



2-2017

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Recommended Citation

Bugueno, J., Li, W., Salat, P., Qin, L., & Akintoye, S. O. (2017). Canine Mesenchymal Stem Cell Bone Regenerative Capacity is Regulated by Site-Specific Multi-Lineage Differentiation. *Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology*, 123 (2), 163-172. <http://dx.doi.org/10.1016/j.oooo.2016.09.011>

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Canine Mesenchymal Stem Cell Bone Regenerative Capacity is Regulated by Site-Specific Multi-Lineage Differentiation

Abstract

Objectives

Mesenchymal stem cells (MSCs) are promising therapies in dentistry due to their multipotent properties. Selecting donor MSCs is crucial because beagle dogs (canines) commonly used in pre-clinical studies have shown variable outcomes and it is unclear whether canine MSCs (cMSCs) are skeletal site-specific. This study tested whether jaw and long bone cMSCs have disparate *in vitro* and *in vivo* multilineage differentiation capabilities.

Study Design

Primary cMSCs were isolated from mandible (M-cMSCs) and femur (F-cMSCs) of four healthy Beagle dogs. Femur served as non-oral control. Clonogenic and proliferative abilities were assessed. *In vitro* osteogenic, chondrogenic, adipogenic and neural multilineage differentiation were correlated with *in vivo* bone regeneration and potential for clinical applications.

Results

M-cMSCs displayed two-fold increase in clonogenic and proliferative capacities relative to F-cMSCs ($p = 0.006$). M-cMSCs *in vitro* osteogenesis based on alkaline phosphatase ($p = 0.04$), bone sialoprotein ($p = 0.05$), and osteocalcin ($p = 0.03$), as well as adipogenesis ($p = 0.007$), and chondrogenesis ($p = 0.009$) were relatively higher and correlated with enhanced M-cMSC bone regenerative capacity. Neural expression markers, nestin and β III-tubulin were not significantly different.

Conclusions

The enhanced differentiation and bone regenerative capacity of mandible MSCs may make them favorable donor graft materials for site-specific jaw bone regeneration.

Keywords

Jaw bone, mesenchymal stem cells, tissue regeneration, dogs

Disciplines

Dentistry



HHS Public Access

Author manuscript

Oral Surg Oral Med Oral Pathol Oral Radiol. Author manuscript; available in PMC 2018 February 01.

Published in final edited form as:

Oral Surg Oral Med Oral Pathol Oral Radiol. 2017 February ; 123(2): 163–172. doi:10.1016/j.oooo.2016.09.011.

Canine mesenchymal stem cell bone regenerative capacity is regulated by site-specific multi-lineage differentiation

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Abstract

Objectives—Mesenchymal stem cells (MSCs) are promising therapies in dentistry due to their multipotent properties. Selecting donor MSCs is crucial because beagle dogs (canines) commonly used in pre-clinical studies have shown variable outcomes and it is unclear whether canine MSCs (cMSCs) are skeletal site-specific. This study tested whether jaw and long bone cMSCs have disparate *in vitro* and *in vivo* multilineage differentiation capabilities.

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Conflicts of Interest statement:

The authors have no conflicts of interest to declare.

Keywords

Jaw bone; mesenchymal stem cells; tissue regeneration; dogs

Introduction

Careful selection of donor tissue for oral bone regeneration is vital for successful graft therapy. Translational studies aimed at improving clinical outcomes commonly use beagle dogs as experimental animals for tissue transplant studies because of their docile nature¹⁻³. Selecting optimum donor graft material for orofacial bone regeneration is still a challenge that causes unpredictable clinical outcomes^{4,5}. While different donor graft materials have been tested, the modulatory effects induced by skeletal site-specific multilineage differentiation capabilities of jaw-specific orofacial mesenchymal stem cells (MSCs) are yet to be fully clarified. MSCs are a unique population of multipotent postnatal stem cells that can be isolated from different tissues.⁶ MSCs have the ability to form multiple tissue types such as bone, cartilage, muscle, nerve, tendon and fat. However, they also show significant differences in *ex vivo* expansion potential and functions based on age and skeletal-site of origin.⁷⁻⁹ Current pre-clinical applications of MSCs have focused extensively on human, mouse and rat MSCs although MSCs have also been isolated from unconventional animal models like dogs, pigs, cats, sheep and goat.¹⁰ Interestingly, phenotypic and functional skeletal site-disparities have been reported in human and murine MSCs.^{7,10-12} These previous studies demonstrated that orofacial MSCs isolated from the jaw display superior osteogenic capacities relative to those isolated from the hip and long bones. The MSC functional site disparities were alluded to evolutionary adaptations at each skeletal site and neuro-ectodermal developmental origin of jaw bones that is distinct from mesodermal origin of spine and hip bones.^{7,11} How these modulate oral bone regeneration is yet to be fully elucidated.

The skeletal site-specific functional differences of MSCs are not limited to humans and rodents as other animal models such as dogs may display similar site-disparity. Also, it is unclear if dog (canine) MSCs inherently display skeletal site-specific functional differences. Although beagle dogs have been used in pre-clinical studies to model oral bone loss or regeneration, the effects of jaw-specific properties of cMSCs have not been clearly defined. This study tested the hypothesis that canine MSCs from the jaw and long bones have disparate *in vitro* and *in vivo* multilineage differentiation capabilities. It is expected that further understanding of underlying biological and genetic differences would enhance MSC-based donor graft selection for bone regeneration.¹³⁻¹⁶

Materials and Methods

Isolation and culture of canine mesenchymal stem cells

Freshly isolated trabecular bone samples were obtained from both the mandible and femur of four female 3-week-old normal healthy beagle dogs from an in-house breeding colony cared for according to National Institutes of Health (NIH) and United States Department of Agriculture (USDA) guidelines of the care and use of research animals. The Institutional

Animal Care and Use Committee of University of Pennsylvania Office of Regulatory Affairs approved all animal protocols. Primary culture of canine mesenchymal stem cells (cMSCs) were established in culture from the mandible and femur as previously described⁷ using α -modified Minimum Essential Medium (α -MEM, Life Technologies, Grand Island NY) supplemented with 20% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 100 U/ml penicillin, 100 mg/ml streptomycin sulfate and 2 mM glutamine. The culture was maintained at 37°C humidified atmosphere of 5% CO₂ and air. Primary cMSCs isolated from the mandible (M-cMSCs) and femur (F-cMSCs) were further sub-cultured, expanded and stored in liquid nitrogen until used. The cMSCs used for all experiments were within passages 2 to 5. M-cMSCs and F-cMSCs for each individual animal were tested together.

Colony forming efficiency and survival of cMSCs

Colony forming efficiency based on colony forming units-fibroblasts (CFU-F) was assessed as previously described by seeding 10¹, 10² and 10³ passage 2 F-cMSCs and M-cMSCs in triplicate 25cm² plastic culture flasks.^{7, 16} At 14 days, the cells were fixed in 100% methanol, stained with methyl violet and colonies of 50 or more cell aggregates representing CFU-Fs were counted. Cell proliferation was assessed based on growth curve analysis of cMSCs plated in 6-well plates at 9.5 × 10³ cells/cm². The cells were trypsinized and counted on days 1, 3, 6, 9, 12, and 15 to plot a growth curve. Proliferation was analyzed using non-linear regression curve fitting of surviving F-cMSCs and M-cMSCs (GraphPad Prism v6 (GraphPad Software Inc. La Jolla CA)).

Long-term survival of cMSCs was assessed by population doublings (PD) as previously described.⁷ Both cMSCs types were plated in T-25 flasks at 1 × 10⁶ cells/flask, and PD was calculated based on cell number after repeated cell passage at 1:10 split ratio until the cells attained replicative senescence. Nuclear extracts at each serial passage were isolated with Nuclei EZ Prep® (Cat # NUC-101, Sigma-Aldrich, St Louis, MO) following the manufacturer's protocol. Telomerase activity was determined by western blotting of equal protein amounts immunoreacted with rabbit anti-cTERT primary antibody (1: 1000 dilution, Cat # NB110-89471, Novus Biologicals, Littleton, CO). Rabbit anti- β -actin (1:2000) (Cat # 4967, Cell Signaling Technology, Danvers MA) served as loading control. Bound antibodies were detected with horseradish peroxidase linked donkey anti-rabbit IgG as secondary antibody (1: 2000 dilution, Cat # NA934V, GE Healthcare Life Sciences, Piscataway, NJ). Immunoreactive bands were digitized and analyzed with ImageJ v1.49g (National Institutes of Health, Bethesda MD).

In vitro osteogenic differentiation

Osteogenic differentiation was performed as previously described.^{15, 16} F-cMSCs and M-cMSCs were cultured at 1 × 10⁴ cells/cm² in ten 60mm dishes (Corning Life Sciences, Tewksbury, MA). Five of the dishes were pre-coated with poly-L-lysine (Sigma-Aldrich) to enhance plastic adherence under long-term culture. At confluence, the cMSCs seeded in coated dishes were switched to α -MEM medium supplemented with 100 ng/ml of human bone morphogenetic protein-2 (BMP-2, GenScript, Piscataway, NJ, USA) and 100 μ M L-Ascorbic acid 2-phosphate (10⁻⁴ M). Medium was changed twice weekly for 7 and 14 days after which protein lysate and RNA were collected in parallel experimental culture dishes.

Total protein was determined using the bicinchoninic acid protein assay (Pierce™ BCA Protein Assay Kit, Thermo Scientific, Rockford IL). Equal protein amount was loaded on a 4 – 20% gradient gel, transferred to nitrocellulose membrane for western blotting and probed with the following primary antibodies: rabbit anti-bone sialoprotein (BSP) polyclonal antibody (Bioss Inc. Woburn MA) at 1:200; rabbit anti-osteocalcin (OCN) polyclonal antibody (Bioss Inc. Woburn MA) at 1:200, and rabbit anti-alkaline phosphatase (ALP) antibody (Novus Biologicals) at 1:800. Either anti-β-actin (1:1000) or anti-α-tubulin (1:200) served as loading control. Secondary antibodies included anti-mouse or anti-rabbit antibodies at concentrations ranging from 1:1000 – 1:3000. Immunoreactive bands were digitized followed by quantification with ImageJ v1.49g (National Institutes of Health, Bethesda MD). Real time PCR was performed with ABI 7300 Real-Time PCR System using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) as previously described.¹⁷ Total RNA was isolated and first strand cDNA was prepared using custom designed primers that include: canine BSP (forward primer 5'-TTG CTC AGC ATT TTG GGA ATG G-3'; reverse primer 5'-AAC GTG GCC GAT ACT TAA AGA CC-3'); canine OCN (forward primer 5'-CTG GTC CAG CAG ATG CAA AG-3'; reverse primer 5'-CCG CTT GGA CAC GAA GGT T-3'); and canine ALP (forward primer 5'-TTC AAA CCG AGA CAC AAG CAC T-3'; reverse primer 5'-GGG TCA GTC ACG TTG TTC CTG T-3'). Gene expression levels were normalized to the housekeeping gene: canine β2 microglubulin (forward primer 5'-TCA CGA CAC CCA GCA GAG AA-3'; reverse primer 5'-GGA ACC CTG ACA CGT AGC AGT T-3').

***In vivo* osteogenic differentiation**

Bone regenerative capacity of F-cMSCs and M-cMSCs was assessed by transplantation of 1×10^6 cells attached to hydroxyapatite-tricalcium phosphate (HA/TCP, Zimmer Inc. Warsaw, IN) into the subcutis of 6-week old immunocompromised mice (NIH-III NU, Charles River Laboratories, Wilmington, MA) as previously described.⁷ At 12 weeks, transplants were harvested, fixed in 4% paraformaldehyde, decalcified in 10% EDTA (pH 8.0) and paraffin-embedded for histological analysis. Images were captured with Nikon Eclipse80i fluorescent microscope (Nikon Instruments, Melville, NJ) and bone regeneration was assessed using an established semi-quantitative bone scoring system.⁷

Adipogenic differentiation

F-cMSCs and M-cMSCs were cultured at 1.8×10^3 cells/cm² in 4-well chamber slides (Coming Life Sciences, Acton, MA) and at confluence, adipogenic differentiation was induced as previously described^{7, 16} using adipogenic differentiation medium composed of α-MEM supplemented with 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 (10%) for 15 days. Control cells were not induced with adipogenic medium. Medium was refreshed twice weekly. At day 15, the cells were fixed with 4% paraformaldehyde, stained with 0.3% Oil Red O and counterstained with 1% Fast green dye. Lipid laden cells were evaluated and quantified microscopically.

Chondrogenesis

F-cMSCs and M-cMSCs were cultured in 75 cm² flasks at 75×10^4 cells/cm² until 80–90% confluent. Subsequently, 2×10^6 cells were transferred and pelleted in a 15 ml polypropylene tube as previously described.^{16, 18, 19} The pelleted cells were induced with chondrogenic medium consisting of α -MEM supplemented with 10^{-8} M dexamethasone, 1% ITS⁺, 10^{-4} M L-Ascorbic acid 2-phosphate, 10 ng/ml transforming growth factor-beta 3, 10 mM β -glycerophosphate, 2 mM, glutamine, 100 U/ml penicillin-streptomycin sulfate, 2 mM pyruvate and medium change every 3 days. Pelleted control cells were exposed to α -MEM without chondrogenic inducers. The pellets were harvested after 4 and 8 weeks, fixed with 4% paraformaldehyde, and paraffin-embedded 5 μ m sections were stained with Alcian blue plus counterstain of nuclear fast red for histological analysis.

Neural differentiation

Collagen coated 8-well chamber slides (Corning® BioCoat™, Corning Life Sciences, Tewksbury, MA) were seeded with F-cMSCs and M-cMSCs at 4×10^3 cells/cm² using α -MEM growth medium until confluent. Control cells were continuously maintained in the same growth medium, but the neurogenically induced cMSCs were switched to a pre-induction medium consisting of α -MEM fortified with 10 ng/ml β -fibroblast growth factor (β -FGF, BD Biosciences, San Jose CA). After 24 hours, the pre-induction medium was switched to neuronal induction medium consisting of α -MEM supplemented with 20% fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin sulfate, 2 mM glutamine, 2% dimethyl sulfoxide, 10 ng/ml β -FGF, 200 μ M butylated hydroxyanisole, 10 μ M forskolin, 25 mM KCl, 2 mM valproic acid and 5 μ g/mL insulin. Neural differentiation was evaluated at 7 and 14 days by immunofluorescent staining using primary antibodies of rabbit anti-nestin (1:200) (LifeSpan BioSciences, Inc. Seattle WA) and rabbit anti- β III-tubulin (1:200) (Bioss, Woburn MA) as primary antibodies. Alexa Fluor® 555 Goat Anti-Rabbit IgG (Life Technologies, Grand Island NY) served as secondary antibody and nuclei were stained with 1 μ g/ml of Hoechst 33342. Images were captured with Nikon Eclipse80i fluorescent microscope (Nikon Instruments, Melville, NJ).

Statistical analysis

Each cell type was plated in triplicates with appropriate controls. Each experiment was performed independently and repeated at least three times. Results were expressed as mean \pm standard deviation. Effects of differentiation media were presented as fold-change relative to control un-induced cMSCs. Although M-cMSCs and F-cMSCs for each individual animal were tested together, data from the animals (n = 4) were pooled for statistical analysis performed with GraphPad Prism v6 (GraphPad Software Inc. La Jolla CA). Comparative analysis of the animals' F-cMSCs and M-cMSCs differential responses was performed by two-way analysis of variance (ANOVA) followed by post-hoc comparisons with Holm-Sidak test and statistical significance was set at $p < 0.05$.

Results

We isolated cMSCs from the mandible and femur trabecular bone samples obtained from beagle dogs commonly used for translational studies¹⁵ The monolayer of primary F-cMSCs

and M-cMSCs demonstrated heterogeneous fibroblast-like morphological shapes and sizes characteristic of MSCs (data not shown). M-cMSCs displayed significantly higher time-dependent proliferation ($p = 0.006$) that peaked on day 12 compared with F-MSC proliferation that peaked on day 9 (Figure 1A). The colony forming capacities based on CFU-Fs were not different between the two cell types (Figure 1B), however, population doubling showed there were more doubling M-cMSCs at early passages than F-cMSCs (Figure 1C). The F-cMSCs also underwent complete senescence after passage 6 unlike M-cMSCs that displayed gradual senescence up to passage 12. The apparently delayed senescence of M-cMSCs was supported by higher expression levels of cTERT (Figure 1D and E).

Analysis of osteogenic proteins that include ALP, BSP, and OCN by western blotting at 14 days post-induction (Figures 2) showed strong immunoreactivity suggestive of a strong osteogenic responsiveness of cMSCs from both skeletal sites. The amounts of ALP (Figures 2C), BSP (Figures 2D) and OCN (Figure 2E) were disproportionately higher in M-cMSCs relative to F-cMSCs (ALP, $p = 0.04$; BSP, $p = 0.05$ and OCN, $p = 0.03$). At the mRNA level, the M-cMSC transcripts of osteogenic markers ALP ($p < 0.001$), BSP ($p < 0.001$) and OCN ($p < 0.001$) (Figures 2F – H respectively) were even much more upregulated relative to F-cMSCs. Analysis of *in vivo* bone regenerative ability of F-cMSCs (Figure 3A, B, E) and M-cMSCs (Figures 3C, D, F) was assessed semi-quantitatively on a scale of 0 to 4 based on a previously established scoring system.⁷ This showed that M-cMSCs can regenerate appreciably more bone quantitatively than F-cMSCs with or without the addition of osteogenic inducers (Figures 3E and F). Hence, the F-cMSCs needed osteogenic induction to regenerate the similar quantitatively appreciable bone (Figure 3E, $p < 0.05$) as unstimulated M-cMSCs. Also, exposure of F-cMSCs to osteogenic medium induced formation of marrow components (hematopoiesis and adipocytes, Figure 3B) while bone formed by M-cMSCs were within a fibrous tissue bed (Figure 3D).

After induction with adipogenic medium, the M-cMSCs responded by displaying more lipid-laden cytoplasmic contents ($p = 0.007$) based on Oil-Red O staining (Figure 4A – E). Similarly, assessment of chondrogenesis by the pellet culture method clearly indicated that M-cMSCs were more responsive to chondrogenic stimulation ($p = 0.009$) based on Alcian blue staining of chondrocyte-like cells (Figure 4F – J). After exposure to neuronal differentiation medium, both F-cMSCs (Figures 5A and C) and M-cMSCs (Figures 5B and D) displayed strong immunoreactivity to anti-nestin (at 7 days) and anti- β -III tubulin (at 14 days) without appreciable differences between the two cell types. Nestin immunoreactivity (Figure 5A, B) revealed that the cells adopted spindle shaped morphology with stretched-out dendrite-like cytoplasmic projections.

Discussion

MSCs have been isolated from bone and other tissues such as canine adipose tissue,^{20–25} umbilical cord blood and tissue,²⁶ dental pulp,²⁷ periodontal ligament,²⁸ amniotic fluid,²⁹ muscle,²⁰ and periosteum.²⁰ However, direct comparison of two different skeletal sites in dogs and clinical implications have not been conclusively evaluated. We used plastic adherence method to successfully isolate a population of cMSCs from mandible and femur

of the same dogs³⁰. Although cMSCs isolated from both sites displayed heterogeneous fibroblast-like morphology, cell surface labeling and flow cytometric analysis were not carried out due to limited starting tissue samples from each animal.³¹ To expand MSCs for clinical applications, clonogenic capacity is a common MSC characteristic that affects multilineage differentiation. We found no significant clonogenic differences between F-cMSCs and M-cMSCs unlike previously reported human MSC studies,⁷. However, M-cMSCs displayed higher survival and population doubling properties that were associated with a more sustained telomerase expression consistent with previous reports on both human and murine OFMSCs.^{32,33}

The case for use of M-cMSC as viable donor graft for oral bone regeneration is strongly supported by the fact that M-cMSCs differentiated much more readily into osteogenic, chondrogenic and adipogenic lineages than F-cMSCs in spite of similar neuronal differentiation. Additionally, osteogenesis of M-cMSCs appeared to be higher than that of F-cMSCs based on protein levels and transcripts of osteogenic markers ALP, BSP, and OCN.³⁴

Osteoresponsiveness of cMSCs was also better activated when dexamethasone/ascorbate combination in the osteoinductive medium was replaced with combination of BMP-2/ascorbate. This is in line with previous report that the combination of BMP/ascorbate effectively induces alkaline phosphatase in MSCs isolated from young dogs.¹⁵ A strong *in vitro* osteogenesis of human OFMSCs has also been shown to translate into high *in vivo* bone regenerative capacity.^{7, 11} Similarly, *in vivo* bone regenerative capacity of M-cMSCs was slightly enhanced with or without stimulation. This indicates that M-cMSCs are inherently osteogenic without the need for pre-induction, a factor that favors their use as donor grafts for oral bone regeneration.^{15, 16, 35} The fact that *in vivo* bone regenerated by transplanted F-cMSCs displayed similar histological features of hematopoiesis and adipogenesis as normal femur bone further point to the site-specificity of MSCs and added functional demand on these cells.^{7, 11} This also emphasizes that based on functional demand, MSCs formed bone similar to their site of harvest which makes M-cMSCs more favorable for oral bone regeneration

Both adipogenesis and chondrogenesis were higher in M-cMSCs relative to F-cMSCs in sharp contrast to higher adipogenic differentiation of human iliac crest MSCs relative to mandible and maxilla MSCs previously reported.⁷ Since chondrogenesis was not addressed in the earlier studies^{7, 11, 36} follow up studies on site-dependent expression levels of genetic markers of adipogenesis and chondrogenesis should give further insights into cMSC site-selectivity.^{16, 22, 25, 26}

A higher propensity of M-cMSCs for neuronal differentiation would have been logical since mandible bone in which the M-cMSCs reside develops embryologically from neuroectoderm, but there were no differences in the neuronal differentiation properties of F-cMSCs and M-cMSCs. This is an indication that some similarities also exist between the two cell types. MSC neuronal morphological changes can be confused with cellular changes in response to chemical stress and cytotoxic effects of the induction medium.³⁷ To minimize this confounding effect, we used the two-step neuronal induction protocols that included

pre-induction initially with β -fibroblast growth factor (β -FGF) to minimize MSC damage by chemical stress.^{38,39} Additionally, we confirmed the neuronal differentiation based on positive immunoreactivity with nestin and β III-tubulin, two known markers of neurogenesis. As rodent MSCs have been shown to spontaneously express nestin;^{40,41} it was not surprising that non-induced cMSCs also displayed some degree of immunoreactivity to these markers (data not shown).

Several factors make a case for assessing skeletal site-specificity of MSCs and their species-to-species differences. The unique neuroectodermal origin of orofacial bones, and intramembranous ossification pattern of mandible coupled with endochondral contributions from Meckel's, coronoid and condylar cartilages make the jaw bones developmentally different.⁴² Furthermore, several bone pathologies display unique radiological and histological features in the jaw. These include fibrous dysplasia of bone,⁴³ cherubism⁴⁴ and hyperparathyroid jaw tumor syndrome.⁴⁵ Additionally, long-term use of bone antiresorptives is often complicated by jaw osteonecrosis while non-oral bones are spared.⁴⁶ Since dogs readily develop jaw osteoradionecrosis,^{47,48} it is not unlikely that they may be susceptible to the same pathological features as humans because dogs are exposed to similar external and environmental factors as humans.

The results presented in this study have some limitations and therefore represents a pilot analysis. Firstly, the cMSCs characterized were isolated from a convenient sample of healthy dogs in an unrelated research project; secondly, the number of tissue samples was limited; and thirdly, a single breed of dogs was evaluated. Therefore, accessibility to cMSCs from a large number of study samples from different breeds of dogs will shed more light on the site-specificity of MSCs characteristics in dogs. Although, the actions of the components of the *in vitro* osteogenesis-inducing medium have been well defined,⁴⁹ it is still unclear if these really recapitulate the native environment that promotes *in vivo* osteogenesis.⁵⁰ For example, the MSC/HATCP grafted in the subcutis of immunocompromised hosts purportedly promotes secretion of vascular endothelial growth factor that induces formation of vasculature, which in turn invades the graft. While tissue vascularization is important for MSC survival and subsequent osteogenesis, these sequences of events are yet to be clearly defined.^{13,51}

In spite of the study limitations, these results are consistent with data from studies focused on human, mouse and rat MSCs.^{7,11,36,52} They enhance our understanding of cMSCs including skeletal site-specificity of MSC in general. Our data demonstrated that cryopreserved cMSCs could be expanded and differentiated. They also showed that M-cMSCs are relatively more responsive to multi-lineage differentiation than F-cMSCs and represent superior donor graft materials for oral bone regeneration.

Acknowledgments

Funding Support:

This study was supported in part by grants # K22CA169089 (awarded to SOA), R21DE022826 (awarded to SOA) and R01DK095803 (awarded to LQ) by United States Department of Health and Human Services/National Institutes of Health, Bethesda MD.

The authors thank Dr. Mark Haskins, University of Pennsylvania School of Veterinary Medicine for provision of tissue samples from the dog genetic colony at University of Pennsylvania

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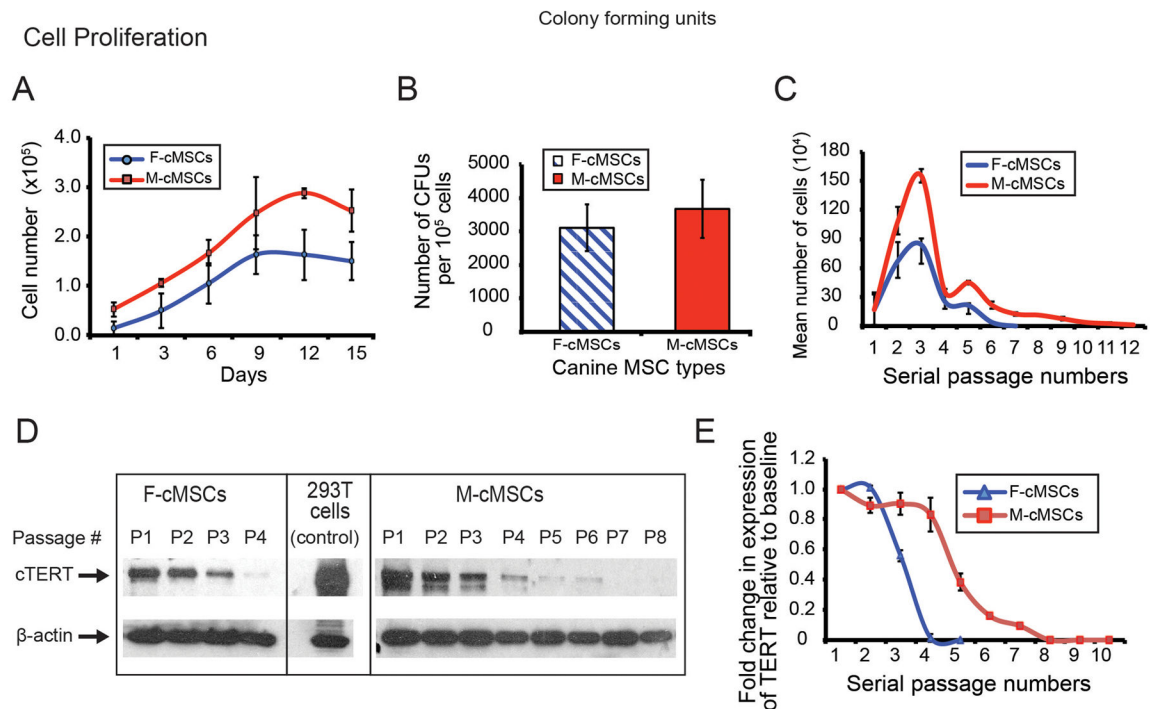


Figure 1. Proliferation and survival of canine MSCs

M-cMSCs display significantly higher proliferation ($p=0.006$) before peaking on day 12 compared with F-cMSCs that peaked on day 9 (A). The colony forming capacities based on CFU-Fs were not different between the two cell types (B), population doubling capacity of M-cMSCs showed there were more doubling cells in the early passages (C). Also, F-cMSCs underwent complete senescence after passage 6 unlike M-cMSCs that did not completely senesce until passage 12. The delayed senescence of M-cMSCs was supported by higher expression levels of cTERT (D and E).

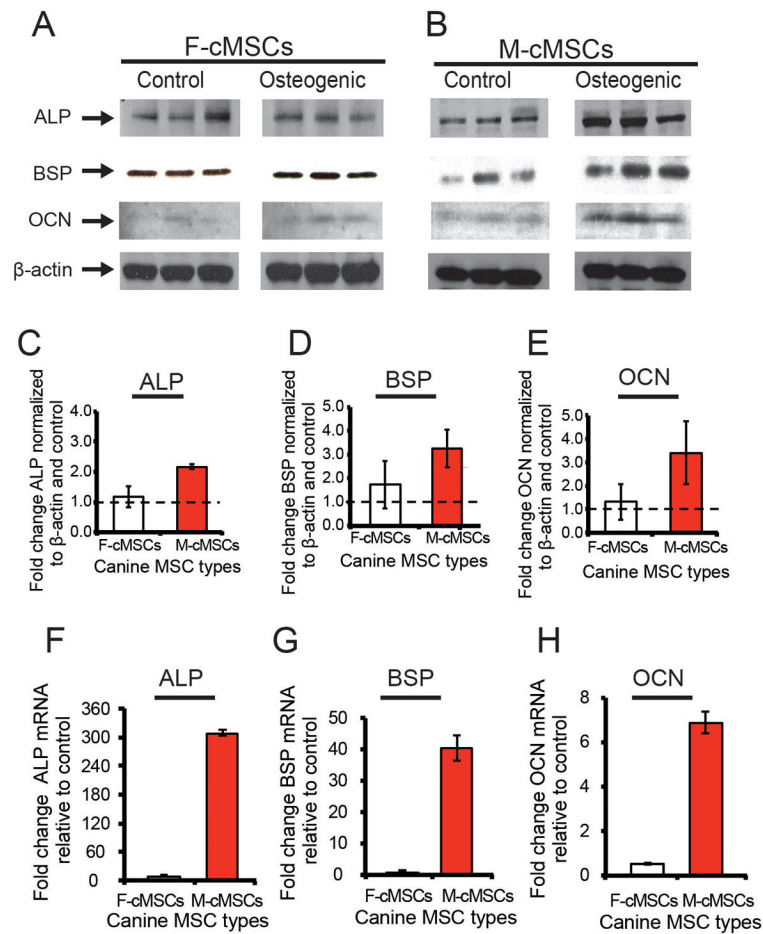


Figure 2. *In vitro* osteogenic responsiveness of cMSCs

Analysis of osteogenic proteins by western blotting 14 days post-induction (A, B) showed strong immunoreactivity suggestive of osteogenic responsiveness of cMSCs. M-cMSC expression levels of three markers of osteogenesis ALP (C), BSP (D) and OCN (E) were higher relative to F-cMSCs (ALP, $p = 0.04$; BSP, $p = 0.05$ and OCN, $p = 0.03$). At the mRNA level, the M-cMSC transcripts of ALP ($p < 0.001$; F), BSP ($p < 0.001$; G) and OCN ($p < 0.001$; H) were also significantly upregulated. [ALP = alkaline phosphatase; BSP = bonesialoprotein; OCN = osteocalcin].

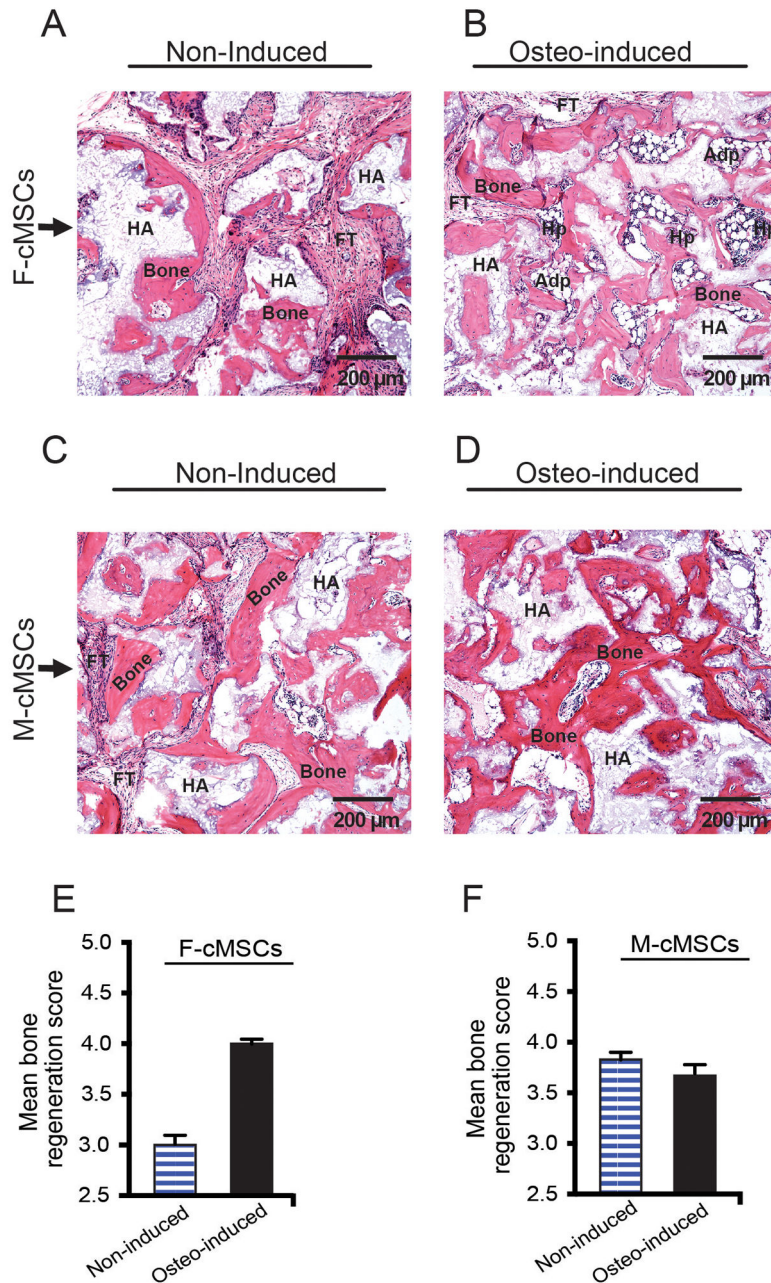


Figure 3. *In vivo* osteogenic responsiveness of cMSCs

Both F-cMSCs (A, B) and M-cMSCs (C, D) formed histologically appreciable *in vivo* bone when transplanted into subcutis of immunocompromised host. Semi-quantitative analysis (E, F) confirmed that F-cMSCs needed exposure to osteogenic medium to form quantitatively appreciable bone ($p < 0.05$) as non-induced M-cMSCs. The exposure of F-MSCs to osteogenic medium induced formation of marrow components [hematopoiesis (Hp) and adipocytes (Adp)] (B) while bone formed by M-cMSCs were within a fibrous tissue (FT) bed (D).

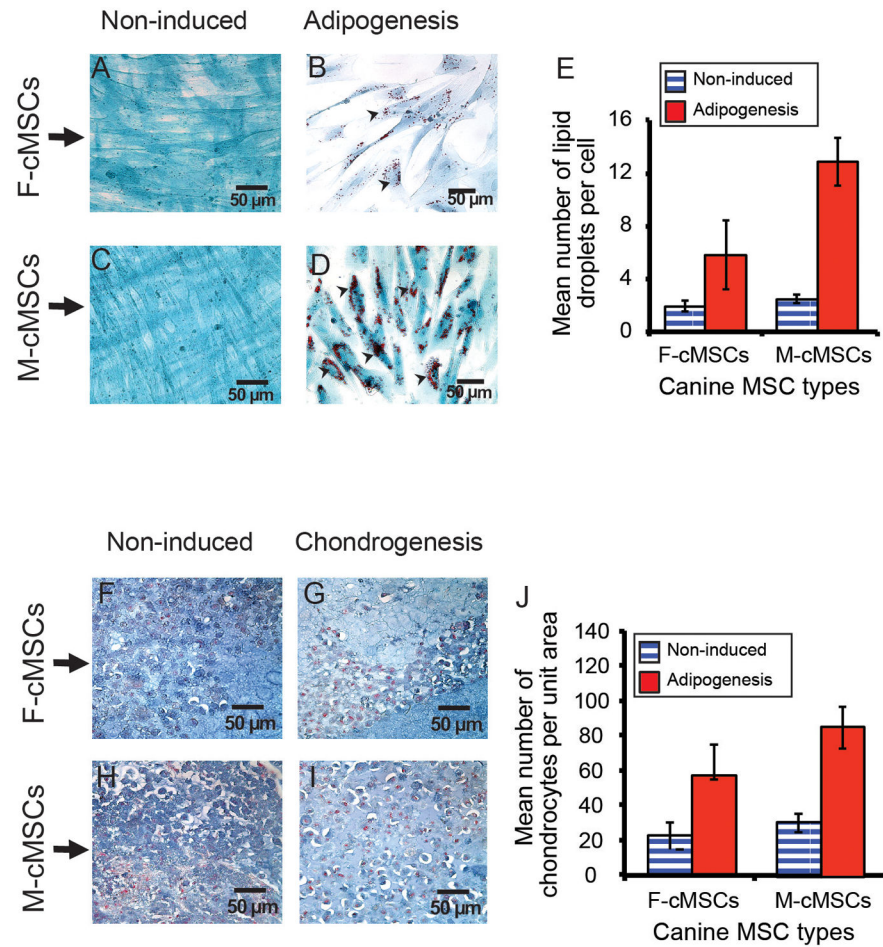


Figure 4. Adipogenic and chondrogenic differentiation of cMSCs

Following induction with adipogenic medium, the M-cMSCs responded by showing more lipid-laden cells ($p=0.007$) based on Oil-Red O staining (Figure 4A – E). Similarly, assessment of chondrogenesis using the pellet culture method clearly indicated that M-cMSCs were more responsive to chondrogenic stimulation ($p=0.009$) based on Alcian blue staining of chondrocyte-like cells (Figure 4F – J). [Representative images are presented in A, B, C, D, F, G, H, and I].

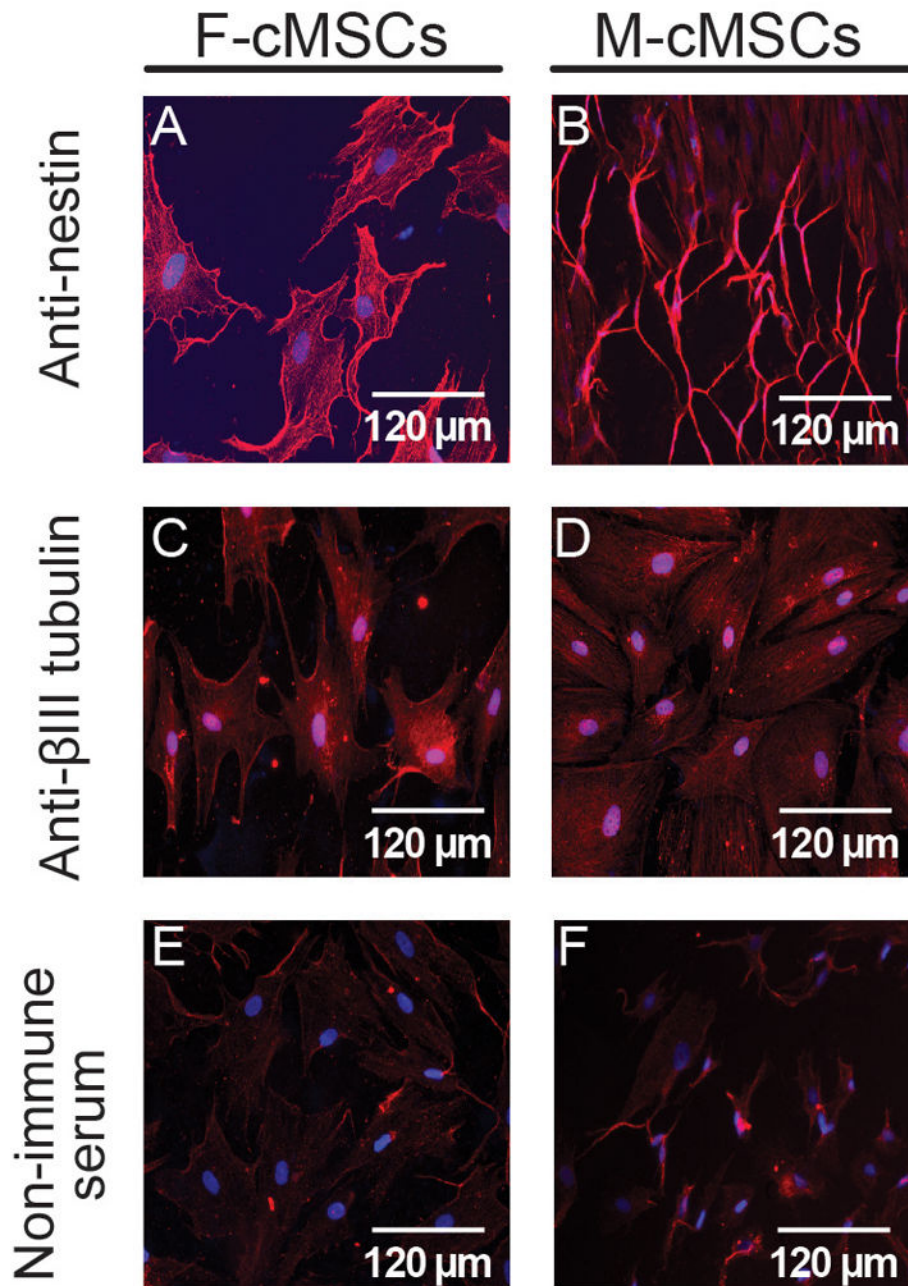


Figure 5. Neuronal differentiation

After exposure to neuronal differentiation medium both F-cMSCs (A, C) and M-cMSCs (B, D) were strongly immunoreactive to antibodies to nestin (day 7) and β -III tubulin (day 14). Based on nestin immunoreactivity (A, B), both cell types displayed spindle shaped morphology with stretched-out dendrite-like cytoplasmic projections [Representative immunostaining images are presented].