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Abstract

Objectives

Mesenchymal stem cells (MSCs) offer a promising therapy in dentistry because of their multipotent properties. Selecting donor MSCs is crucial because Beagle dogs (canines) commonly used in preclinical 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 the mandible (M-cMSCs) and femur (F-cMSCs) of four healthy Beagle dogs. The femur served as the 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 = .006). M-cMSCs in vitro osteogenesis based on alkaline phosphatase (P = .04), bone sialoprotein (P = .05), and osteocalcin (P = .03), as well as adipogenesis (P = .007) and chondrogenesis (P = .009), were relatively higher and correlated with enhanced M-cMSC bone regenerative capacity. Neural expression markers, nestin and βIII-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.

Disciplines

Dentistry

The bone regenerative capacity of canine mesenchymal stem cells is regulated by site-specific multilineage differentiation

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Objectives. Mesenchymal stem cells (MSCs) offer a promising therapy in dentistry because of their multipotent properties. Selecting donor MSCs is crucial because Beagle dogs (canines) commonly used in preclinical 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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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 nature.[1-3] Selecting the optimal donor graft material for orofacial bone regeneration is still a challenge that causes unpredictable clinical outcomes.[4,5] Although 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) have yet to be fully clarified.

MSCs are 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 the donor's age and the skeletal site of origin.[7-9] Current preclinical applications of MSCs have focused extensively on human, mouse, and rat MSCs, although MSCs have also been isolated from unconventional animal models, such as 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 neuroectodermal developmental origin of jaw bones that is distinct from the mesodermal origin of the 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 canine MSCs (cMSCs) inherently display skeletal site−specific functional differences. Although Beagle dogs have been used in preclinical studies to model oral bone loss or regeneration, the effects of the jaw-specific properties of cMSCs have not been clearly defined. This study tested the hypothesis that canine MSCs from the jaw and long

Statement of Clinical Relevance

Mesenchymal stem cells offer a promising graft therapy in dentistry because of their multipotent properties. Careful donor graft selection is vital for success of preclinical models of oral bone regeneration.

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bones have disparate in vitro and in vivo multilineage differentiation capabilities. It is expected that further understanding of underlying biologic 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 and United States Department of Agriculture 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 cMSCs were established in culture from the mandible and femur, as previously described,[7] by 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% carbon dioxide (CO_2) and air. Primary cMSCs isolated from the mandible (M-cMSCs) and femur (F-cMSCs) were further subcultured, 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 10^1, 10^2, and 10^3 passage 2 F-cMSCs and M-cMSCs in triplicate 25-cm^2 plastic culture flasks.[7,16] At 14 days, the cells were fixed in 100% methanol and 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 six-well plates at 9.5×10^3 cells/cm^2. The cells were trypsinized and counted on days 1, 3, 6, 9, 12, and 15 to plot a growth curve. Proliferation was analyzed by using nonlinear 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 as previously described.[7] Both cMSCs types were plated in T-25 flasks at 1×10^6 cells/flask, and population doublings was calculated on the basis of 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 immunoglobulin G as secondary antibody (1:2000 dilution, Cat # NA934 V; GE Healthcare Life Sciences, Piscataway, NJ). Immunoreactive bands were digitized and analyzed with ImageJ v1.49 g (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 1×10^4 cells/cm^2 in ten 60-mm dishes (Corning Life Sciences, Tewksbury, MA). Five of the dishes were precoated 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) and 100 μM L-ascorbic acid 2-phosphate (10^{-4} M). The 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; ThermoScientific, Rockford, IL). Equal protein amount was loaded on a 4% to 20% gradient gel, transferred to nitrocellulose membrane for Western blotting, and probed with the following primary antibodies: rabbit antibone sialoprotein (BSP) polyclonal antibody (Bioss Inc., Worburn, MA) at 1:200; rabbit antiosteocalcin (OCN) polyclonal antibody (Bioss Inc.) at 1:200, and rabbit antialkaline phosphatase (ALP) antibody (Novus Biologicals, Littleton, CO) at 1:800. Either anti-β-actin (1:1000) or anti-α-tubulin (1:200) served as loading control.

Secondary antibodies included antimouse or antirabbit antibodies at concentrations ranging from 1:1000 to 1:3000. Immunoreactive bands were digitized followed by quantification with ImageJ v1.49 g (National Institutes of Health, Bethesda, MD). Real-time polymerase chain reaction (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 β_2-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

The bone regenerative capacity of F-cMSCs and M-cMSCs was assessed by transplantation of 1×10^6 cells attached to hydroxyapatite-tricalcium phosphate (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, the transplants were harvested, fixed in 4% paraformaldehyde, decalcified in 10% ethylenediaminetetraacetic acid (EDTA; pH 8.0) and paraffin-embedded for histologic analysis. Images were captured with Nikon Eclipse80 i fluorescent microscope (Nikon Instruments, Melville, NJ), and bone regeneration was assessed using an established semiquantitative bone scoring system.[7]

Adipogenic differentiation

F-cMSCs and M-cMSCs were cultured at 1.8×10^3 cells/cm^2 in four-well chamber slides (Coming Life Sciences, Acton, MA), and at confluence, adipogenic differentiation was induced, as previously described,[7,16] by using adipogenic differentiation medium composed of α-MEM supplemented with 10^{-8} M dexamethasone, insulin (1 μg/mL), 1-methyl-3-isobutylxanthine (5×10^{-8} M), indomethacin (10^{-4} M), and fetal bovine serum (10%) for 15 days. The control cells were not induced with adipogenic medium. The 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 cm^2 flasks at 75×10^4 cells/cm^2 until 80% to 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-β_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 and fixed with 4% paraformaldehyde, and paraffin-embedded 5-μm sections were stained with Alcian blue plus counterstain of nuclear fast red for histologic analysis.

Neural differentiation

Collagen coated eight-well chamber slides (Corning BioCoat; Corning Life Sciences, Tewksbury, MA) were seeded with F-cMSCs and M-cMSCs at 4×10^3 cells/cm^2 by using the 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 preinduction 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 preinduction medium was switched to a neuronal induction medium, which consisted 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 μM butylated hydroxyanisole, 10 μM forskolin, 25 mM potassium chloride, 2 mM valproic acid, and 5 μg/mL insulin. Neural differentiation was evaluated at 7 and 14 days by immunofluorescent staining by using primary antibodies of rabbit antinestin (1:200) (LifeSpan BioSciences, Inc., Seattle, WA) and rabbit anti-βIII-tubulin (1:200) (Bioss) as primary antibodies. Alexa Fluor 555 Goat Anti-Rabbit immunoglobulin G (Life Technologies) served as secondary antibody, and nuclei were stained with 1 μg/mL of Hoechst 33342. Images were captured with Nikon Eclipse80 i fluorescent microscope (Nikon Instruments).

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 ± 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

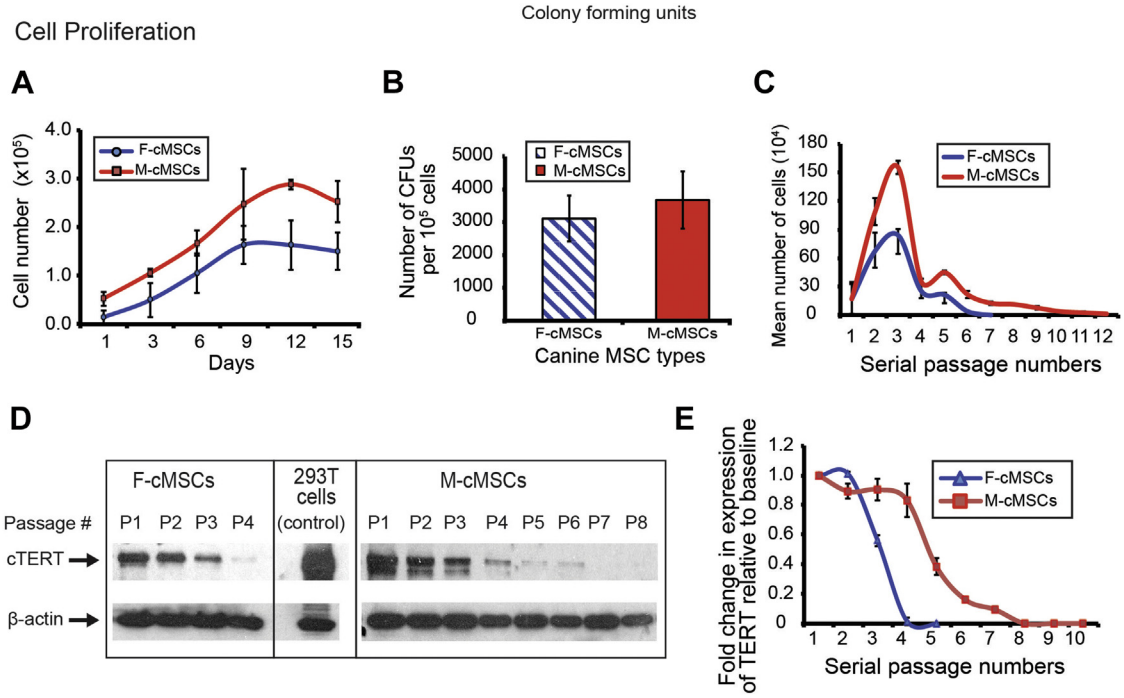

Fig. 1. Proliferation and survival of canine mesenchymal stem cells (MSCs). Mandibular canine MSCs (M-cMSCs) display significantly higher proliferation ($P = .006$) before peaking on day 12 compared with femur cMSCs (F-cMSCs) that peaked on day 9 **(A)**. The colony-forming capacities based on colony-forming units—fibroblasts (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, which did not completely senesce until passage 12. The delayed senescence of M-cMSCs was supported by higher expression levels of cTERT **(D and E)**.

two-way analysis of variance followed by post hoc comparisons with Holm-Sidak test; statistical significance was set at $P < .05$.

RESULTS

We isolated cMSCs from the mandible and femur trabecular bone samples obtained from Beagle dogs commonly used for translational studies.[15] The monolayer of primary F-cMSCs and M-cMSCs demonstrated heterogeneous fibroblast-like morphologic shapes and sizes characteristic of MSCs (data not shown). M-cMSCs displayed significantly higher time-dependent proliferation ($P = .006$) 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 that there were more doubling M-cMSCs than F-cMSCs at early passages (Figure 1C). The F-cMSCs also underwent complete senescence after passage 6, unlike M-cMSCs, which displayed gradual senescence up to passage 12. The apparently delayed senescence of M-cMSCs was supported by higher expression levels of cTERT (Figures 1D and 1E).

Analysis of osteogenic proteins that include ALP, BSP, and OCN by Western blotting at 14 days after induction (Figure 2A and 2B) showed strong immunoreactivity suggestive of a strong osteogenic responsiveness of cMSCs from both skeletal sites. The amounts of ALP (Figure 2C), BSP (Figure 2D) and OCN (Figure 2E) were disproportionately higher in M-cMSCs relative to F-cMSCs (ALP, $P = .04$; BSP, $P = .05$; and OCN, $P = .03$). At the mRNA level, the M-cMSC transcripts of osteogenic markers ALP ($P < .001$), BSP ($P < .001$), and OCN ($P < .001$) (Figures 2F to 2H, respectively) were even much more upregulated relative to F-cMSCs.

Analysis of in vivo bone regenerative ability of F-cMSCs (Figures 3A, 3B, and 3E) and M-cMSCs (Figures 3C, 3D, and 3F) was assessed semiquantitatively 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 compared with F-cMSCs with or without the addition of osteogenic inducers (Figures 3E and 3F). Hence, the F-CMSCs needed osteogenic induction to regenerate the similar quantitatively appreciable bone (see Figure 3E; $P < .05$) as unstimulated M-cMSCs. Also, exposure of F-MSCs to osteogenic medium induced formation of marrow components (hematopoiesis and adipocytes; see Figure 3B), whereas bone formed by M-cMSCs were within a fibrous tissue bed (see Figure 3D).

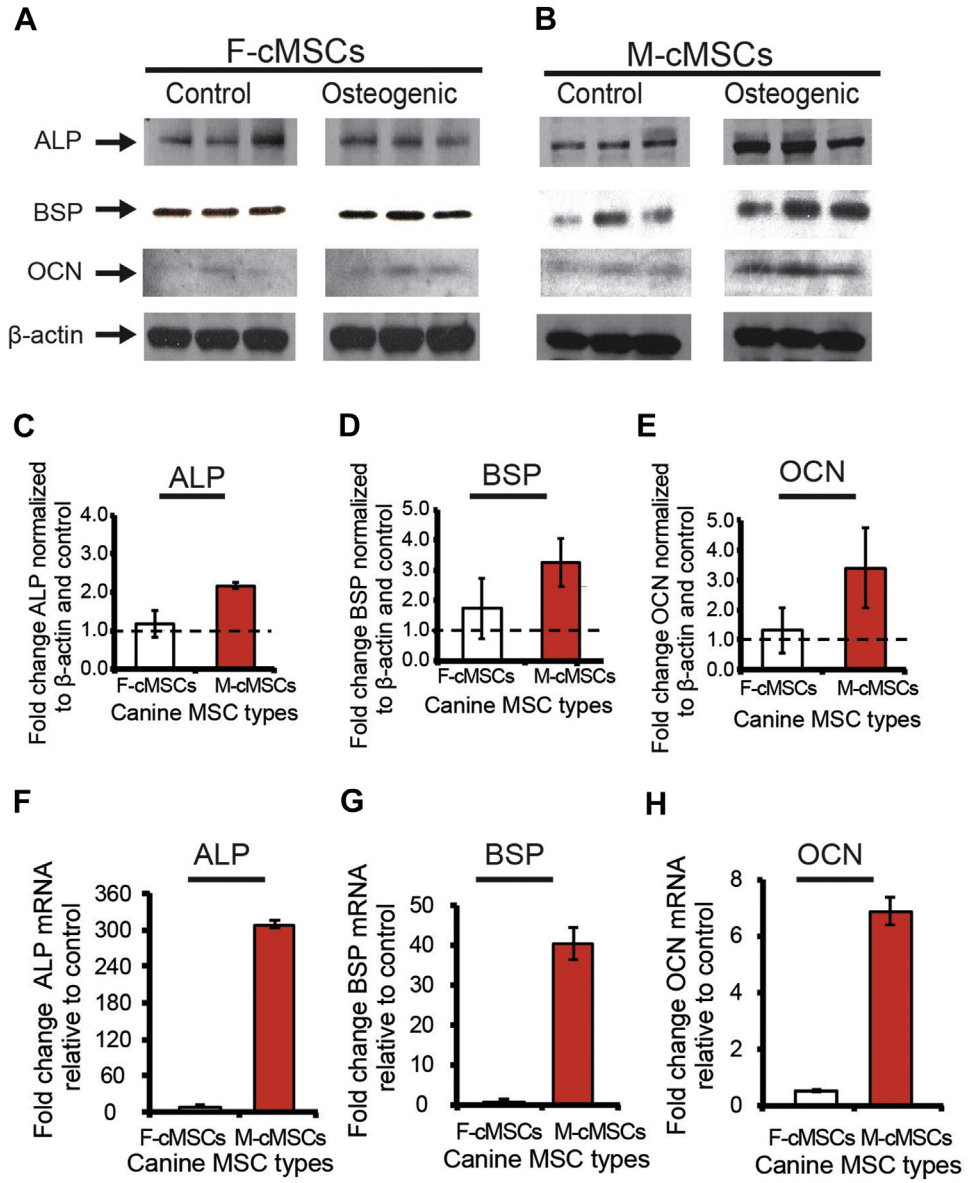

Fig. 2. In vitro osteogenic responsiveness of canine mesenchymal stem cells (cMSCs). Analysis of osteogenic proteins by Western blotting 14 days after induction **(A, B)** showed strong immunoreactivity suggestive of osteogenic responsiveness of cMSCs. Mandibular cMSC (M-cMSC) expression levels of three markers of osteogenesis ALP **(C)**, BSP **(D)**, and OCN **(E)**, were higher relative to femur cMSCs (F-cMSCs) (ALP, $P = .04$; BSP, $P = .05$; and OCN, $P = .03$). At the mRNA level, the M-cMSC transcripts of ALP ($P < .001$; **F**), BSP ($P < .001$; **G**) and OCN ($P < .001$; **H**) were also significantly upregulated. ALP, Alkaline phosphatase; BSP, bone sialoprotein; OCN, osteocalcin.

After induction with adipogenic medium, the M-cMSCs responded by displaying more lipid-laden cytoplasmic contents ($P = .007$) based on Oil-Red O staining (Figures 4A to 4E). Similarly, assessment of chondrogenesis by the pellet culture method clearly indicated that M-cMSCs were more responsive to chondrogenic stimulation ($P = .009$) based on Alcian blue staining of chondrocyte-like cells (Figures 4F to 4J). After exposure to neuronal differentiation medium, both F-cMSCs (Figures 5A and 5C) and M-cMSCs (Figures 5B and 5D) displayed strong immunoreactivity to antinestin (at 7 days) and anti-β-III tubulin (at 14 days) without appreciable differences between the two cell types. Nestin immunoreactivity (see Figures 5A and 5B) 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] the periodontal ligament,[28]

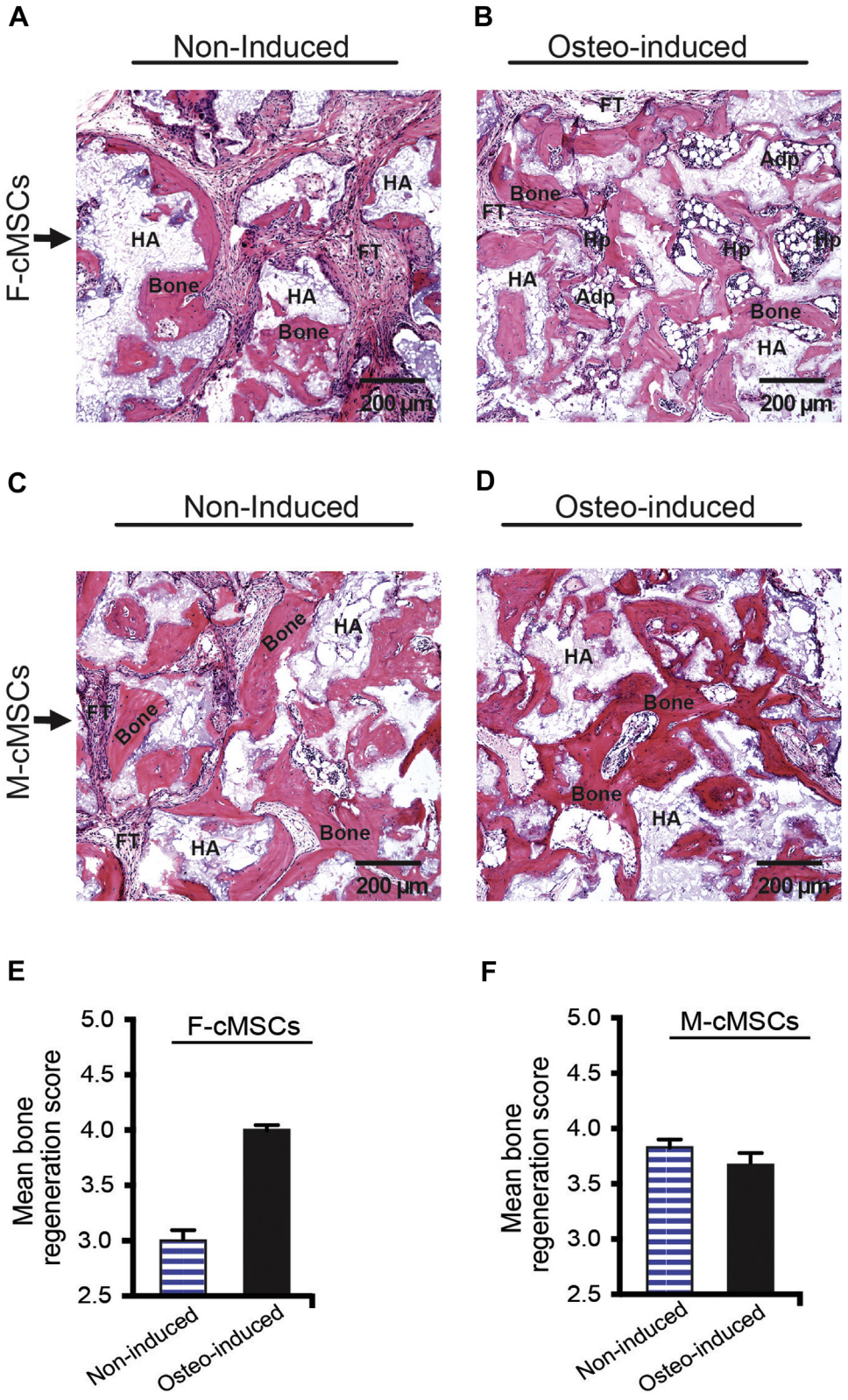

Fig. 3. In vivo osteogenic responsiveness of canine mesenchymal stem cells (cMSCs). Both femur and mandibular cMSCs [F-cMSCs **(A, B)** and M-cMSCs **(C, D)**] formed histologically appreciable in vivo bone when transplanted into subcutis of the immunocompromised host. Semiquantitative analysis **(E, F)** confirmed that F-cMSCs needed exposure to osteogenic medium to form quantitatively appreciable bone ($P < .05$) as noninduced M-cMSCs. The exposure of F-MSCs to osteogenic medium induced formation of marrow components [hematopoiesis (Hp) and adipocytes (Adp)] **(B)**, whereas bone formed by M-cMSCs were within a fibrous tissue (FT) bed **(D)**.

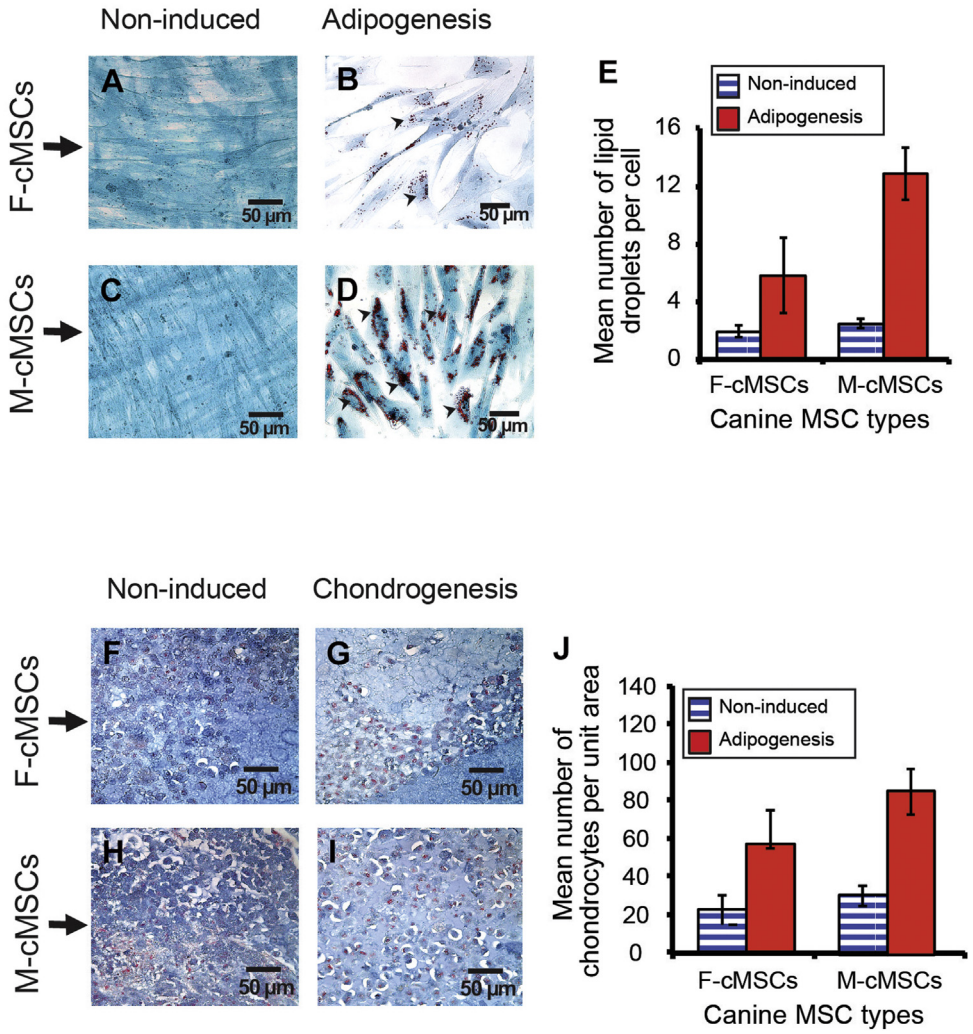

Fig. 4. Adipogenic and chondrogenic differentiation of canine mesenchymal stem cells (cMSCs). Following induction with adipogenic medium, the mandibular cMSCs (M-cMSCs) responded by showing more lipid-laden cells ($P = .007$) based on Oil-Red O staining **(A-E)**. Similarly, assessment of chondrogenesis using the pellet culture method clearly indicated that M-cMSCs were more responsive to chondrogenic stimulation ($P = .009$) based on Alcian blue staining of chondrocyte-like cells **(F-J)**. [Representative images are presented in A, B, C, D, F, G, H, and I.]

amniotic fluid,[29] muscle,[20] and the periosteum.[20] However, direct comparison of two different skeletal sites in dogs and the clinical implications have not been conclusively evaluated. We used plastic adherence method to successfully isolate a population of cMSCs from the mandible and femur of the same dogs.[30] Although the cMSCs isolated from both sites displayed heterogeneous fibroblast-like morphology, cell surface labeling and flow cytometric analysis were not carried out because of 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, in contrast to the 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, which is consistent with the findings of previous studies 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 compared with 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]

The osteoresponsiveness of cMSCs was also better activated when the dexamethasone and ascorbate

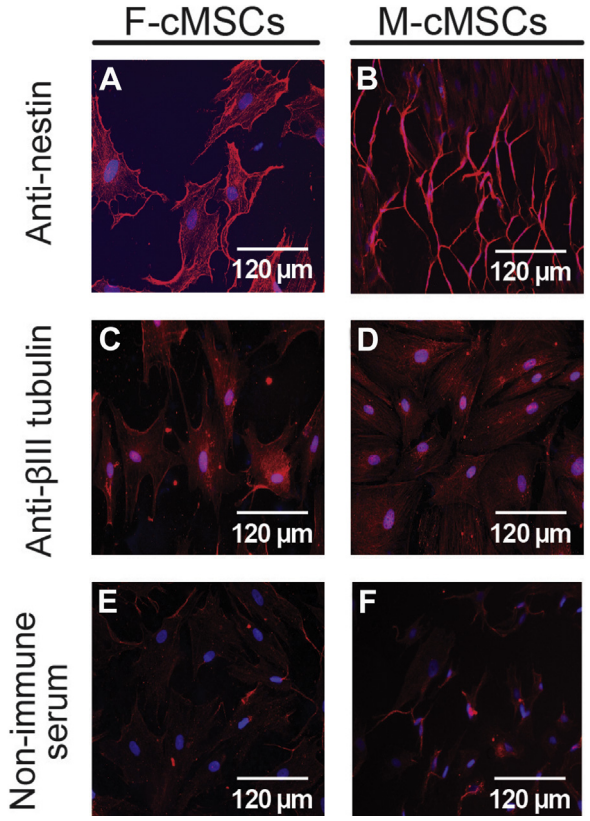

Fig. 5. Neuronal differentiation. After exposure to neuronal differentiation medium both femur canine mesenchymal stem cells (F-cMSCs) **(A, C)** and mandibular cMSCs (M-cMSCs) **(B, D)** were strongly immunoreactive to antibodies to nestin (day 7) and β-III tubulin (day 14). Based on nestin immunoreactivity **(A, B)**, both cell types displayed spindle shaped morphology with stretched-out dendrite-like cytoplasmic projections. **E** and **F** are control cells stained with non-immune serum. [Representative immunostaining images are presented.]

combination in the osteoinductive medium was replaced with a combination of BMP-2 and ascorbate. This is in line with previous reports that the combination of BMP and 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, the 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 preinduction, 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 histologic features of hematopoiesis and adipogenesis as normal femur bone further points to the site-specificity of MSCs and to the 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 mandibular and maxillary MSCs, as 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 mandibular bone in which the M-cMSCs reside develops embryologically from the 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 morphologic 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 preinduction initially with β-FGF to minimize MSC damage by chemical stress.[38,39] Additionally, we confirmed the neuronal differentiation based on positive immunoreactivity with nestin and βIII-tubulin, two known markers of neurogenesis. As rodent MSCs have been shown to spontaneously express nestin,[40,41] it was not surprising that noninduced cMSCs also displayed some degree of immunoreactivity to these markers (data not shown).

Several factors make a case for assessing the skeletal site-specificity of MSCs and their species-to-species differences. The unique neuroectodermal origin of orofacial bones and the intramembranous ossification pattern of the mandible, coupled with the endochondral contributions from Meckel's, coronoid, and condylar cartilages, make the jaws developmentally different.[42] Furthermore, several bone pathologies display unique radiologic and histologic features in the jaw. These include fibrous dysplasia of bone,[43] cherubism,[44] and hyperparathyroid jaw tumor syndrome.[45] Additionally, long-term use of bone antiresorptive medications is often complicated by jaw osteonecrosis, whereas 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 pathologic 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, the study represents a pilot analysis. First, the cMSCs characterized were isolated from a convenient sample of healthy dogs in an unrelated

research project; second, the number of tissue samples was limited; and third, 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 MSC 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 the vascular endothelial growth factor that induces formation of vasculature, which, in turn, invades the graft. Although tissue vascularization is important for MSC survival and subsequent osteogenesis, these sequences of events have yet to be clearly defined.[13,51]

CONCLUSIONS

In spite of the limitations of this study, these results are consistent with data from the studies that focused on human, mouse, and rat MSCs.[7,11,36,52] These findings 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 multilineage differentiation compared with F-cMSCs and represent superior donor graft materials for oral bone regeneration.

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