Mesenchymal Stem Cells Derived From Canine Umbilical Cord Vein—A Novel Source for Cell Therapy Studies

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The canine model provides a large animal system to evaluate many treatment modalities using stem cells (SCs). However, only bone marrow (BM) protocols have been widely used in dogs for preclinical approaches. BM donation consists of an invasive procedure and the number and differentiation potential of its mesenchymal stem cells (MSCs) decline with age. More recently, umbilical cord was introduced as an alternative source to BM since it is obtained from a sample that is routinely discarded. Here, we describe the isolation of MSCs from canine umbilical cord vein (cUCV). These cells can be obtained from every cord received and grow successfully in culture. Their multipotent plasticity was demonstrated by their capacity to differentiate in adipocytic, chondrocytic, and osteocytic lineages. Furthermore, our results open possibilities to use cUCV cells in preclinical trials for many well-characterized canine model conditions homologs to human diseases.

Introduction

Researches aimed at regenerative medicine have developed fast in recent years and are causing great expectation among scientists and the general public. Current approaches are being developed to repair and restore the function of damaged or disease tissues. Animal models are of immense importance in this field and most of the knowledge and advances achieved came from preclinical studies.

Historically, the dog has been an useful model for studying mechanisms and testing new therapies of several human pathologies including prostate cancer, cardiovascular diseases, bone regeneration, diabetes, leukemia, spinal cord injury, as well as solid organ transplantation [1–7]. Dogs are also a source of many well-characterized genetic diseases homologs to human, such as X-linked severe combined immunodeficiency (XSCID) and Duchenne muscular dystrophy [8–10]. In addition, they are an ideal large animal model to study various treatment modalities such as gene therapy and stem cell (SC) transplantation.

Studies carried out over the last decades suggest that SCs could be of great value for the development of new therapies. SCs are defined by their ability to self-renewal and to differentiate into multiple cell types and by their capacity of in vivo reconstitution of a given tissue [11]. They can be derived from the inner cell mass of an early embryo as well as numerous postnatal sources, including bone marrow (BM), umbilical cord (UC), placental tissue, and dental pulp [12–16]. Much of the knowledge concerning canine SCs comes from the numerous studies of hematopoietic and mesenchymal components from BM [17–21]. In recent reports, adult and embryonic canine SC lines were established from novel sources such as the blood vessels from muscle biopsies, termed mesoangioblasts, and from embryo blastocysts, respectively [22–25]. Moreover, dog somatic cell nuclear transfer demonstrated by Lee and colleagues will provide new possibilities to evaluate the concept of therapeutic cloning in this animal model [26].

Given that many advances achieved in the dog are directly transferable to humans since it constitutes a large animal model, it could be of great value for the development of new therapies. Isolating SCs in the dog model will provide the opportunity to evaluate the efficacy and safety of gene therapy and cell transplantation. Here, for the first time, we isolated canine mesenchymal stem cells (MSCs) from UC. These cells, termed canine umbilical cord vein (cUCV), are simple to harvest in large amount without risk to the donor, may be expanded in vitro, cryogenic stored, and thawed. We also demonstrated that cUCV express mesenchymal markers

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and were able to differentiate into, at least, adipocytes, chondrocytes, and osteocytes.

**Materials and Methods**

*Isolation and culture of canine adherent cells from UCV*

All experimental protocols were approved by the ethical committee of animal use of Institute of Biosciences, University of São Paulo. For this study, Golden Retriever newborn dogs were obtained from Peter White Kennel, São Paulo and the Brazilian Colony of Golden Retriever Muscular Dystrophy, Faculty of Veterinary Medicine and Zootechny of University of São Paulo. Full-term UCVs were collected immediately after birth and washed with phosphate-buffered saline (PBS) containing 300 U/mL penicillin and 300 μg/mL streptomycin (Gibco) and placed in Dulbecco’s modified Eagle’s medium low glucose (DMEM-LG; Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, Gibco), 300 U/mL penicillin, and 300 μg/mL streptomycin. All samples were processed within 10–12 h after collection.

In the laboratory, the UCVs were filled with 0.1% collagenase (Sigma, St. Louis, MO) in PBS and incubated at 37°C for 15 min. Then, the UCVs were washed internally with proliferation medium consisting of DMEM-LG supplemented with 10% FBS and 100 μg/mL penicillin and 100 μg/mL streptomycin. Detached cells were harvested after gentle massage of the UC and centrifuged at 300g for 10 min. Cells were resuspended in proliferation medium, seeded in 25 cm² flasks, and maintained at 37°C in a humidified atmosphere containing 5% CO₂. After 24 h incubation, nonadherent cells were removed and culture medium was replaced every 3 days. Adherent cUCV cells were obtained from Peter White Kennel, São Paulo and the Brazilian Colony of Golden Retriever Muscular Dystrophy, Faculty of Veterinary Medicine and Zootechny of University of São Paulo. Full-term UCVs were collected immediately after birth and washed with phosphate-buffered saline (PBS) containing 300 U/mL penicillin and 300 μg/mL streptomycin (Gibco) and placed in Dulbecco’s modified Eagle’s medium low glucose (DMEM-LG; Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, Gibco), 300 U/mL penicillin, and 300 μg/mL streptomycin. All samples were processed within 10–12 h after collection.

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**Immunophenotyping**

Canine umbilical cord vein (cUCV) cells at passage 3 were seeded onto chamber slides and allowed to grow until reaching 70%–80% confluence. For antibody staining, growth medium was removed and the cells were fixed with 4% paraformaldehyde for 15 min. Blocking was performed using 1% bovine serum albumin (BSA, Sigma, St. Louis, MO) in PBS-Tween (PBST) for 30 min. The cells were then incubated with the following primary antibodies (15 μg/mL): anti-canine CD29 (Veterinary Medical Research & Development, Inc., Pullman, WA) or anti-canine CD90 (Becton Dickinson, Franklin Lakes, NJ) for 1 h at room temperature. The secondary antibody anti-mouse IgG-Cy3 conjugated (Millipore, Billerica, MA), diluted 1:100, was incubated in the same conditions described earlier. Negative control samples were incubated with PBS instead of primary antibody or only with secondary antibody. Cells were then counterstained with 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA) and analyzed on Axiovert 200 microscope (Carl Zeiss, Thornwood, NY) using appropriate filters. Images were captured using Axiovision 3.1 software (Carl Zeiss).

**Expansion characteristics**

cUCV cells at passage 2 were monitored for 28 days to determine their cumulative population doubling level (cpdl). Cell plating density was set at 1 × 10⁶ cells in a 35-mm dish and cultivated 4 days in proliferation medium until near confluence. Each passage, in a total of 7, was performed using TrypLE and recovered cells were replated at initial density as passage 2 cells and thereafter. Cell number was determined by 3 independent direct counts in Neubauer chamber. The population doubling (pd) at each subculture was calculated by using the following equation: pd = ln (Nf/ Ni)/ln 2, where N_i and N_f are initial and final cell numbers, respectively, and ln is the natural log. The pds of continuous subculture were added to obtain the cpdl.

**Karyotype analysis**

For evaluation of any abnormal karyotype at latter passages, chromosomes preparations were performed in cUCV cells cultures. Briefly, metaphase cells were arrested with 0.1 μg/mL colchicine (Sigma) for 20 min. Then, cUCV cells were detached from cultures flasks using TrypLE (Gibco), resuspended in a hypotonic solution (0.075 M KCl), and incubated for 20 min at 37°C. Cells were pelleted at 1,000 rpm for 10 min and fixed by washing 3 times in methanol:acetic glacial acid (3:1). Chromosome spreads were obtained by pipetting suspension drops onto clean glass slides and air-dried. The best metaphases were captured with Axioplan 2 microscope (Zeiss) and analyzed using Ikaros 3 software (Zeiss).

**Differentiation studies**

cUCV cells were subjected to differentiation protocols to evaluate their MSC property. For this purpose, adipogenic, chondrogenic, and osteogenic inductions were assessed in the third passage cultures. Noninduced control samples were maintained in proliferation medium during all the differentiation study.

**Adipogenesis.** Subconfluent cells (90%) were cultured in DMEM high glucose medium (Gibco, Grand Island, NY) supplemented with 5% rabbit serum (Gibco), 5 μM rosiglitazone (GSK), 1 μM dexamethasone (Sigma, St. Louis, MO), and 5 μg/mL insulin (Sigma). Adipogenic differentiation was confirmed on day 21 by intracellular accumulation of lipid-rich vacuoles stainable with Oil Red O (Sigma). For the Oil Red O stain, cells were fixed with 4% paraformaldehyde for 30 min, washed, and stained with a working solution of 0.16% Oil Red O for 20 min.

**Chondrogenesis.** For chondrogenic differentiation, cells were cultured as monolayer or cell sphere. For the last one,
The results reported in this work were representative of 30 cUCV cell lineages established in our laboratory. We were able to generate primary adherent cell culture from all UC samples with a 100% yield. These cells were obtained as follows: 2.5 × 10^5 cells were centrifuged in a 15-mL polypropylene tube at 500g for 5 min and the pellet resuspended in 10 mL of basal medium consisting of DMEM-LG supplemented with 100 nM dexamethasone, 50 μM ascorbic acid-2 phosphate (Sigma, St. Louis, MO), 1 mM sodium pyruvate (Gibco), and 1% ITS-Premix (Becton Dickinson, Franklin Lakes, NJ). Without disturbing the pellet, the cells were resuspended in 0.5 mL of chondrogenic differentiation medium consisting of basal medium supplemented with 10 ng/mL transforming growth factor beta 1 (TGF-β1) (R&D Systems, Minneapolis, MN). On day 1, the tubes were flipped gently to acquire a single floating cell sphere. To perform monolayer cell culture, subconfluent cells (90%) were also cultured in chondrogenic differentiation medium. In both cases, the medium was changed every 3–4 days, and then cells were fixed on day 21 with 4% paraformaldehyde. Cryosections (10 μm thick) of cell spheres or monolayer cultures were stained with Toluidine Blue to demonstrate extracellular matrix mucopolysaccharides. Staining solution was prepared by adding 1% of Toluidine Blue dissolved in distilled water containing 1% of sodium borate, followed by filtering. One drop of this solution was added to each sample for 1 min and then washed with distilled water and left to air-dry.

### Osteogenesis

To promote osteogenic differentiation, subconfluent (90%) cUCV cells were treated with proliferation medium supplemented with 50 μM ascorbate-2 phosphate, 10 mM β-glycerophosphate (Sigma), and 0.1 μM dexamethasone for 21 days. Osteogenesis was demonstrated by accumulation of mineralized calcium phosphate assessed by von Kossa stain and Alizarin Red. Briefly, for von Kossa stain, the cells were stained with 1% silver nitrate (Sigma) for 45 min under ultraviolet light, followed by 3% sodium thiosulfate (Sigma) for 5 min, and then counterstained with van Gieson. To perform Alizarin Red stain, ethanol (50%) fixed cells were incubated for 30 min at room temperature (RT) in a solution containing 1% Alizarin Red and 1% ammonium hydroxide. Cells were rinsed twice with distilled water and allowed to dry completely.

### Total RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNAs were isolated from cells using Trizol (Invitrogen) under conditions recommended by the manufacturer. Extracted RNA was dissolved in diethylpyrocarbonate-treated water (DEPC water) and cDNAs were synthesized from 0.2 μg total RNA using Superscript III reverse transcriptase (Invitrogen). Polymerase chain reaction (PCR) was performed using specific primers to amplify the target sites listed in Table 1. Twenty-five microliters of PCR were prepared with 1 μL cDNA, 10 pmol of each primer, 1 unit of Taq polymerase (GE Healthcare, Piscataway, NJ), 1× PCR buffer (GE Healthcare), and final concentration of 160 μM dNTPs. Cycle conditions were as follows: 94°C for 4 min; 30–35 cycles at 94°C for 1 min; optimal annealing temperature (Table 1) for 1 min; 72°C for 1 min; followed by 72°C for 5 min. The PCR products were separated on 6% polyacrylamide gel by electrophoresis, stained with ethidium bromide, and visualized under UV light. Digital images were captured with ImageQuant (GE Healthcare).

### Results

#### Isolation and morphological characteristics of cUCV cells

The results reported in this work were representative of 30 cUCV cell lineages established in our laboratory. We were able to generate primary adherent cell culture from all UC samples with a 100% yield. These cells were obtained...

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### Table 1: Primers Used in RT-PCR

<table>
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<tr>
<th>Markers</th>
<th>Gene</th>
<th>Primer sequence (5′–3′)</th>
<th>Amplicon size</th>
<th>Annealing temp. (°C)</th>
<th>Reference</th>
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<td>Adipocytes</td>
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<td>Forward ATCAGTGAAACGGGGATGTG</td>
<td>117</td>
<td>60</td>
<td>[42]</td>
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<td></td>
<td></td>
<td>Reverse GACTTTTCTGTCATCAGCATTA</td>
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<tr>
<td></td>
<td>LEPTIN</td>
<td>Forward CTAATGTCGGCTGGAAGCTG</td>
<td>102</td>
<td>60</td>
<td>[42]</td>
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<td>LPL</td>
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<td></td>
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<td></td>
<td>BSP</td>
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<td>Reverse AACGGTTGCGATACACTAAAGAC</td>
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<td></td>
<td>Reverse TTCTCCATGGTGAGAGAC</td>
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</table>
Expansion characteristics

When cultivated in proliferation medium, cUCV cells presented a cpdl of 15 in 28 days. These cells could be cultured for at least 7 passages without changing their morphology or proliferation capacity (Fig. 2A). Furthermore, cUCV could be frozen and thawed without losing their viability and expansion characteristics (data not shown). At both early and late passages, cells maintained a diploid karyotype of 78 chromosomes (Fig. 2B).

Immunophenotypic characterization of cUCV cells

To characterize the adherent cells population derived from cUCV, expression of typical surface proteins were examined by immunocytochemistry and flow cytometry. Passage 3 cUCV cells were positively labeled with CD29 (β1 integrin) and CD90 (Thy1) adhesion molecules (Fig. 3A and B). No signal was observed in the negative control sample incubated only with the secondary antibody (Fig. 3C). By flow cytometry, all adherent cells derived from cUCV did not express hematopoietic lineage markers (CD14, CD34, CD45, and CD117) but express the adhesion molecule CD44 (Supplementary Fig. 1; Supplementary materials are available online at http://www.liebertpub.com). As these surface markers are not sufficient for the identification or definition of MSC, cUCV cells were subjected to differentiation studies for further confirmation of their MSC property.

Multilineage differentiation potential

The plasticity of adherent cells obtained from cUCV was investigated under conditions favorable to adipogenic, chondrogenic, and osteogenic inductions. Under the influence of adipogenic differentiation medium, cUCV cells achieved an adipocytic phenotype by the end of the third week. The presence of multized intracytoplasmic lipid droplets was confirmed by Oil Red O staining only in induced cultures (Fig. 4B), but not in noninduced cultures (Fig. 4A). Expression of FABP4 and LPL was seen only in adipo-induced cells (Fig. 5A). On the other hand, basal level of LEPTIN mRNA was observed in noninduced control cells, and the expression level

![FIG. 1. Adherent cells isolated from canine umbilical cord vein (cUCV) (A and B) Morphology of cells after 3 days in culture. (A) Cells with a wide cytoplasm (arrow) dispersed among fibroblast-like cells. (B) Cluster of cells with endothelial appearance (arrow). (C) cUCV cells after 6 days in culture. (D) Cells at passage 5. Scale bars = 500 μm.](image)

![FIG. 2. Proliferation characteristics of canine umbilical cord vein (cUCV). (A) Mean values of cumulative population doublings from cUCV lineage cultured for 7 passages in proliferation medium. (B) Karyotype of cUCV lineage after 7 passages, showing an euploid number of chromosomes.](image)
was increased following adipogenic induction. To verify the chondrogenic ability, cUCV cells were subjected to monolayer (Fig. 4C and D) or cell sphere (Fig. 4E and F) culture systems. Induced (Fig. 4D–F) and noninduced (Fig. 4C) cultures were stained with Toluidine Blue to confirm chondrogenic differentiation. As demonstrated in Figure 4E, cUCV cells originated a single floating spherical cell mass. After staining, these sections showed irregular groups of cells embedded in a mucopolysaccharide extracellular matrix surrounded by a thin capsule (Fig. 4F). Chondrogenic treatment resulted in specific expression of COL2A, SOX9, and AGGREGAN, all of which were undetected or had basal expression in non-induced cells (Fig. 5B). Finally, cUCV cells exposed to osteogenic medium for 3 weeks showed direct evidences of calcium mineralization seen by Alizarin Red (Fig. 4H) and von Kossa staining (Fig. 4J). Noninduced control cultures did not show spontaneous differentiation for osteogenic lineages (Fig. 4G and I), respectively. Expression of OSTEOPONTIN, COL1A1, and BSP was observed only in induced cells, with no basal expression in control cells (Fig. 5C). In short, these results confirmed the mesenchymal nature of the adherent cells isolated from cUCV and their multipotent property.

FIG. 3. Immunophenotyping of canine umbilical cord vein (cUCV) cells. The majority of cells labeled positively, in red, for (A) CD29 and (B) CD90. Negative control was performed after staining with anti-mouse Cy3 secondary antibody alone (C). Counterstaining with DAPI (blue) was used to identify all nuclei present. Scale bars = 100 μm.

FIG. 4. Differentiation potential of canine umbilical cord vein (cUCV) cells. (A and B) Adipogenic differentiation. (A) Noninduced cUCV cells stained with Oil Red O. (B) Induced cUCV cells. Adipogenesis was detected by the formation of intracytoplasmic lipid droplets stained with Oil Red O. (C–F) Chondrogenic differentiation. Noninduced monolayer culture (C), induced monolayer culture (D), and 3D cell sphere-induced culture (E) stained with Toluidine Blue to confirm chondrogenic differentiation. (F) Higher magnification of (E) showing mucopolysaccharide-rich extracellular matrix in pinkish metachromatic areas. A clear capsule surrounding the entire sphere was also evident (arrow). (G–J) Osteogenic differentiation. Osteogenesis was demonstrated by accumulation of mineralized calcium phosphate assessed by Alizarin Red (G and H) and von Kossa stain (I and J). Noninduced cells are shown in G and I and induced cells in H and J. Scale bars = 100 μm (B and F); Scale bars = 200 μm (A, C, and D); Scale bars = 500 μm (E, G–J).
Discussion

Umbilical cord stem cells (UCSCs) have been used as an alternative source to BM for both experimental and clinical applications [14,27–34]. UCSCs are obtained after full-term delivery of the newborn, from a sample that is routinely discarded. In the case of dogs, after each puppy delivery, UC was collected and then the mother could ingest the amniotic sac and placenta, replenishing the nutrients and energy lost during the partition. The process is noninvasive, as opposed to the obtention of other sources of adult SCs, and does not cause any harm to the mother or the newborn.

Mesenchymal stem cells from human UC have been isolated from umbilical vein, UC matrix, perivascular region, and cord blood [14,32–36]. The last one is a controversial source of MSCs as described by some researchers [14,37,38]. Recently, our group compared the efficiency of MSCs isolation from UC tissue and UC blood from the same patient and under the same conditions. We demonstrated that the UC tissue and not the blood is the most abundant source of MSCs [39]. On the other hand, cord blood is rich in hematopoietic SCs that have already been proven useful to treat various hematological disorders [27–30].

The results obtained here suggest that cUCV contains a substantial amount of MSCs that could be isolated by a fast and simple procedure using a rapid enzymatic digestion. Using this methodology, we were able to isolate adherent cells from all 30 UC obtained, yielding a 100% of success. Other groups had already reported a harvesting efficiency of 100% using human UC. Such results were described by Sarugasger et al. [32] who examined the perivascular cell population of human UC; Weiss et al. [34] who isolated cells from human UC matrix; and Secco et al. who studied the human UC tissue [39].

During the first day of cultivation some cells with endothelial appearance were present in our cultures but they spread weakly and practically did not proliferate. This result is in accordance with Romanov et al. [14] who isolated MSCs from human umbilical vein. Their methodology was used in this study, with few modifications, to isolate and establish our cUCV lineages. After 3 weeks of cultivation, attached cells from cUCV had a fairly uniform fibroblast-like morphology commonly described in MSCs populations from a variety of sources.

Adherent cells were obtained from cUCV and could be expanded in vitro, up to numbers that would be sufficient for a preclinical assay, without any numeric chromosome alteration. The proliferation potential of cUCV cells (cpdl = 15 in 28 days) was very similar to canine adipose tissue-derived MSC reported by Neupane et al. [40] (~15 cpdl in 30 days) although quite inferior to human UC blood MSC reported by Bieback et al. [36] and Kern et al. [41] (both cpdl = 20 in 7 passages).

As reported by Kern et al. [41] and Wagner et al. [42] as well as by many other groups, MSCs isolated from different tissues express a well-defined set of cell-surface markers that include CD29, CD44, CD90, SH2 (CD105), and SH3 (CD73). To characterize the adherent cells obtained from cUCV, we performed immunocytochemistry to investigate the expression of CD29 and CD90. Both adhesion markers were positively labeled in passage 3 cUCV cells. By flow cytometry we could also demonstrate that these cells do not express any hematopoietic marker but they express CD44 as shown in dog mesoangioblasts described by Sampaolesi et al. [22]. However, as reported previously by Wagner et al. [42] no obvious difference in the expression of these surface antigens could be observed between MSCs and fibroblasts. Their results show that this panel of surface markers is not itself sufficient to define or identify MSCs. In order to evaluate the MSC property of cUCV cells, we subjected them to differentiation studies.

SCs are defined by their ability to self-renewal and their capacity to generate committed cells in vitro and in vivo [11]. MSCs can be induced to differentiate along the adipogenic, chondrogenic, and osteogenic lineages using specific culture medium [41,42]. In all 6 of our 30 lineages obtained from cUCV, we demonstrated their multipotency and plasticity. Here, adipogenic, chondrogenic, and osteogenic differentiation were confirmed by the appearance of lipid vacuoles, mucopolysaccharide-rich extracellular matrix, and calcium deposits, respectively. Furthermore, the differentiation studies were confirmed by expression of specific mesoderm lineage markers.

Results from the present study demonstrate that adherent cells isolated from cUCV could be defined as multipotent MSC with ability to give rise to at least 3 mesodermal lineages. This is the first report of UCMSC isolated from dogs. In addition to canine bone marrow mesenchymal stem cell (BMMSC), other sources had previously been described in this model, such as embryonic stem cells, mesoangioblasts, and adipose-derived MSC [17–25,40].

FIG. 5. mRNA expression of specific differentiation markers. (A) Adipogenic markers; FABP4, LEPTIN, and LPL. (B) Chondrogenic markers; COL2A, SOX9, and AGGRECAN (AGC). (C) Osteogenic markers; OSTEOPONTIN (OPN), COL1A1, BONE SIALOPROTEIN (BSP). The expression of GAPDH was used as reference for evaluating the quality of mRNA. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
Traditionally, rodent models of human diseases have been widely used in preclinical studies since they are easy to be manipulated. However, large animal models, such as the dog, are more faithful to reproduce human pathologies. Dogs represent a source of many well-characterized genetic diseases homologous to human making them ideal for in vivo approaches, such as heterologous stem cells transplantation or autologous genetically corrected stem cells transplantation [8–10,22]. Furthermore, stem cell lineages established from dogs with genetic disease will represent a valuable tool for in vitro and in vivo screening of therapeutic drugs. Also, cell therapy for many others pathologies such as bone fracture, medullar lesion, and myocardial infarction could be tested in this model.

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Author Disclosure Statement

The authors indicate no potential conflicts of interest.

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