutting edge veterinary therapies (de Bakker *et al*, 2014; Volk *et al*, 2012). However, with respect to canine medicine and MSC-based therapies, there are still many unknown factors. For example, studies on ideal number of cells in transplantation and skeletal site-specific characterization of canine stem cells from the orofacial region and axial/appendicular bones have not been addressed yet.

In vitro and *in vivo* trials with cMSCs have used different tissues and anatomic regions as donor sites: adipose tissue (Kisiel *et al*, 2012; Vieira *et al*, 2010; Martinello *et al*, 2011; Neupane *et al*, 2008; Reich *et al*, 2012; Requicha *et al*, 2012), umbilical cord tissue (Seo *et al*, 2009), umbilical cord blood (Seo *et al*, 2009), bone marrow (Csaki *et al*, 2007; Kisiel *et al*, 2012, Volk *et al*, 2012, Volk *et al*, 2005, Eslaminejad and Taghiyar, 2010, Mathieu *et al*, 2009, Tharasanit *et al*,

2011), dental pulp (Dissanayaka *et al*, 2011), periodontal; ligament (Wang *et al*, 2012), amniotic fluid (Uranio *et al*, 2011,), muscle (Kisiel *et al*, 2012), and periosteum (Kisiel *et al*, 2012). Isolation and characterization of cMSCs from bone marrow have used donor sites such as long bones but rarely orofacial region. Moreover, studies have focused their attention on the osteogenic potential of cMSCs *in vitro* and *in vivo* (Kadiyala *et al*, 1997, Kang *et al*, 2012, Guercio *et al*, 2012).

Assessing site-specific differences in cMSCs is of interest, since there are no studies comparing, for instance, differentiation capacity of cMSCs between orofacial region and appendicular/axial bones. Two studies have found a significant increase in osteogenic response from bone marrow-derived human MSCs from the orofacial region compared to those harvested from the iliac crest (Akintoye et al, 2006; Osyczka *et al*, 2009). Our study tested a similar hypothesis that cMSCs are skeletally site-specific. In support of previous studies (Csaki *et al*, 2007, Kisiel *et al*, 2012, Volk *et al*, 2012); our results demonstrated that cMSCs, that were previously collected and cryopreserved, were successfully expanded in culture flasks. The cells were able to adhere to plastic surfaces, grew uniformly on monolayers and adopted a fibroblastic-like morphology. These properties, plus their multi-lineage differentiation capacity exhibited through the expression of some osteogenic markers and morphological observations, are in accordance with the two criteria established by the International Society for Cellular Therapy to characterize MSCs from animal sources (Dominici *et al*, 2006). Previous studies have determined that cMSCs can be cryopreserved and

still maintain their viability and be induced to differentiate along multiple lineages (Kraus and Kirker-Head, 2006, Zhu *et al*, 2013).

Proliferative capacity of cMSCs

Mandible cMSCs displayed higher proliferative rates than those of the femur. Mean number of mandible cells was consistently higher than femur cells at all-time points. Similar higher numbers were observed in the population doubling experiment for M-cMSCs. The increased proliferative capacities of M-cMSCs indicate more self-renewal ability than those of F-cMSCs. Unlike long bones, bones originating from the neural crest cells, such as maxilla and mandible, do not contain prominent hematopoietic components (McCauley and Somerman, 2012). This fact could explain the higher proliferation and population doubling of M-cMSCs since stromal cells of non-hematopoietic marrow divide more actively than hematopoietic cells, which are usually mitotically latent (Bianco *et al*, 1999). Furthermore, F-cMSCs underwent cellular senescence earlier than M-cMSCs. Expression of cTERT confirmed our previous findings since this enzyme was downregulated in agreement to the increasing cell passages. The use of TERT in our study was based on the knowledge that the tissue distribution of telomerase activity in dogs is similar to that in humans, where it is basically restrained to malignant cells or cells with high proliferative potential such as MSCs, and not found in normal somatic tissues (Zavlaris et *al*, 2009; Argyle and Nasir, 2003). The high specificity of the rabbit polyclonal antibody to cTERT that we used has been shown to be as high as 92% according to Zavlaris *et al* (Zavlaris et *al*, 2009) using tumor samples Similarly Akintoye *et al* (Akintoye et al, 2006) has

reported higher proliferation rate, population doubling and telomerase expression in human orofacial MSCs relative to those of iliac crest in same individuals. The higher telomerase expression of M-cMSCs also correlates with similarly higher colony forming efficiency (CFE) relative to F-cMSCs. These site-dependent differences have also been reported between cMSCs from ilium that displayed relatively higher CFE than femur or humerus (Volk *et al*, 2012).

Differentiation of cMSCs

Diverse differentiation pathways of M-cMSCs and F-cMSCs were assessed including osteogenic, adipogenic, chondrogenic and neurogenic lineages.

In vitro osteogenesis of cMSCs

The capacity of MSCs to undergo osteogenic differentiation *in vitro* is well established and they externalize markers known to be expressed by bone forming osteoblast. According to Huang *et al* (Huang *et al*, 2007) three different stages has been observed in the cell growth of osteoprogenitors *in vitro* (Figure 37)*:* (i) the first 4 days are characterized by cell proliferation where a DNA peak is observed, (ii) from day 5 to day 14, there is an early cell differentiation where the main osteoprogenitor cell marker is ALP. After this initial peak of ALP its level starts to drop. Also found at an early stage is the expression of BSP, (iii) the third stage, which occurs from day 15 to day 28, is distinguished by terminal differentiation and matrix maturation. The main markers at this stage are osteocalcin and osteopontin, followed by calcium and phosphate deposition. In general, ALP rises initially before decreasing when mineralization has far progressed; BSP is momentarily expressed at an early stage and then

upregulated again during bone formation by differentiated osteoblasts; and osteocalcin is associated with mineralization (Aubin, 2001).

Figure 37 Osteogenic differentiation in vitro
(Adapted from Huang et al, Tissue Engineering, 2007)

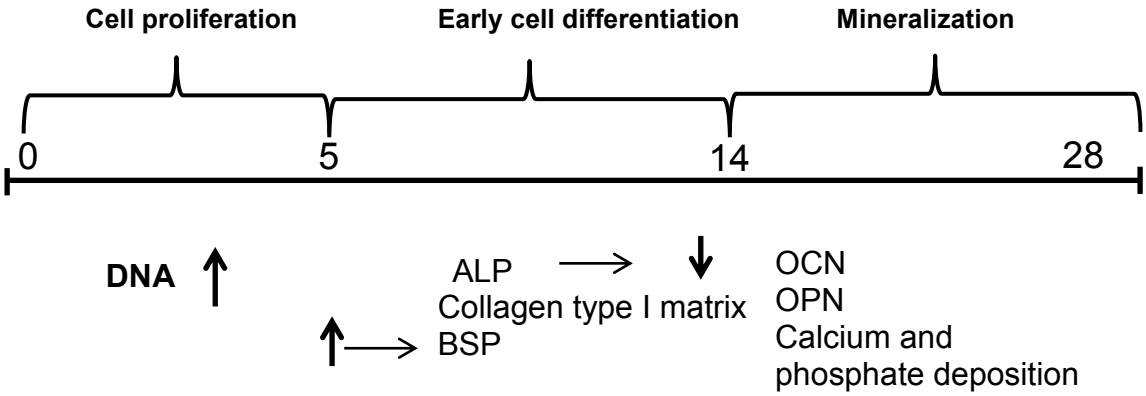


Figure 37 Osteogenic differentiation in vitro and expression of bone markers at different periods.

As ALP is one of the earliest markers of osteoblastic cell differentiation (Choi *et al*, 2011), the active expression of this marker in our study, specifically in M-cMSCs, on day 7 showed their responsiveness to the initiation of osteogenic differentiation. Additionally, on day 14, M-cMSCs displayed a statistically significant upregulation of ALP compared with F-cMSCs ($p<0.05$). As expected, both cell types displayed maximal ALP expression levels on day 7 before the slight decline on day 14. A similar trend was displayed also by BSP expression levels.

Osteocalcin is an important non-collagenous protein component of bone extracellular matrix, it is considered indicator of osteoblast differentiation, and it is produced and secreted by osteoblast in the late stage of maturation (Sila-Asna *et al*, 2007; Kaveh *et al*, 2011). Accordingly, our observations demonstrated a significant OC upregulation from M-cMSCs relative to F-cMSCs, and this increase was more pronounced at 14 days of induction of mandible cells.

There were no changes at days 7 and 14 for both types of cMSCs, and compared with the cells under non-induced media with regards to the expression of OPN. This data could be due to possible presence of intracellular OPN. It was first reported by Zohar *et al* (Zohar *et al*, 1997) in osteogenic cultures of fetal rat calvarial cells. During MSC differentiation, as intracellular OPN has an effect on the activation of mitogen-activated protein kinase (MAPK) signaling; it is possible that OPN has effect on MSCs survival and differentiation.

In vitro osteogenic differentiation of MSCs fundamentally depends on the culture conditions. Two common components of osteogenic induction medium are bone morphogenetic proteins (BMPs) and glucorticoids particularly, dexamethasone. Apparently, MSC responsiveness to osteogenic inducers is specie-specific. . According to Volk *et a*l (Volk *et al*, 2005), BMPs are effective inducers cMSC osteogenesis.. On the other hand, dexamethasone looks to have no osteoinductive effect and reduces BMP-stimulated osteogenesis. Moreover, the addition of ascorbate which promotes formation of a collagen-rich matrix, to BMP-containing medium seems to be necessary when MSCs from young dogs are used. In this study, the authors found that combination of BMP and ascorbate

resulted in a significant increase of ALP activity, whereas the combination of dexamethasone and ascorbate was unsuccessful in inducing osteogenesis. These findings are consistent with our first set of osteogenesis trials where we used medium containing dexamethasone and ascorbate and the expression of ALP and BSP were not increased in mandible and femur MSCs compared with the cells maintained under non-osteogenic conditions (data not shown). But switching to BMP-containing osteogenic medium thereafter improved osteogenic responsiveness of cMSCs..

In vivo osteogenesis of cMSCs

While *in vitro* osteogenesis of M-cMSCs was apparently higher than that of F-cMSCs, there were no site-related differences in *in vivo* bone formation by both cell type whether induced osteogenically or not. In a study by Kang et al (Kang *et al*, 2012), where osteogenic potential of cMSCs from adipose tissue, bone marrow, umbilical cord blood, and Wharton's jelly were compared, it was found that the *in vitro* osteogenic potential presented differences among the cell types without any significant differences in bone formation *in vivo*. These outcomes suggest the osteogenic potential observed *in vitro* and *in vivo* can be slightly different for each type of MSCs. This hypothesis is supported by Cho *et al* (Cho *et al*, 2010) who found discrepancies of differentiation potential between *in vitro* and in *vivo* results of differentiation in several types of MSCs.

Tissue vascularization plays a vital role in osteogenesis to support osteoprogenitor cell survival.(Kang *et al*, 2012). On the other hand, the formation of blood vessels can be induced by the initial presence of MSCs (Kaigher *et al*,

2003) because MSCs also secrete vascular endothelial growth factor (VEGF) that plays a central role in angiogenic response. Kang et al (Kang *et al*, 2012) speculated that the results obtained in the *in vivo* assay where new bone formation was similar in different types of MSCs, could be influenced by the capacity of MSCs to promote neovascularization. Furthermore, the biochemical and mechanical factors affecting the destiny of MSCs in their stem cell niche are different from those used in the *in vitro* techniques (Birmingham *et al*, 2012). While the functions of the inducers during osteogenesis *in vitro* has been elucidated (Vater *et al*, 2011), the *in vivo* biochemical environment and the driving source for the osteogenic differentiation of MSCs in their native habitat remains unclear (Birmingham *et al*, 2012).

Adipogenic differentiation

Adipogenic differentiation was confirmed by the presence of Oil Red O stained lipid vacuoles within cells cultured in adipogenic medium. These observations are consistent with previous reports of cMSCs (Vieira *et al*, 2010; Csaki *et al*, 2007, Kisiel *et al*, 2012). Cells from the mandible area seemed to have greater adipogenic potential based on a subjectively greater number of lipid clusters within the cells, compared with cells from femur. This is in contrast to the study by Akintoye *et al* (Akintoye *et al*, 2006) where human MSCs from appendicular bone such as iliac crest showed a more pronounced differentiation to adipocytes compared with cells from the orofacial area (maxilla and mandible). Other studies (Seo *et al*, 2009: Neupane *et al*, 2008) have reported the inability of isolated and cultured cMSCs, in one case from adipose tissue and from umbilical cord blood

in the other, to differentiate towards the adipogenic lineage. In the study by Neupane *et al* (Neupane *et al*, 2008), cMSCs were found to be refractory to the commonly used adipogenic induction media for human MSCs. After replacement of fetal bovine serum with rabbit serum and addition of higher glucose concentration to the medium, adipogenic differentiation was enhanced. As demonstrated by Csaki *et al (*Csaki *et al*, 2007), in our study, adipogenesis was induced with insulin, dexamethasone and 1-methyl-3-isobutylxanthine (IBMX). Some investigators (Gregoire *et al*, 1998) have proposed that although the full complement of inducing agents required for differentiation varies with each cell culture model, insulin/insulin-like growth factor I (IGF-I), cyclic adenosine monophosphate and glucocorticoids are generally considered necessary for the induction of adipogenic differentiation either in serum-containing or in serum-free media.

Chondrogenic differentiation

Our attempt at differentiating cMSCs into the chondrogenic lineage using induction medium with TGFβ-3 was considered successful. Recovery of the cell pellet culture after 4 and 8 weeks followed by Alcian blue staining revealed intense staining of a high content of cartilage specific proteoglycans. The presence of chondrocytes was noticed by pink or red staining of their respective nuclei and the cells adopting rounded shape. Similar findings were described by Csaki *et al* (Csaki *et al*, 2007), where they induced chondrogenic differentiation in bone marrow-derived MSCs. In contrast to this, control cultures showed little or no alcian blue staining. Despite these results, Kisiel *et al* (Kisiel *et al*, 2012)

reported unsuccessful attempts at differentiating cMSCs from different tissues along the chondrogenic lineage. It would be interesting to validate the expression pattern of genes associated with chondrogenic markers such as collagen type II, aggrecan, and sex-determining region Y box 9 (SOX9) to confirm differentiation of cMSCs towards chondrogenesis when morphological and histochemical results remain inconclusive (Neupane *et al*, 2008; Seo *et al*, 2012; Volk *et al*, 2012, Vieira *et al*, 2010). In our study, the addition of dexamethasone and TGFβ-3 to the chondrogenic medium played an important role in chondrogenesis: TGFβ-3 works by upregulating the expression of extracellular matrix genes (Dong *et al*, 2005), and dexamethasone, by also increasing the expression of extracellular matrix genes and/or enhancing their TGFβ-3 –mediated expression (Derfoul *et al*, 2006)

Neurogenesis differentiation

In our study, cMSCs were induced neurogenically for different time points ranging from 24 hours to 4, 7, and 14 days to assess how soon they promote formation of neuronal precursor cells. As previously reported (Kim *et al*, 2014; Jang *et al*, 2010.) we used the two-step neurogenic induction protocol: the first step was preinduction with fetal bovine serum and β-fibroblast growth factor (β-FGF); the second step was induction phase with medium supplemented with butylated hydroxyanisole (BHA), forskolin, valproic acid, and insulin. The purpose of these two steps is to decrease environmental damage to cells after adding supplemented medium for neuronal induction (Kim *et al*, 2014). Previous studies have used numerous reagents to differentiate neural precursor cells such as

dibutyryl-cyclic adenosine monophosphate (db-cAMP), 3-isobutyl-1-methylxanthine (IBMX), and retinoic acid (RA) (Tio *et al*, 2010); a cocktail of IBMX, indomethacin, and insulin (Fujimura *et al*, 2005); neural growth factor (Kamishina *et al*, 2008), and β-FGF and forskolin (Jang *et al*, 2010). IBMX and db-cAMP upregulate intracellular cAMP levels, which possibly activate protein kinase A (PKA). Wang *et al* (Wang *et al*, 2007) stated that PKA mediates neural differentiation of human cord blood-derived MSCs. β-FGF has a substantial capacity for neuronal differentiation by producing neuronal precursor cells. Forskolin is a regularly used agent to upregulate the intracellular levels of cAMP, which eventually activates the protein kinase A (PKA) signaling pathway. Additionally, forskolin induces the neuron-like morphology and expression of some neural specific genes in human MSCs (Jang *et al*, 2010).

The observed neuronal-like morphological changes in the induced cMSCs were consistent with previous studies on cMSCs (Oda *et al*, 2013; Kamishina *et al*, 2006), umbilical cord blood and amniotic membrane-derived cMSCs (Seo *et al*, 2009-Q; Park *et al*, 2012). In fact, Kamishina et al (Kamishina *et al*, 2006) reported that cMSCs had neuron-like morphologic characteristics as early as 3 hours after the induction of neural differentiation. The interpretation of the significance of these *in vitro* neuronal changes should not be overestimated as previously mentioned by other researchers (Lu *et al*, 2004; Neuhiber *et al*, 2004),. These investigators have expressed that the morphological and immunocytochemical changes observed after neuronal induction could be the result of cytotoxic effects of the reagents in the induction medium, which leads to

cell shrinkage and actin cytoskeleton retraction. Also, these changes might be a response to chemical stress, because similar cellular modifications have been observed in the presence of Triton X-100 or sodium hydroxide (Deng *et al*, 2006). We also analyzed the neural-specific proteins nestin and βIII-tubulin by immunostaining and found that both non-induced and induced cMSCs expressed these neuron-specific markers. The spontaneous expression of these neural-specific proteins by cMSCs, under normal culture conditions has also been previously reported. Deng *et al* (Deng *et al*, 2006) found that nearly 100% of mice MSCs cultures spontaneously expressed the intermediate filament protein nestin, In addition, the cells in their study were also positive for several neuron-specific proteins, including βIII-tubulin and medium weight neurofilament (NFM), but negative for the astrocyte-specific glial fibrillary acidic protein (GFAP) and vimentin. Kamishina *et al* (Kamishina *et al*, 2006), studying neuronal differentiation of cMSCs from iliac crest bone marrow, found that immunocytochemical and western blot analyses revealed that untreated cMSCs strongly expressed βIII-tubulin and GFAP. The authors concluded that, if cMSCs are positive for βIII-tubulin, they probably have inherent potential to differentiate into neuronal cells under appropriate conditions.

The spontaneous attainment of neural properties by non-induced MSCs. may be explained by the neural differentiation propensity of stem cell reflected in the development of the nervous system during embryogenesis. Undetermined ectoderm cells differentiate into neural lineage by default unless inhibited by ventralizing factors, such as bone morphogenetic protein-4 (BMP4) (Wilson and

Hemmati-Brivanlou 1995). Therefore, it is likely that MSCs, as multipotent stem cells, may exhibit a neural property in their default state of differentiation *in vitro*, where there are no pro-mesoderm inhibitors such as BMP4 (Deng *et al*, 2006). Since our studies show inconsistencies in site-specific neuronal differentiation of cMSCs, it will be more informative to examine this further at the genetic level and with longer induction periods.

6 CONCLUSIONS

Dogs offer not only a valuable experimental model but also represent a clinically relevant and superior animal model compared with other organisms. Previous studies have successfully isolated cMSCs from different tissues, and *in vitro* differentiation capacities have also been reported. Undifferentiated cMSCs have been characterized morphologically, immunophenotypically, and by their gene expression. However, in marked contrast with human MSCs, basic biology of cMSCs is yet to be fully elucidated, and so far no uniform characterization criteria are available for MSCs from canine origin. Only a limited number of trials have attempted to identify a panel of cell surface markers and transcription factor profiles for these stem cells. While the current study tested cMSCs from a restricted number of subjects, it enhanced our understanding of cMSCs and their skeletal site-specific characteristics. Our results demonstrated that cryopreserved cMSCs could be expanded and differentiated, *in vitro,* at least into the three main differentiation lineages: osteogenic, adipogenic, and chondrogenic, as well as neurogenic. In addition, the impressive osteogenic potential of cMSCs, in this

study also showed that M-cMSCs are apparently more responsive to multi-lineage differentiation relative to F-cMSCs. These are consistent with data from studies using human, mouse and rat MSCs (Akintoye *et al*, 2006; Yoshimura *et al*, 2007; Aghaloo *et al*, 2010; Lee *et al*, 2011).

One prospective future direction is to confirm these results by using a larger population of MSC donors; therefore, the inter-animal variability would be minimized. Since dog breeds exhibit an extremely wide range of body types, it would be interesting to research MSCs from different canine breeds. Another avenue of investigation of cMSCs would be the refinement of *in vitro* expansion strategies as well as detailed comprehension of donor characteristics. Future pre-clinical and clinical studies regarding cMSCs is definitely required not only to motivate, but also to appropriately translate the potential therapeutic use of these cells in both veterinary and human medicine.

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